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LIST OF ABBREVIATIONS

AE	Assimilation efficiency
APnEO	Alkylphenol(n)polyethoxylate
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BMF	Biomagnification factor
BP	Bisphenol A
CAS No	Chemical Abstracts Service registered number
DCM	Dichloromethane
d.w.	Dry weight
EDCs	Endocrine disrupting chemicals
E1	Estrone
E2	17β-estradiol
HNO_3	Nitric acid
$HClO_4$	Perchloric acid
k2	Elimination rate
kd	Uptake rate
log K_{ow}	Octanol–water partitioning coefficient
log K_{oc}	Organic carbon-water partitioning coefficient
n.d	Not detected
n.a	Not available
N_2	Nitrogen gas
NaCl	Sodium chloride
NP	Nonylphenol
NPnEO	Nonylphenol(n)ethoxylate
OP	Octylphenol
OPnEO	Octylphenol(n)ethoxylate
$t_{1/2}$	Half-life
UDPGT	Uridine diphospo-glucuronosyltransferase
v/v	Volume over volume
w.w.	Wet weight

ABSTRACT

Endocrine Disrupting Chemicals (EDCs) are known to impair the reproduction in fish by intersex, altered mating behavior and reduced testicular growth. Target EDCs in this study consist of natural (E1 and E2) and estrogenic chemicals (NP, OP and BP) which were reported as potentially estrogenic to aquatic organisms.

This study consists of five experiments examining the bioaccumulation of EDCs through food chain which representing by commercial diet, polychaete and benthic fish. In experiment 1, bioaccumulation of EDCs was determined in polychaete through dietary exposure. Biomagnification factor (BMF) values indicated EDCs were not biomagnified in polychaete. Besides, E1 concentration was below detection limit and speculated to be biotransformed. In experiment 2, EDCs concentration was measured in wild polychaete collected from Osaka Bay. EDCs concentrations were unexpectedly high in polychaete compared to sediment; thus, predicted to biomagnify the compounds from the sediment and possibly transfers through food chain. Therefore, bioaccumulation of EDCs was determined in benthic fish, *Pleuronectes yokohamae* through dietary exposure in experiment 3. BMF values also demonstrated no biomagnifications and this finding were verified by no induction of vitellogenin in fish serum. In each exposure experiments, higher EDCs concentration were observed in exposed groups compared to control suggesting the assimilation in *P. yokohamae*. This assumption has been affirmed in experiment 4 by the high assimilation efficiencies (AE) computed in *P. yokohamae* by dietary exposure with percentage of over 88–96% (except NP). Therefore, low bioaccumulation of EDCs in homogenate fish tissues and presence of compounds concentration below detection limit (BP, E1 and E2) in this study were probably due to intensive metabolism. EDCs had been reported to be metabolized and biotransformed into glucuronide conjugates in fish; hence

glucuronidation activity was analyzed in the microsomal of intestine and liver of *P. yokohamae* in experiment 5. High UGT activity in the microsomes of intestine and liver suggesting efficient metabolism and elimination of BP form the *P. yokohamae* body. Thus, it can be justified that BP was not bioaccumulated/biomagnified in the previous dietary exposure due to glucuronidation. The other target EDCs were assumed to be glucuronidated as well based on the verification by other authors who conducted studies specifically on fish.

In the present study, EDCs were not bioaccumulated through the food chain. This finding has been verified by high glucuronidation activities in intestine (first-pass metabolism organ) of *P. yokohamae*.

ABSTRACT

魚類の内分泌を攪乱し、再生産に影響を及ぼす可能性のあり、海底質中に残留する内分泌攪乱化学物質（以下EDCs）の食物連鎖経由生物濃縮とその影響について、5つの実験を実施して研究した。

第1の実験では人口底質で飼育した底生動物（イソゴカイ、*Perinereis nuntia*）に、EDCs（ノニルフェノール（NP）、オクチルフェノール（OP）、ビスフェノールA（BP）、17β-エルトラジオール（E2）およびエストロン（E1））を添加した餌を投与し、それらの生物濃縮を調べた。その結果、ゴカイ中EDCs濃度は上昇するものの餌中の濃度を上回ることはなく、生物濃縮することはなかった。第2の実験では底質中EDCs濃度が既知の大阪湾で採取した底生動物中EDCs濃度を調べた。その結果、多毛類の1種（*Paraprionospio* sp.）で底質中濃度を上回るEDCsが検出され、底質→底生動物→底生魚類の食物連鎖経由でのEDCs生物濃縮の可能性が示唆された。そこで第3の実験では底生魚種であるマコガレイ（*Pleuronectes yokohamae*）稚魚にEDCsを添加した餌を投与し、餌からのEDCsの生物濃縮を検証した。しかし、EDCs濃度の若干の上昇は観察されたものの、いずれの魚体中濃度とも餌のそれらを上回ることはなく、餌からのEDCs生物濃縮は認められなかった。これは同実験のマコガレイ中に、メス特有の卵黄前駆物質でEDCs暴露のバイオマーカーであるvitellogenin（Vg）の誘導がほとんど認められなかったことからも、EDCsの体内へほとんど蓄積しなかったことがうかがえる。さらに第4の実験として、EDCsを添加した底質を敷いた水槽でマコガレイを飼育し、底質からのEDCs移行を確かめた。その結果、餌投与実験と同様にEDCsの体内濃度上昇はほとんど認められなかった。一方、第5の実験でマコガレイ消化管からの餌中EDCs消化吸収率を測定した結果、NPで50%、その他のEDCsで88～96%の消化吸収率が得られた。

以上の結果、底質中EDCsは一部の底生動物中に生物濃縮されることから、これらを餌とする底生魚類に食物連鎖経由で移行することが考えられた。一方、EDCs添加餌のマコガレイへの投与実験およびEDCs添加底質での飼育実験から、EDCsの食物連鎖経由あるいは底質経由での魚類への生物濃縮の可能性の低いことが明らかとなった。マコガレイによる餌中EDCs消化吸収率の高かったことから考えて、消化管から吸収される際にEDCsは何らかの代謝を受け、それらの代謝物として体内に吸収されたと考えられる。さらにこれらの代謝物にはVgを誘導するような女性ホルモン作用がほとんどなかったことが実験で確認された。

CHAPTER 1

GENERAL INTRODUCTION

Background of EDC study

In the early 1980s, sexually disruption in fish was first reported in England (Tyler et al. 2008). They found low incidence of intersex, or hermaphroditism in populations of wild roach (*Rutilus rutilus*), a freshwater cyprinid living in a sewage effluent settlement lagoon from sewage treatment works (STW) (Sweeting 1981). This finding was very unusual because roach are gonochoristic (they have either an ovary or a testis) (Schultz 1996). The presence of intersex roach at the sites have raised the possibility that compounds in STW effluent might cause disruption in sexual development. By late 1980s, a field study began in which caged rainbow trout were placed in effluents from sewage treatment works. From the study they discovered that at a few sites, the effluent was lethal to rainbow trout, but the remaining sites were found to strongly estrogenic (Purdom et al. 1994). The similar studies were also conducted in other fish species such as bream in Germany (Hecker et al. 2002), chub in France (Flammarion et al. 2000) and common carp in Portugal (Diniz et al. 2005). These are among the initial studies of EDCs which triggers the importance of this issue to other researchers.

What is EDC?

U.S. Environmental Protection Agency defines Endocrine Disrupting Chemicals (EDCs) as exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body, that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (Crisp et

al. 1997). The endocrine system is a complex communication system between chemical signals and their targets, and is responsible for regulating internal functions of the body. Basically, any substance that alters the function of this system is known as an endocrine disrupting chemical or endocrine disrupter (Vasquez-Duhalt et al. 2005).

Commission of the European Communities (2001) has listed out approximately 560 substances of EDCs in Annex 1 of the CEC report. These compounds are able to act as estrogens, like natural (e.g. 17β-estradiol, E2; estriol, E3; estrone, E1) and synthetic hormones (e.g. ethinylestradiol, EE2; diethylstilbestrol, DES), phyto- and mycoestrogens, pharmaceutical or therapeutic agents and industrial chemicals (e.g. nonylphenol, NP; octylphenol, OP; bisphenol A, BP). Other EDC could act as antiestrogens like chlorinated dioxin and –furanes, PCB-congeners and PAH; as androgens like tributyltin, or as antiandrogens like p.p'-DDE or the fungicide vinclozolin (Matthiesen et al. 1996). They do not have many structural similarity or common chemical properties, so they can exert their effects by different mechanisms by mimicking or antagonizing the effects of hormones, by altering the pattern of synthesis and metabolism of hormones and by modifying hormone receptor levels (Depledge and Billinghurst 1999).

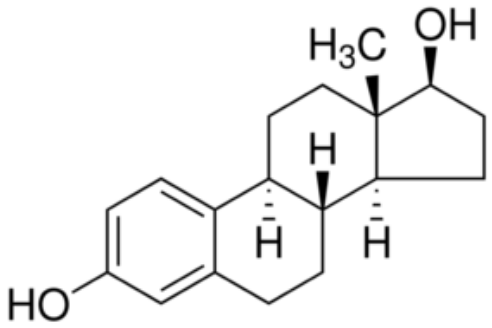
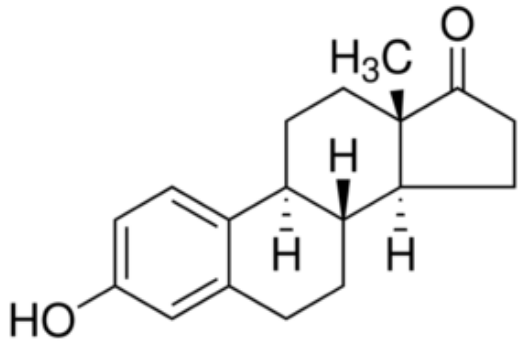
In a review article, Esplugas et al. (2007) describe the associate effects with the presence of EDCs in the environment are: (i) feminization of male fish; (ii) some problems in the reproductive system in fishes, reptiles, birds and mammals and; (iii) changes in the immunologic system of marine mammals.

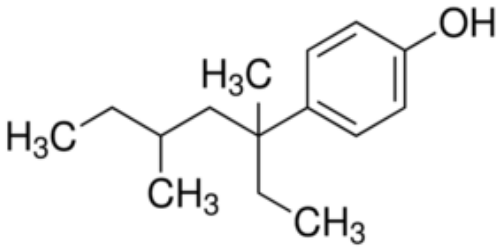
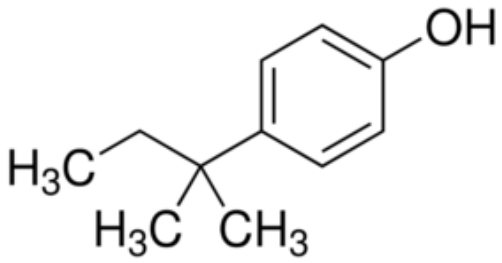
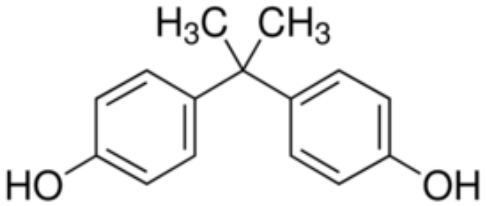
Target EDCs

Currently, the most concern EDCs is estrogenic compounds including natural estrogens and product of industrial chemicals. Natural estrogens produced by women and livestock excretion are extremely potent endocrine receptor modulators, while the industrial chemicals interfere with normal hormonal activity by mimicking and blocking the action of natural estrogens. Natural estrogens included in this study were the female hormones estrone (E1) and 17β-estradiol (E2) and the industrial chemicals are alkylphenols such as nonylphenol (NP), octylphenol (OP) and product of plasticizers, bisphenol A (BP). In Table 1.1 the physico-chemical properties, chemical structures and some other relevant data of natural estrogens and industrial chemicals are compiled.

Natural estrogens are contributed predominantly by human and livestock through their feces and urine. A number of aquatic species for example turtles, trout and minnows were sexually inhibited or reversed by the presence of natural estrogens (Jobling et al. 1998; Panter et al. 1998; Irwin et al. 2001). Desbrow et al. (1998) had found E1 and E2 to be the most significant estrogenically active substances in seven domestic effluents with concentration level of 1–80 ng/L and 1–50 ng/L, respectively. This situation was signified by the occurrence of intersex in fishes (Taylor et al. 2005). In zebrafish for instance, 49 ng/L of E1 and 52 ng/L of E2 were reported to significantly alter sex ratio in zebrafish (Holbech et al. 2006). Similar situation had been observed in roach by Taylor et al. (2005). Intersex in fishes can lead to feminization which induces vitellogenesis (Thorpe et al. 2001; Routledge et al. 1998; Nimrod and Benson 1998) at dissolved concentrations as low as 1–10 ng/L (Thorpe et al. 2001; Routledge et al. 1998). Effect concentrations for vitellogenin (Vg) induction (a sensitive biomarker for exposure to estrogens) have been reported as low as 5 ng/L

Table 1.1 Common name, abbreviation, IUPAC name, CAS No., chemical structure, molecular formula, molecular mass, water solubility, n-octanol/water partition coefficient (log K_{ow}), soil organic carbon-water partitioning coefficient (log K_{oc})

Chemical (common name, abbreviation and IUPAC name)	CAS no	Chemical structure	Molecular formula and molecular mass (g/mol)	Water solubility (mg/L)	log K_{ow}	log K_{oc}
1. Natural estrogens 17β-estradiol (E2) 1,3,5(10)-Estratiene-3,17β-diol	50-28-2		$C_{18}H_{24}O_2$ 272.39	1.7-4.7[a]	4.0[b]	2.94[d]
Estrone (E1) 3-Hydroxy-1,3,5(10)-estratriene-17-one	53-16-7		$C_{18}H_{22}O_2$ 270.37	12.42 [a]	4.10 [a]	2.99[d]

2. Industrial chemicals Nonyphenol (NP) 4-Nonylphenol (technical grade)	25154-52-3		$C_{15}H_{24}O$ 220.36	5.4 [a]	4.48[c]	4.7[e]
Octylphenol (OP) 4-*tert*-Octylphenol	140-66-9		$C_{14}H_{22}O$ 206.32	12.6 [a]	4.12[c]	3.44[f]
Bisphenol-A (BP) 2,2-Bis-(4-hydroxyphenyl)propane	80-05-7		$C_{15}H_{16}O_2$ 228.29	0.12 (pH7, t=20-25°C) [a]	3.32-3.40 [a]	2.31[g]

[a] Geyer et al. 2000; [b] Schweinfurth et al. 1997; [c] Ahel and Giger 1993; [d] Casey et al. 2005; [e] Sekela et al. 1999; [f] Brooke et al. 2005; [g] Clara et al. 2004

for E2 (Tabata et al. 2001) and 3.2 ng/L for E1 (Thorpe et al. 2001) and their effects are additive (Routledge et al. 1998). Other estrogenic effects observed included altered mating behavior (Wenzel et al. 2001), reduced testicular growth (Jobling et al. 1996) and reduce reproductive output (Van den Belt et al. 2001). Therefore, these compounds should be considered as potential and significant subject of further study.

Industrial chemicals such as alkylphenols were among the first anthropogenic compounds shown to mimic the natural female hormone, E2 (Laws et al. 2000; Routledge et al. 1998; Jobling et al. 1996). The common biodegradation products of alkyphenols are NP and OP. Recently, studies have indicated alkylphenol compounds possess estrogenic activity. For example, investigations with male rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) have confirmed that alkylphenols are capable of inducing synthesis of yolk protein Vg (Jobling et al. 1996; Nimrod and Benson 1996; Sumpter and Jobling 1995). These compounds also have the ability to inhibit testicular growth in fishes and induce the formation of egg cells in testis (Gimeno et al. 1996; Gray and Metcalfe 1997). Besides, histopathology of testes has revealed the severely suppressed of spermatogenesis in swordtail fish, *Xiphophorus helleri* exposed to 100 ppb NP (Kwak et al. 2001). Similar observations have been made in medaka exposed to OP (Gronen et al. 1999), and 86% incidence of testis-ova was demonstrated following exposure to 50 μg/L of NP (Gray and Metcalfe 1997).

BP is another weak estrogen mimicker but responsible for testes growth inhibition and retardation of maturation, lower semen quality, reduced ovulation and occurrence of intersex gonads (Lahnsteiner et al. 2005). In fish, common carp males exposed to BP showed an alteration of testes structure and increase in plasma of Vg (Mandich et al. 2007). Yokota et al. (2000) demonstrated that BP adversely affects the early life stage of Japanese medaka by the presence of oocytes in the testis at

concentration of 1820 μg/L. In addition, the mixture of 100 ppb NP and 10 ppm BP was found to cause apoptotic masses in interstitial area as well as the germ cell death in swordtail fish (Kwak et al. 2001). These compounds were widely reported as potential estrogenic chemicals in environment and could impair the reproduction in fish deem further investigation.

Potential sources of exposure

The general sources of emergent pollutants in environment was concluded as in Figure 1.1 The aquatic environment has been known as "the ultimate sink" for natural estrogens and industrial chemicals (Sumpter 1998); which EDCs have been found in freshwater (Kolpin et al. 2002), estuarine and marine environments (King County 2007; Koyama et al. 2013; Reddy and Brownawell 2005; Schlenk et al 2005; Zuo et al. 2006), raising the possibility that EDCs impact organisms living in these aquatic environments.

Particularly, sewage plant effluents appear to be one of the major routes of natural estrogens released in the environment (Johnson and Sumpter 2001; Purdom et al. 1994). The occurrence of EDCs in sewage treatment plant has raised attention of the regulatory agencies about their impact in environment. Both natural and industrial estrogens have been frequently found in effluent as byproducts of incomplete breakdown of their respective parent compounds (Johnson and Sumpter 2001).

Natural estrogens such as E2 and E1 have been found in sewage effluent in low ng/L concentrations (Desbrow et al. 1998; Snyder et al. 1999; Baronti et al. 2000). These estrogens bind with estrogens receptors in exposed organisms with an affinity

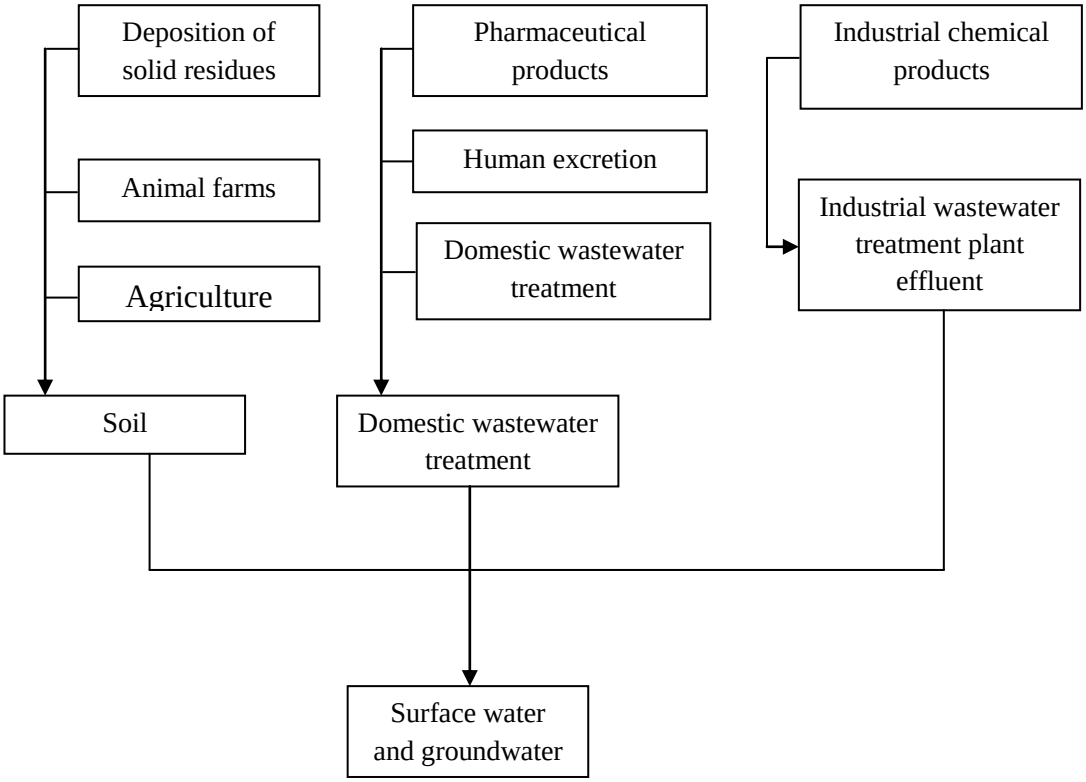


Figure 1.1 Possible route of exposition for emergent estrogenic compounds (Esplugas et al. 2007)

identical or similar to endogenous estrogen hormone, E2, and have the potential to exert effects at extremely low concentrations.

The target industrial chemicals are alkylphenols and BP. Biodegradation products of alkylphenols in detergents, such as NP and OP, are also found in sewage effluent and wastewater from septic systems, but at high ng/L to low μg/L concentrations (Lye et al. 1999; Rudel et al. 1998). Some alkylphenols have been shown to bind with the estrogen receptor and stimulate a biological response similar to E2, although concentrations at least 1,000-fold more are required (Jobling and Sumpter 1993; White et al. 1994; Körner et al. 2000). Meanwhile, BP has been detected in

sewage effluent (Körner et al. 2000) and septic system wastewater (Rudel et al. 1998) in low μg/L concentrations. BP exhibits estrogenic activity in both an in vitro cell proliferation assay with human breast cancer cells (Brotons et al. 1995) and an estrogen-receptor yeast-based assay (Metcalfe et al. 2001).

Potential environmental transports

In the aquatic ecosystems, estrogenic EDCs have been found in wastewater effluent, surface water, sediment, groundwater, and even drinking water as shown in Table 1.2 (Campbell et al. 2006). Low concentration of NP and OP were also reported in the atmosphere within 0.01-81 ng/m^3 and 0.01-2.5 ng/m^3, respectively (Ying et al. 2002; Rudel et al. 1998). Levels of estrogenic EDCs reported in the environment is presented in Table 1.2.

Wastewater effluent is known as the main environmental transport of estrogenic EDCs (Kolpin et al. 2002; Legler et al. 2002a; Snyder et al. 2003). In the waterways, EDCs may undergo a series of processes, such as dilution, photolysis, biodegradation and sorption to bed sediments. Due to low polarity of the target EDCs, with octanol-water partition coefficient (log K_{ow}) between 3.0-4.48, they not remain in soluble form and end up in suspended organic material and sorbed onto sediment. In the sediments there is potential for biological uptake, degradation and transformation to less or more mobile forms (Campbell et al. 2006). Therefore, sediment acts as a potential secondary source of EDCs within fluvial systems (see Jurgens et al. 1999).

Table 1.2 EDCs in wastewater effluent, surface water, sediment, groundwater and drinking water (*Source:* Campbell et al. 2006)

EDC	Wastewater effluent (ng/L)	Surface water (ng/L)	Sediment (μg/g)	Groundwater (ng/L)	Drinking water (ng/L)
E2	650 [1] 4.5-8.6 [6]	1.9-6.0 [2] 0.15-3.6 [5]	220-2,480 [3] 50-530 [7]	13-80 [4]	0.20-2.1 [5]
E1	0.35-18 [5] 1.2-19 [8]	0.10-4.1 [5] <0.1-17 [1]	160-1,170 [3] 70-2,520 [7]		0.20-0.60 [5]
NP	25-770 [5] 18-185 [11]	<100-15,000 [9] <100-7,300 [12]	0.03-9.05 [9] 6.4-154 [13]	200-780 [10]	2.50-16 [5] 10-2,700 [12]
OP	2.2-73 [5] 281-358 [14]	<10-190 [9] <100-13,000 [9]	<0.01-1.08 [9] 1.8-8.8 [13]		0.20-4.9 [5]
BP	4.8-47 [5] 15-258 [14]	85-250 [11] <3-230 [16]		3-1,410 [15] 20-44 [15]	0.50-2.0 [5] 20-44 [15]

1] Kolodziej et al. 2004; [2] Dorabawila and Gupta 2005; [3] Braga et al. 2005b; [4] Wicks et al. 2004; [5] Kuch and Ballschmiter 2001; [6] Cargouët et al. 2004; [7] Reddy and Brownawell 2005; [8] Pawlowski et al. 2003; [9] Petrović et al. 2004; [10] Ahel et al. 1996; [11] Heisterkamp et al. 2004; [12] Shao et al. 2005; [13] Hilscherova et al. 2002; [14] Körner et al. 2000; [15] Rudel et al. 1998; [16] Suzuki et al. 2004

EDCs transfer in aquatic organisms

EDCs in the sediments are expected to undergo low photodecomposition and biodegradation (Petrović et al. 2001) and may persist for long periods (Lai et al. 2000). A number of studies have demonstrated that estrogenic compounds persist in sediment with concentration up to 1,000 times than overlying water column (Peck et al. 2004; Williams et al. 2003; Petrović et al. 2002). Thus, benthic organisms have high possibility to be effected by EDCs through the sediment. Both in vitro and in vivo studies found that sediments can exhibit high estrogenic activity towards benthic organisms (Legler et al. 2002b; Duft et al. 2003; Koyama et al. 2013). EDCs have been

detected in several benthic organisms such as polychaete, amphipod, mussels and clams (Mäenpää and Kukkonen 2006; Zulkosky et al. 2002; Ferrara et al. 2001).

Despite benthic, EDCs were measured in other aquatic organisms like shrimps, prawns, squids, octopus, crabs and fishes (Ferrara et al. 2001, 2005, 2008; Basheer et al. 2004; Hu et al. 2005; Tsuda et al. 2000a; Ishibashi et al. 2006; Kashiwada et al. 2002). EDCs have been associated with reduced fecundity, reproductive failure and population level effects in a range of aquatic organisms (Jobling et al. 2002; Matthiessen and Gibbs 1998; Nash et al. 2004). High doses of E2 administered to juvenile rainbow trout were observed to caused kidney failure and mortality (Herman and Kincaid, 1988). In U.K. rivers, widespread feminization has been demonstrated in cyprinid fish, the roach (*Rutilus rutilus*) associated with the detection of estrogenic compounds in the sewage effluent (Jobling et al. 1998). Feminization in a wild fish, the gudgeon (*Gobio gobio*) was reported in the same rives (Van Aerle et al. 2000) by the indication of intersexual gonads and/or aberrant reproductive ducts (female like) in male fishes (Rodgers-Gray et al. 2000).

As reported by previous researches, EDCs have been detected in a wide range of aquatic organisms and environments. Thus, there is possibility that EDCs were transferred from the environmental media (water and sediment) to aquatic organisms through the food chain. Fent et al. (2010) had analyzed estrogenic chemicals in forty-eight macro invertebrates and fish samples, suggesting the chemicals most probably accumulated through food chain. A principal component analysis of chemical concentrations in the Detroit River food webs indicated the accumulation patterns associate with the chemical transfer through food chain (Russell et al. 1999). Therefore, EDCs transfer from sediment to aquatic organisms through food chain should be investigated further.

Bioaccumulation and biomagnification

Many of the estrogenic chemicals present in the aquatic environment are hydrophobic, thus have a strong tendency to bioaccumulate through food chain (Sumpter 1995).

Bioaccumulation is the process which causes an increased chemical concentration in an aquatic organism compared to their exposure routes (Mackay and Fraser 2000). EDCs may accumulate in aquatic organisms through different mechanisms: via the direct uptake from water by gills or skin, via uptake of suspended particles (ingestion) and via the consumption of contaminated food. The terminology is defined as the following:

(a) Bioaccumulation is referred as the uptake of substances from both food and water (Geyer et al. 2000).

(b) The term biomagnification is used for the dietary uptake via contaminated food.

Biomagnification can be regarded as a special case of bioaccumulation in which the chemical concentration in the organism exceeds that in the organism's diet due to dietary absorption (Mackay and Fraser 2000). A biomagnification factor (BMF) can be defined as the ratio of the concentration of chemical in the organism to that in the organism's diet at steady state (Sijm et al. 1992). BMF may be expressed on wet, dry, or lipid basis.

If EDCs input to the aquatic ecosystem is slow release from polluted sediments then it is plausible that uptake by benthic organisms followed by predation by larger organisms such as fish could be a significant source of bioaccumulation (Farrington 1991). Bioaccumulation of certain hydrophobic compounds cannot be explained by simple partitioning processes between sediment, water and fish (Van der Oost et al. 1988; Thomann 1989), it is likely that the uptake via contaminated food

(biomagnification) contributes significantly to the bioaccumulation of these contaminants in fish. Laboratory experiments with fish demonstrated that food digestion and absorption in the gastrointestinal tract provide a mechanism by which the chemical fugacity can be raised when one organism is consumed by another (Gobas et al. 1993). This was supported by Connolly and Pedersen (1988) that chemical fugacities of hydrophobic chemicals increase with every step in the food chain. Thus, EDCs bioaccumulation in the aquatic organisms should be examined via contaminated food which practically termed as biomagnifications.

Assimilation efficiencies (AE)

Aquatic animals are exposed to chemicals from both the particulate and dissolved phases. Recently, the trophic transfers of chemicals in aquatic food chains have been increasingly recognized as important source for contaminant accumulation (Luoma and Fisher 1997; Fisher and Wang 1998; Reinfelder et al. 1998). So, one critical parameter in understanding the bioaccumulation/biomagnifications of chemicals in aquatic systems is the assimilation efficiency (AE) (Landrum et al. 1992). Assimilation can be defined as the quantity of compound incorporated into the tissue of test organism following the process of digestion, hence indicates the uptake efficiency of EDCs.

Previous studies treat AE as important parameter to determine the bioaccumulation/biomagnifications of chemicals from dietary exposure (Landrum et al. 1992; Wang and Fisher 1999). AE can estimate the rate of ingested EDCs across organism gut lining which possibly could interfere with the normal metabolic processes. Brett and Groves (1979) computed AE as the percentage of food ration that is assimilated after loss to feces. Given the difficulties in quantifying chemical

bioavailability from food in previous studies involving long-term exposures (Wang and Fisher 1999), measurement of AE is an important approach to address the bioaccumulation of EDCs in aquatic animals (Luoma and Fisher 1997).

Vitellogenin (Vg)

To date, regulation of vitellogenesis in oviparous vertebrate has been widely used for the evaluation of estrogenic effects (Arukwe et al. 2002; Kordes et al. 2002; Zhong et al. 2004). Vitellogenin (Vg) is a female-specific estradiol-inducible protein, present in hemolymph and plasma of both maturing invertebrates and oviparous vertebrates (Pan et al. 1969). In fishes, Vg is synthesized in the liver, transported via the vascular system to the ovary and incorporated in developing oocytes (Mommsen and Walsh 1988). Following sequestration by oocytes, Vg is proteolytically cleaved to form yolk proteins, lipovitellin and phosvitin which major source of nutrition for developing embryo.

There are a number of techniques for measuring Vg. Non-specific measure of Vg can be made through assaying alkaline-labile protein-bound phosphorous in plasma fish (Kramer et al. 1998; Craik and Harvey 1984). More specific measurement techniques for Vg include radioimmunoassay and enzyme-linked immunosorbent assays (ELISA) which was successfully applied to fathead minnow (Tyler et al. 1999; Parks et al. 1999). The vitellogenin protein is specific for each individual species and, thus, often requires the production of specific antibodies for the selected species.

The presence of Vg in fish was chosen as a biomarker of exposure to EDCs (Donohoe and Curtis 1996; Kime et al. 1999; Folmar et al. 2000). The induction of Vg has been measured in field and laboratory studies. Field studies in United Kingdom (Purdom et al. 1994; Harries et al. 1996) and U.S. (Folmar et al. 1996) have reported

significant elevations of serum Vg in caged male carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) captured near the outfall of sewage treatment. In Japan, evidence of Vg in the serum of wild male fish was measured in carp (*Cyprinus carpio*) captured from Tama River, Tokyo during preliminary survey from July 1997 to June 1998 (A. Hara, unpublished data). Hashimoto et al. (2000) also reported high levels of Vg associated with gonadal abnormality in male marine fish, marbled sole (*Pleuronectes yokohamae*) captured from Tokyo Bay. Besides, *in vivo* studies also described the increase of plasma Vg of fish exposed to NP (Christiansen et al. 1998; Jobling et al. 1996). Thus, Vg could be a good bioindicator to describe the bioaccumulation/biomagnification of EDCs in fish.

Metabolism of EDCs by glucuronidation

Besides bioaccumulate/biomagnify, fish is capable of metabolizing compound to assist the elimination. Metabolisms are known to affect to the bioaccumulation/biomagnifications of EDCs as these processes will biotransform the parent EDCs into the metabolite form. Active compound such EDCs typically are lipid soluble. For several EDCs, the first step in the metabolism is through the phase I pathway of monooxygenation. The main site in the fish body of steroid catabolism is the liver with some contribution of intestine, kidney and gill (James 2011). The following phase II of metabolism known as glucuronidation, glutathione and sulfotransferase conjugation.

Glucuronidation is one of the major metabolic pathways involved in the elimination of endogenous and exogenous compounds from organisms (Dutton 1980; Evans and Relling 1999; Turgeon et al. 2001). The presence of glucuronide conjugate has been reported in microsome of intestine prepared from carp organs (Yokota et al.

2002) after being treated with BP. This finding has affirmed glucuronidation as one of detoxification pathways in fish which need further examination. Glucuronidation is catalyzed by uridine diphosphate glucuronosyl transferases (UGTs) which are family of isoenzymes that play a key role in excretion of xenobiotic compounds (George 1994). The enzymes have been detected in vertebrate tissues such as liver, gut epithelia, gills and gonads (Clarke et al. 1991).

UGTs enzymes act to transfer the glucuronosyl group from the UDP-glucuronic acid to several lipophilic compounds such as bile acids, bilirubin, steroid hormones, environmental pollutants, drugs and dietary components (Tephly and Burchell 1990; Mackenzie et al. 1992). The conjugated derivatives are more polar and water soluble than the precursor, thus improves compound ability to be excreted into the bile and/or urine (Mackenzie et al. 1997; Van Heek et al. 2000; Hanselman et al. 2003). In other word, glucuronidation is one of mechanism that favors the elimination of EDCs from tissue of organisms. This metabolism process will reduce and prevent the bioaccumulation of EDCs in the test organisms.

The biotransformation of organic xenobiotics such EDCs is one of the prime factors determining their distribution and retention time in fish (Lech 1974). It is important to know whether EDCs are metabolized in the organs of fishes for estimation of toxicity of these chemicals (Hites and Biemann 1972; Marx 1974; Yokota et al. 2002). In fish, glucuronidation is quantitatively the most important pathway for detoxification and excretion of xenobiotics (Melancon and Lech 1976; Varanasi and Gmur 1981; George 1994).

Previous and recent studies have shown the estrogens and alkylphenols were glucuronidated in the fish bile prior to excretion (Truscott 1979; Truscott 1983; Ferreira-Leach and Hill 2001; Pedersen and Hill 2002). In rainbow trout, 90% of the

estrogens were excreted as E2-glucuronides (Forlin and Haux 1985) and NP have been conjugated with glucuronic acid in the bile (Thibaut et al. 1998; Coldham et al. 1998). The purpose of conjugation of a substance with glucuronic acid is to produce strongly acidic compound which is more water-soluble to facilitate excretion. The glucuronide metabolites can be found in the excretory routes such as liver, bile or intestine. The rapid metabolism of BP into BP glucuronides was reported in rainbow trout liver compared to zebrafish (Lindholst et al. 2003). Bisphenol A was also found to be glucuronidated in the carp intestine (Yokota et al. 2002). Three metabolites of OP glucuronide were identified in liver, kidney, gut and plasma of roach fry (Ferreira-Leach and Hill 2000). Thus, microsomes from the excretory routes organs could be a good test subject to examine the glucuronidation of EDCs in fish.

Objectives of this Study

This study aims to examine the bioaccumulation and biomagnification of EDCs through food chain representing by spiked diet exposed to polychaete and benthic fish. Assimilation efficiencies, vitellogenin induction and glucuronidation were also subjected to study as these parameters will verify the bioaccumulation/biomagnification values.

Overview of this Study

After reviewing several EDCs studies conducted in aquatic environment surrounded of Japan, the concentration were reported higher in sediment compared to water. Due to the hydrophobicity of EDCs, these compounds were suggested to bind relatively longer in the suspended matter and sediment. Thus, benthic organisms such polychaete have the possibility to accumulate this compound from the sediment

through feeding. For that reason, the biomagnifications experiment (Chapter 2) was conducted by exposing EDCs to polychaete through spiked diet. After that, EDCs concentration was determined in the polychaete from Osaka Bay, one of the polluted bays in Japan (Chapter 3). In this field study, EDCs concentration in polychaete was found higher than the concentration in sediment. Therefore, the polychaete seems to biomagnify EDCs and probably might transfer them through the food chain. Consequently, biomagnifications of EDCs in benthic fish through dietary exposure was conducted (Chapter 4) together with the vitellogenin analysis. In order to examine the percentage of EDCs incorporated into fish tissues, assimilation efficiencies study was done (Chapter 5). Glucuronidation frequently reported as the major metabolism occurred in fish. Thus, the identification of glucuronide metabolite in the fish intestine was conducted in the last part of study (Chapter 6).

CHAPTER 2

Bioaccumulation of dietary endocrine disrupting chemicals (EDCs) by the polychaete, *Perinereis nuntia*

ABSTRACT

To investigate the biomagnification factor (BMF) of EDCs by the polychaete, *Perinereis nuntia*, organisms were exposed to EDCs through their diet. BMF values ranged from 0.001 to 0.028 indicating that EDCs were not biomagnified. Elimination rates (0.20–0.25 day^{-1}) were higher than uptake rates (0.0003–0.003 day^{-1}) verifying that EDCs were not biomagnified by *P. nuntia* due to their fast elimination. The calculated half-life of each EDC in this study varies from 2.76 to 3.45 days. Overall, the findings from this study demonstrated that the studied EDCs are not biomagnified in *P. nuntia* but accumulation does occur from the diet.

INTRODUCTION

The endocrine system is a complex communication system between chemical signals and their targets, and is responsible for regulating internal functions of the body. Any substance that alters the function of this system is known as an endocrine disrupting chemical or endocrine disruptor (Vasquez-Duhalt et al. 2005). Generally, estrogenic compounds enter the aquatic ecosystems through the effluent of sewage treatment (Kumar et al. 2011) and can be taken up by organisms in the receiving water body. As suspension and deposit feeders in aquatic ecosystems, polychaetes, such as *Perinereis nuntia* are likely to accumulate EDCs from sediment through dietary uptake (Osborne et al. 1982). Studies on the dietary accumulation of natural and estrogenic chemicals by benthos have received little attention. However, many benthic

invertebrates are important food sources for bottom feeding fishes. As a consequence, benthic invertebrates are key in the study of biomagnification and trophic transfer of contaminants in the aquatic environment (Reynoldson 1987). Moreover, EDCs accumulation from sediments by benthos may be important, as evidenced by the relatively high concentrations measured in sediment from Osaka Bay (Japanese Ministry of Environment 2002; Koyama et al. 2013) and Tokyo Bay (Hashimoto et al. 2005a; Isobe et al. 2001, 2006). Therefore, it is necessary to study the biomagnifications of EDCs from sediment by benthic invertebrates in term of dietary uptake. This study aimed to determine the biomagnification factors of estrogenic chemicals (nonylphenol, NP; octylphenol, OP; bisphenol A, BP) and natural estrogens (estrone, E1; 17β-estradiol, E2) by *P. nuntia* from dietary uptake. Uptake and elimination rates as well as half-lives of each compound were also evaluated.

MATERIALS AND METHODS

Generally, following are the list of chemical standards, reagents and analytical instruments used in the full-scale of study which included in each chapter. The manufacturer is cited next to each reagent and instrument.

List of chemical standards

1. p-Nonylphenol (NP), analytical grade (99.0%): Kanto Chemical Co. Inc., Japan
2. Octylphenol (OP), analytical grade (98.6%): Tokyo Chemical Ind. Co., Japan
3. Bisphenol A (BP), analytical grade (97.0%): Wako Pure Chemical Ind., Japan
4. Estrone (E1), analytical grade (99.0%): Wako Pure Chemical Ind., Japan
5. 17β-estradiol (E2), analytical grade (99.9%): Nacalai Tesque Inc., Kyoto, Japan
6. E2-16,16,17-d3 (E2-d3): Wako Pure Chemical Ind., Japan – Internal standard

7. p-n-NP-d4 (NP-d4): Wako Pure Chemical Ind., Japan – External standard
8. Bisphenol A mono-β-D-glucuronide (BPG): Sigma-Aldrich, Japan

List of chemical reagents

1. Acetone, pesticide grade: Wako Pure Chemical Ind., Japan
2. Acetonitrile, HPLC grade: Kanto Chemical Co. Inc., Japan
3. Ammonium acetate, HPLC grade: Wako Pure Chemical Ind., Japan
4. Aprotinin from bovine lung, ultrapure 95%: Wako Pure Chemical Ind. Co., Japan
5. CHAPs (3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate, detergent of solubilizing membrane proteins: Dojindo Molecular Technologies, Inc., Japan.
6. Chromium (III) oxide (Cr_20_3), 99%: Wako Pure Chemical Ind. Co., Japan
7. Cyclohexane, HPLC grade: Wako Pure Chemical Ind. Co., Japan
8. Dichloromethane (DCM), pesticide grade: Kanto Chemical Co. Inc., Japan
9. Diethyl ether, pesticide grade: Wako Pure Chemical Ind. Co., Japan
10. Ethyl acetate, pesticide grade: Wako Pure Chemical Ind. Co., Japan
11. Ethylenediaminetetraacetic acid disodium salt (EDTA.2NA), >99%: Dojindo Molecular Technologies, Inc., Japan.
12. Florisil PR for chromatography: Wako Pure Chemical Ind. Co., Japan
13. Hexane, pesticide grade: Wako Pure Chemical Ind., Japan
14. Hydrochloric acid (HCl), for poisonous metal analysis: Wako Pure Chemical Ind. Co., Japan
15. Isopropyl alcohol (2-propanol), spectrophotometric grade: Wako Pure Chemical Ind. Co., Japan

16. Magnesium chloride ($MgCl_2$) anhydrous, 99.9%, Wako Pure Chemical Ind. Co., Japan
17. Methanol, spectrophotometric grade: Wako Pure Chemical Ind. Co., Japan
18. Nitric acid, 98%: Wako Pure Chemical Ind. Co., Japan
19. Perchloric acid ($HClO_4$), for poisonous metal analysis: Wako Pure Chemical Ind. Co., Japan
20. 2-Phenoxyethanol, 94%: Wako Pure Chemical Ind. Co., Japan
21. Potassium chloride (KCl) for analysis, 99%: Wako Pure Chemical Ind. Co., Japan
22. Sodium acetate trihydrate, 99.0%: Wako Pure Chemical Ind. Co., Japan
23. Sodium azide (NaN_3), 99%: Wako Pure Chemical Ind. Co., Japan
24. Sodium chloride (NaCl), 99.5%: Wako Pure Chemical Ind. Co., Japan
25. Sodium sulfate anhydrous, 99.9%: Wako Pure Chemical Ind. Co., Japan
26. Triethylamine, 99%: Wako Pure Chemical Ind. Co., Japan
27. Tris(2-amino-2hydroxymethyl)-1,3-propanediol, hydrochloride reagent grade: Wako Pure Chemical Ind. Co., Japan
28. Uridine 5'-diphosphoglucuronic acid trisodium salt, 98-100%: Sigma-Aldrich, Japan.

List of analytical instrument

1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS-ESI) Agilent 1200 LC system with API-2000
2. Shimadzu 1600 UV-Visible Spectrophotometer
3. Ultra-High performance liquid chromatography (U-HPLC) Merck Hitachi LaChromUltra

4. Microplate reader (MTP-32, Corona Electric Co.)

Test organism (*P. nuntia*)

The polychaete, *P. nuntia* were purchased from a private aquaculture facility in the Kochi prefecture, Japan and were acclimatized in the laboratory condition for at least 2 weeks prior to use. They were kept in a container bedded with glass bead as the substrate and maintained in a water-flow-through system. The polychaete were fed using commercial diet (Nippon Formula Feed Manufacturing Co., Japan) with salinity, dissolved oxygen, pH and water temperature recorded periodically.

Preparation of EDCs spiked diet

The mixture of EDCs was prepared according to test group concentrations (low and high) as shown in Table 2.1. These nominal concentrations were decided after reviewing previous studies on EDC which conducted in water, sediment and various test organisms (e.g Diehl et al. 2012; Hayashi et al. 2008; Japanese Ministry of Environment 2002; Koyama et al. 2013). EDCs were dissolved in diethyl ether and spiked to the 30 g of commercial diet (for yellowtail) in stainless tray to aid drying process. Spiking was done by pasteur pipette while mixing well the solvent and diet. The spiked diet was left in draft chamber overnight prior to use with gentle mixing for every 3-4 hours.

Table 2.1 Nominal concentrations of mix EDCs spiked onto the diet

EDC	Concentration (ng/g)	
	Low	High
NP	500	5,000
OP	50	500
BP	50	500
E1	50	500
E2	20	100

Exposure scheme

In this experiment, glass beads (1.5 cm in diameter) were bedded to 6 cm height in a polypropylene vessel (16.5 x 9 x 10 cm) as substrate for *P. nuntia*. These vessels were then placed into tanks (60 x 46 x 18 cm) and maintained in a water-flow-through system. Test waters were completely replaced 50 litre/10 times/day with natural sand-filtered seawater and aerated well. These were prepared separately for control, low and high test groups. Each tank contains eight polypropylene vessels and all *P. nuntia* in a vessel were collected per sampling. The total body weights of *P. nuntia* in each vessel were from 15-20 g (Figure 2.1).

Figure 2.1 Experimental set-up for control, low and high test groups

Exposures of *P. nuntia* to dietary EDCs were carried out using commercial fish diet (Nippon Formula Feed Manufacturing Co., Japan) spiked with a mixture EDCs

dissolved in diethyl ether. *P. nuntia* were fed twice a day with feeding rate of 2% of their body weight. Prior to feeding, the water level in vessels were reduced so *P. nuntia* would emerged and consumed the diet. The exposure groups were fed with EDCs spiked fish diet for 14 days and unspiked diet for another 14 days. The sampling was done at day 0, 2, 4, 7 and 14 of exposure and elimination period, respectively. The overall average wet weight of individual *P. nuntia,* was 0.646±0.11 g and 0.936±0.04 g, for exposure and control groups, respectively. After sampling, polychaete samples were kept in freezer (-18°C) until analysis. Non-consumed food residues were removed daily. Water parameters such temperature, dissolved oxygen, pH and salinity were measured periodically.

Pre-treatment of samples

After thawing, the polychaetes from each vessel were weighed into a glass bottle to record the wet weights. The polychaetes were then freeze dried at -48°C and 7.8 Pa for 4 days. Dry weights of polychaetes were recorded right after the process.

Preparation of Florisil column (3% water) and Triethylamine (0.01%)

Florisil column (3% water) was prepared for the EDCs analysis in diet, polychaete and fish. This column was used for the clean-up purpose. To prepare this column, florisil PR was filled into Erlenmeyer flask up to one-quarter full and the weight was recorded. The florisil was heated in a furnace in 400°C for 13 hours. Florisil weight after heating was measured three hours after the process. The 3% volume of mili-Q water to be added into the florisil was determined based on the weight after heating. In order to mix the mili-Q water with florisil well, the mixture was

shook for 1 hour by shaker. Prior to use, the florisil 3% water was kept overnight at room temperature.

Triethylamine (0.01%) was prepared as the mobile phase B in LC-MS/MS-ESI measurement. To prepare this mobile phase, 0.1 mL of trietylamine was added into 1,000 mL mili-Q water and ultrasonicated for 1 hour to degas the air from the solution.

EDCs analysis

1. Polychaete and Diet

After addition of external standard of *p*-n-NP-d4, target chemicals in the freeze-dried polychaetes (0.6 g) were ultrasonically extracted twice with 20 mL of acetone-hexane (3:7, v/v) for 15 min. After centrifugation (4°C, 760 x g, 10 min), the organic layer was collected and concentrated to about 0.5 mL using a rotary evaporator and gentle stream of nitrogen gas (N_2); the solvent was then completely replaced with hexane. Clean up was performed in a 5 mL glass pipette (6 mm i.d. x 17 cm) filled with florisil (containing 3% water) and prewashed with 30 mL hexane. The target chemicals were eluted with 50 mL of hexane-isopropyl alcohol (9:1, v/v). The elution were then concentrated to 0.5 mL using a rotary evaporator and gentle stream of N_2. Subsequently, Hybrid SPE-Precipitation 30 mg (Supelco, Bellefonte, PA, USA) was applied for the removal of excess lipid which was pretreated with 5 mL acetonitrile followed by concentrated elution and 3 mL acetonitrile. This step was conducted on VacElut 12 manifold glass basin (VacElut manifold) by slowly dropped the eluate to make sure the lipid was retained in the SPE tubes. Finally the eluate was concentrated to 0.2 mL under a gentle stream of N_2 right after additional of internal standard (E2-16,16,17-d3). EDCs in the spiked diet were analyzed in the manner as polychaete.

2. Seawater

EDCs extraction from seawater samples were carried out within 24 h of collection. After addition of external standard of *p*-n-NP-d4, liquid-liquid extraction was performed on 1 L of glass-fiber (GF/C Whatman)-filtered sample using 100 mL hexane and 5 min shaking in separating funnel. After complete separation, the hexane layer was collected while the aqueous portion was subjected to second extraction. The first and second extracts were combined and dehydrated using sodium sulfate anhydrous. Further extraction was done using polar solvent, 100 mL DCM with 5 min shaking. The denser DCM layer was collected into a rotavapor flask by filtering through sodium sulfate anhydrous. The extracted solvent was then concentrated to dryness by a rotary evaporator and gentle blow of N_2 at 40°C, and make up to 0.5 mL by acetonitrile and internal standard of E2-16,16,17-d3.

Measurement using LC-MS/MS-ESI

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was carried out with an Agilent 1200 LC system (Agilent Technologies, USA) coupled to an API-2000 triple stage quadrupole mass spectrometer equipped with electrospray ionization (ESI) source (Applied Biosystems, USA). The ESI was employed in negative mode. The source parameters for the MS detection were optimized with the quantitative function of Analyst (Version 1.4.1, Applied Biosystems) equipped with API-2000. The optimized parameters were set as follows: 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. MS/MS measurements

were performed in the multiple reaction monitoring (MRM) mode and target chemicals were five EDC compounds (Table 2.2).

The EDCs were analyzed by gradient liquid chromatography using a reverse-phase Extend C-18 column (2.1 mm i.d. x 100 mm, 3.5μm of particle size, Agilent) kept at 40°C. The mobile phase consisted of solvents A (100% acetonitrile) and B (water containing 0.01% triethylamine, (v/v)). The analytes were eluted using the following gradient program: 20-95% solvent A in 15 min, and then keeping 95% solvent A in 10 min. The solvent A flow was increased for washing the column until 500 μL/min in 0.1 min and maintained 2 min (not flowing to ESI detector using 6-port switching valve system equipped LC-MS/MS). After washing the column, solvent A was changed back to initial ratio within 0.1 min and held for 8 min in 500 μL/min until the next injection.

Detection limit and recovery test

The detection limit was determined as the minimum concentrations corresponding to those of signal to noise ratio of 3 on the chromatogram of actual sample. The ratio between intensity of signal of each compound was obtained under selected ion monitoring (precursor and daughter ions, m/z) conditions and intensity of noise. The limit of detection of each EDC is shown in Table 2.2.

The recovery test was performed using fish diet spiked with 100 ng/g of each EDC standards and internal standard (E2-16,16,17-d3). The EDCs standard extraction and measurement was done as above procedures. Mean percent recovery, as determined from an estimate of the amount of added and recovered, were between 82.0% and 104.9%. Analysis was conducted in triplicate (n = 3) with error of all analysis was within ±20%.

Table 2.2 Precursor and daughter ions of EDCs and limit of detection by LC-MS/MS-ESI

EDCs	Precursor ions, m/z (amu)	Daughter ions, m/z (amu)	Limit of detection (ng/g)[a]
NP	219.0	132.9	0.3
OP	205.0	132.9	0.3
BP	227.1	211.8	0.2
E1	269.1	145.0	0.3
E2	271.1	144.9	0.4

[a]Detection limit using 3.0 g wet weight sample

Data analysis

In this experiment, BMFs (biomagnifications factors) were calculated as the ratio of concentration in organism to diet at steady state (Kobayashi et al. 2011) as follows: BMF = (EDCs concentration in *P. nuntia* at steady state)/(EDCs concentration in diet). Elimination rate (k2) was calculated according to first-order kinetic model: $C_t = C_0 e^{-k2t}$, where C_t is the EDCs concentrations (ng/g wet weight) at time t and C_0 is the EDCs concentrations (ng/g wet weight) at day 0 of elimination period. The uptake rate (kd) was computed according to Kobayashi et al. (2011) as follows: BMF = kd/k2; kd was computed based on the BMF and k2 values. Half-life (d) is a convenient measure to determine the persistence of a chemical in the environment and computed as follows: $t_{1/2} = \ln 2/k2$.

RESULTS AND DISCUSSION

Water parameters and control diet

Mean water temperature and dissolved oxygen concentration were 13.5±2.5°C and 6.65±0.6 mg/L, respectively, whereas pH and salinity were 8.05±0.4 and 34.2±1.0, respectively. None of EDCs were detected in the water sample. The initial concentrations of each EDC in the spiked fish diet are given in Table 2.3. NP, OP and BP levels in the control diet were 27.9±1.77 ng/g, 3.02±0.34 ng/g and4.16±1.24 ng/g, respectively. Generally, commercial pellet diet ingredient consists of fish meal from wild catches. The pellet feed composition has been reported to contain 36-52% fish meal (Olsen 2011). Thus, it was expected that only low concentration of EDCs would be detected in the control diet.

Table 2.3 EDCs concentration, mean (±SD), ng/g dry weight in control and spiked diet, n=3

EDCs	Control	Low	High
NP	27.9 (±1.77)	336 (±41.30)	2879 (±609)
OP	3.02 (±0.34)	48.1 (±13.30)	326 (±107)
BP	4.16 (±1.24)	24.8 (±11.60)	290 (±80)
E1	n.d[a]	31.1 (±7.82)	325 (±68)
E2	n.d[a]	12.6 (±1.36)	95.1 (±23)

n.d – Not detected
*SD standard deviation

EDCs concentration in P. nuntia

The moisture content of *P. nuntia* was calculated in the range of 23.9-25.6%. In the present study, the values were reported on wet weight basis as a reflection of the natural environment of polychaete. The concentrations of NP, OP, BP and E2 in *P. nuntia* are shown in Figure 2.2. In this study, E1 was not detected in *P. nuntia*. E1 was probably degraded by metabolism activity and biotransform as suggested by Walter et

al. (1988). The biotransformation process known as glucuronidation, by microsomal UDP glucuronosyltransferase, is a major pathway for inactivation and excretion of both endogenous and xenobiotic organic compounds (Clarke et al. 1991).

On day 0, NP, OP and BP were detected in *P. nuntia* at 0.78, 0.32 and 0.21 ng/g wet weight (w.w.), respectively. These EDCs were derived from the unspiked diet, which were fed during the acclimatization period. However, E2 was not detected at day 0, indicating that *P. nuntia* does not produce or accumulate E2 from diet. E2, NP, OP and BP concentrations were observed to increase rapidly from day 0 in both low and high exposure groups. Steady state concentrations for the above mentioned EDCs was achieved at around day 7 of exposure (Figure 2.2). Their maximum concentrations in low and high exposure groups were 1.15 and 3.62 ng/g w.w. for NP, 0.78 and 2.07 ng/g w.w. for OP, 0.77 and 3.33 ng/g w.w. for BP, 0.515 and 1.18 ng/g w.w. for E2, respectively. Therefore, day 7 was defined as the point during which steady state was reached for all EDCs and the concentration at this time point was used for the BMF computation. During the elimination period, *P. nuntia* were fed with unspiked diet. As expected, E2, NP, BP and OP concentrations gradually decreased until the end of the experimental period.

Biomagnification factor (BMF)

BMFs were calculated based on concentration of EDCs at day 7 of exposure relative to the initial concentration of the respective compound in the diet. In the present study, BMF values in both exposure groups (low and high) were <1.0 (Berntssen et al. 2007), suggesting that the studied EDCs were not biomagnified by *P. nuntia* from the diet (Table 2.4). Nevertheless, EDCs were assimilated by *P. nuntia* from the diet as indicated by the increasing EDCs concentrations during the exposure

period (Figure 2.2). The BMF values of NP, OP, BP and E2 in both exposure groups were similar, thus differences in the concentrations of EDCs does not seem to affect the BMF values.

Previous studies have reported the BMFs of NP and OP from oyster, *Crassostrea gigas* (prey) to snail, *Thais clavigera* (predator) with value of 0.7–2.4 and 0.5–4.3, respectively (Cheng et al. 2006). Another study of NP reported a lower value (BMF of 0.2) based on lipid weight from benthic invertebrates (prey) to ghost shrimp, *Neotrypaea californiensis* (predator) (Diehl et al. 2012). BMFs in both studies were estimated from field samples and were higher than the present study. The values were computed by the ratio of concentration in the predator to prey, differ from dietary exposure in the present study. Thus, the predator probably derived additional EDCs concentration from the water and lead to higher BMF values compared to dietary exposure experiment. The other reasons probably due to aquatic organisms in natural ecosystems are continuously exposed to effluent containing EDC for many years as opposed to the shorter exposure time of 14 days used in the present study. However, no BMF data are available from the literature for BP, E1 and E2 in invertebrates.

Uptake rate, elimination rate and half-lives of EDCs

In the present study, the uptake rate for NP was the lowest at 0.0003 day^{-1} compared to E2 (0.003 day^{-1}), BP (0.0022 day^{-1}) and OP (0.0011 day^{-1}) (Table 2.4). There are no studies of uptake rate reported for EDCs through dietary exposure. However, uptake rates of NP from sediment by oligochaete, *L. variegatus* ranged from 0.011 to 0.074 h^{-1} (0.264-1.78 day^{-1}) (Mäenpää and Kukonen 2006). Meanwhile, 4-NP uptake rate of 355 day^{-1} was reported in amphipod, *Eohaustorius estuarius* (Hecht et al. 2004). Both studies showed significant bioaccumulation factors (≥ 1.0) from sediment

to invertebrate. Thus, the lower uptake rate in the present study might suggest low biomagnification of EDCs in *P. nuntia*.

Due to very low concentrations measured in low exposure groups, a statistical fit to the first order kinetic model could not be established. The data were small and too noisy, hence cannot be applied to the proposed model. Thus, the elimination rates of low exposure groups were not determined. The elimination rates for the EDCs evaluated in this study are shown in Table 2.4. The average rate of elimination is between 0.201 and 0.251 day^{-1}. The elimination rate study of 4-NP in oligochaete, *Lumbriculus variegatus*, was reported from 0.002 to 0.008 h^{-1} (0.048-0.192 day^{-1}; Mäenpää and Kukkonen 2006); meanwhile the values were 0.04 day^{-1} and 0.007 h^{-1} (0.168 day^{-1}) in mussel, *Mytilus galloprovincialis* and clam, *Tapes philippinarum*, respectively (Gatidou et al. 2010; Lietti et al. 2007). Besides, similar NP elimination rates were reported in amphipod, *Gammarus pulex*, 0.010 h^{-1} (0.24 day^{-1}) and polychaete, *Capitella teleta*, 0.236 day^{-1} (Gross-Sorokin et al. 2003; Jager and Selck 2011). Compared to the present study, *P. nuntia* had higher elimination rates than other invertebrates, 0.251 day^{-1}. Heinonen et al. (2002) studied BP accumulation in the freshwater clam, *Pisidium amnicum*, and reported a very rapid elimination rate of 0.045 h^{-1} (1.08 day^{-1}). In the brackish water bivalve, *Corpicula japonica*, BP was eliminated at 0.0125 h^{-1} (0.3 day^{-1}) (Hayashi et al. 2008) compared to 0.201 day^{-1} by *P. nuntia*. This shows the slower elimination of BP by *P. nuntia* compared to freshwater clam and brackish water bivalve. However, BP was eliminated at 0.027 day^{-1} from *M. galloprovincialis*, later than *P. nuntia* of the present study. There are no reports for elimination rate of OP and E2 by benthic invertebrates. Consequently, invertebrates have different ability to eliminate various EDCs.

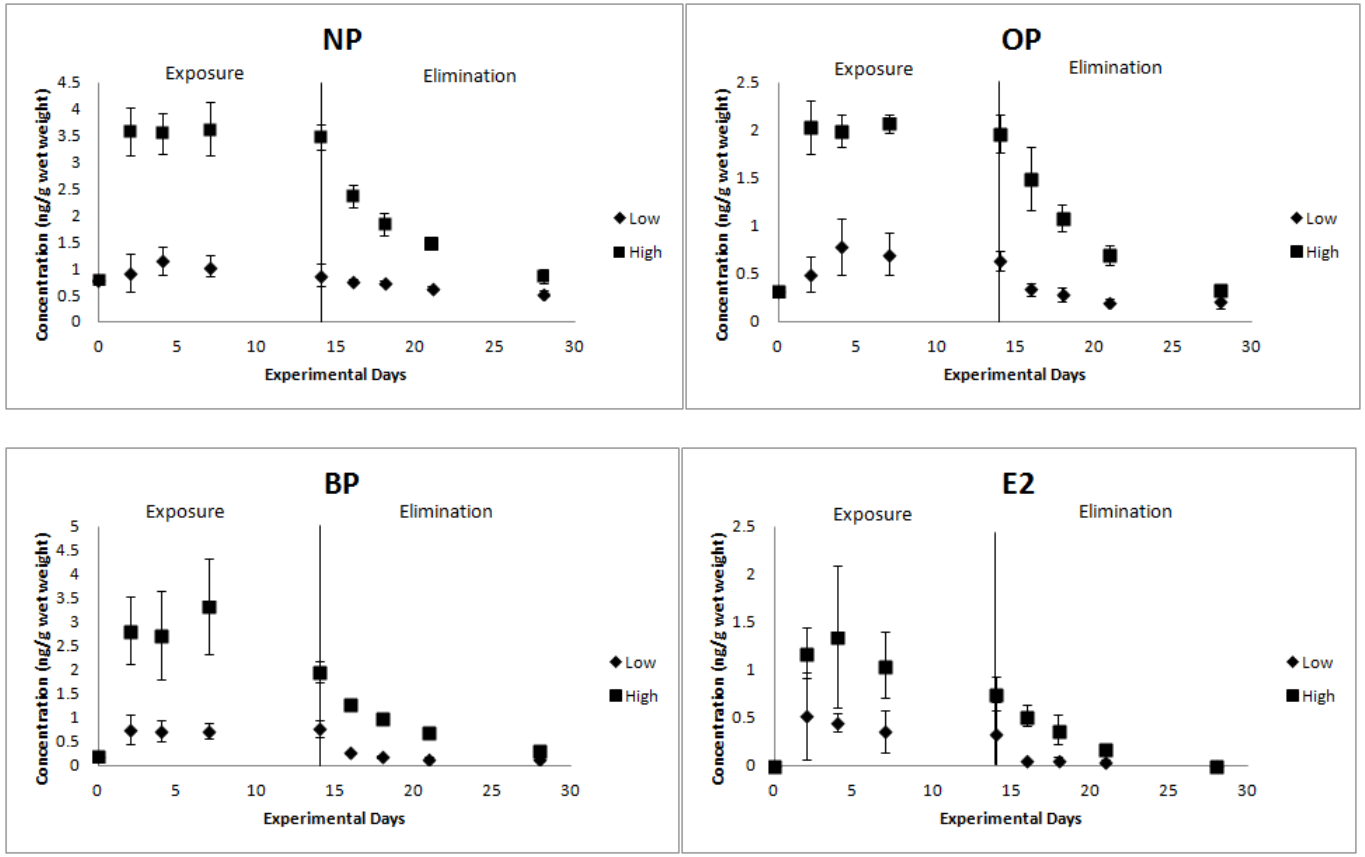


Figure 2.2 Accumulation and elimination of NP, OP, BP and E2 (ng/g wet weight) by *P. nuntia* in low and high exposure groups. Error bars indicate the standard deviation (n=3).

Table 2.4 BMF, k2, kd and $t_{1/2}$ of EDCs in low and high exposure groups

EDCs	Low Exposure				High Exposure			
	BMF[a]	k2[b]	kd[c]	$t_{1/2}$[d]	BMF[a]	k2[b] (day^{-1})	kd[c] (day^{-1})	$t_{1/2}$[d] (day)
NP	0.001	n.a	n.a	n.a	0.001	0.251	0.0003	2.762
OP	0.009	n.a	n.a	n.a	0.005	0.213	0.0011	3.254
BP	0.026	n.a	n.a	n.a	0.011	0.201	0.0022	3.448
E2	0.028	n.a	n.a	n.a	0.011	0.208	0.0030	3.332

[a]Biomagnifications Factor
[b]Elimination rate,
[c]Uptake rate
[d]Half-life
n.a – Not available

The half-life values of each EDC are presented in Table 2.4. Since elimination rate cannot be determined, the half-life values were not measured for the low exposure group. In the present study, NP has the shortest half-life with 2.76 days while the half-lives of OP, BP and E2 are more than 3 days. Several studies have reported the half-lives of NP by invertebrates. For instance, half-lives of NP in the amphipod (*G. pulex*) and clam (*T. philippinarum*) were 69.3 h (2.9 days) (Gross-Sorokin et al. 2003) and 99.0 h (4.1 days) (Lietti et al. 2007), respectively. In the case of the oligochaete, *L. variegatus* and polychaete, *C. teleta*, the half-lives of NP were reported between 3.6-14 days (Mäenpää and Kukkonen 2006) and 2.9 days (Jager and Selck 2011), respectively. In *P. nuntia,* the half-life of NP is 2.76 days similar to *G. pulex* and *C. teleta*. The half-lives of BP reported in freshwater clam, *P. amnicum* at 1.81 (Heinonen et al. 2002) and 2.31 days in brackish water bivalve, *C. japonica* (Hayashi et al. 2008). BP in *P. nuntia* is retained slightly longer, 3.45 days, compared to the freshwater clam and brackish water bivalve. However, no study reported the half-lives of OP and E2 through dietary uptake of invertebrates. Basically, the half-life of NP and BP varies on invertebrates.

Based on the present results and reported information, NP does not seem to be biomagnified by *P. nuntia* as a result of lower uptake and higher elimination rates for this compound. Besides, the higher elimination rate of BP in this species essentially makes it impossible to be biomagnified. In general, a higher elimination than uptake rate, indicates that the bioaccumulation of EDCs by *P. nuntia* are rapidly eliminated and/or biotransformed. As a result, biomagnifications values of NP, OP, BP and E2 are very low (< 0.1).

CONCLUSION

NP, OP, BP and E2 concentrations in *P. nuntia* were increased during exposure period, but the values were lower than spiked diet. Estrone (E1) was not detected in this study, hence speculated to be biotransformed and further investigation is needed in order to verify this hypothesis. BMF values of EDCs are very low and found not to be biomagnified but transferred from the diet. Furthermore, elimination rate values in this study were observed to be higher than uptake rates suggesting the ability of *P. nuntia* to metabolize and/or eliminate the EDCs. Differences in accumulation of EDC between laboratory exposure and field sample can be substantial, leading to large differences in biomagnifications. For that reason, the next objective is to determine the concentration of EDC in the polychaete from field study.

CHAPTER 3

Accumulation of endocrine disrupting chemicals (EDCs) in the polychaete *Paraprionospio* sp. from the Yodo River mouth, Osaka Bay

ABSTRACT

This study presents the levels of endocrine disrupting chemicals (EDCs) accumulated by *Paraprionospio* sp. from the Yodo River mouth, Osaka Bay from April 2007 to June 2009. Since high concentrations of nonylphenol (NP), bisphenol A (BP), octylphenol (OP), estrone (E1), and 17β-estradiol (E2) have been measured in sediment from Osaka Bay, some bioaccumulation could be expected particularly in benthic animals. EDCs were analyzed in *Paraprionospio* sp., a dominant benthic species in Osaka Bay. The results showed that *Paraprionospio* sp. had accumulated varying concentrations of NP at 1,460–4,410 ng/g wet weight (w.w.); BP at 22.5–39.6 ng/g w.w.; OP at 18.9–45.4 ng/g w.w.; E1 at 0.06–2.50 ng/g w.w.; and E2 at 0.89–4.35 ng/g w.w. Accumulation of NP and OP were highest among the samples gathered in summer (July 2008), while concentrations of BP, E2, and E1 did not much differs within 3 years. EDC levels in *Paraprionospio* sp. were apparently greater than those in sediments showing bioaccumulation.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a worldwide concern due to their observed effects on the reproductive system in fish (Imai et al. 2005, 2007; Jobling et al. 1996) as they are able to mimic the normal functions of hormones and change hormone receptor levels. EDCs are either natural or estrogenic; the natural forms include estrone (E1) and 17β-estradiol (E2) excreted by humans and livestock, which

are discharged from sewage treatment works and agricultural run-off into the aquatic ecosystems (Tashiro et al. 2003; Zhang et al. 2008), while estrogenic chemicals such as nonylphenol (NP), octylphenol (OP) and bisphenol A (BP) are mostly industrial byproduct of surfactants in detergents and paints, agricultural dispersing agents, plastic manufacturing, and other industrial applications (Folmar et al. 2001;White et al. 1994). Both types have been reported to adsorb to particles in water and settle to the coastal bottom (Isobe et al. 2001, 2006). Many suspected EDCs are hydrophobic substances with affinity to bind to particles and accumulate on bottom sediments (Yu et al. 2004). The EDCs in this study are hydrophobic with log octanol–water partitioning coefficient (K_{ow}) of 3–4.48 (Ahel and Giger 1993; Jurgens et al. 2002; Ternes et al. 2002). Higher EDCs concentration in sediment versus water were reported in several studies including some in Japan (Campbell et al. 2006; Hashimoto et al. 2005b; Petrovic et al. 2002; Vasquez-Duhalt et al. 2005; Zhang et al. 2009). Therefore, benthic organisms have high possibility to be exposed and accumulate EDCs from sediment especially benthic worm (polychaete) as they feed on deposits and suspensions (Fauchald and Jumars 1979). The spionid polychaete *Paraprionospio pinnata*, first reported by Okuda (1937), is frequently noted in Japanese coastal waters attaining high densities in organically polluted and/or stagnant areas (Yokoyama 1988) and are therefore good specimens for the study of bioaccumulation of estrogenic chemicals. Numerous studies of estrogenic potential of sediment have been reported (Schlenk et al. 2005; Thomas et al. 2004), but little information described on the bioaccumulation by benthic organisms (Fernandes et al. 2008). Therefore, this study aims to determine the accumulation of selected EDCs in *Paraprionospio* sp. (most dominant genus sampled) at the Yodo River mouth, Osaka Bay, one of the polluted inner bays in Japan.

MATERIALS AND METHODS

Study area

Osaka Bay (Figure 3.1) is located at the eastern end of the Seto Inland Sea which connects to the Pacific Ocean through the Kitan Strait and is semi-enclosed by Awaji Island (Tsujimoto et al. 2006). The water temperature ranges from <10 to 25°C throughout the year with highest temperature recorded in August and September. The surface water salinity fluctuates over the year (25 to 30 psu), but the salinity in the bottom water are relatively stable (Osaka Prefectural Fisheries Experimental Station 1973 – 2002). The average and maximum depths are 28 m and 197 m, respectively (Hosono et al. 2010), and 70% of the superficial sediment is composed of silty mud (Tanimoto et al. 1984).

Osaka and Kobe City along Osaka Bay have combined population of around 16 million (Hosono et al. 2010) thus large amounts of household, agricultural and industrial wastes are discharged into Osaka Bay (Tsujimoto et al. 2006). This bay became severely polluted as a result of economic growth during year 1953-1971 (Association for New Social Infrastructure of Osaka Bay 1996). The pollutants dominantly flow into bay through the Yodo River, which constitutes the major source of freshwater (estimated volume of 5.14 km^3/yr) and possibly introduced some EDCs in the area. Thus, the Yodo River mouth is a significant study area as previous studies reported relatively high concentrations of EDCs in estuary (Reddy and Brownawell 2005), inlet (Fernandes et al. 2008) and bay (Zhang et al. 2009).

Samples collection (Paraprionospio sp.)

The polychaete, *Paraprionospio* sp. was collected by members of Education and Research Center for Marine Resources and Environment, Faculty of Fisheries,

Kagoshima University which was funded by Ministry of Environment under EXTEND 2010 program. The sampling was done from April 2007 to June 2009 on a seasonal basis (except for winter) by randomly collecting the polychaetes from the Yodo River mouth area (marked by circle in Figure 3.1) using a Smith McIntyre grab. The polychaetes were collected at 5–10 sampling stations and combined to make it into two replicates per sampling. The samples were then placed on 500-μm stainless sieve and rinsed with seawater. The polychaetes were kept in a deep freezer (-75°C) until analysis. Genus identification was verified before analysis.

Sediment samples were adopted from Fisheries Research Agency, National Research Institute of Fisheries and Environment of Inland Sea, Hiroshima on April 25, 2000. The samples were collected from Osaka Bay, Osaka Prefecture several meters away from polychaetes sampling points (ST17, 18 and 21) by Smith McIntyre grab. After wet sieving with 2-mm mesh size, the samples were kept in freezer (-18°C) until analysis.

Pre-treatment of samples

After thawing, the polychaetes of each sampling were weighed into a glass bottle to record the wet weight. The polychaetes were then freeze dried at -48°C and 7.8 Pa for 4 days. Dry weights of polychaetes were recorded right after the process. Polychaete body lengths ranged from 30–55 mm and approximately 1 mm width. Total wet and dry weights of collected samples were 14.7–26.0 g and 2.70–6.03 g, respectively.

Sediment samples were treated the same way as polychaetes. However, the freeze-dried term was extended to 7 days. Wet weight and dry weight of sediments were recorded before and after freeze dried.

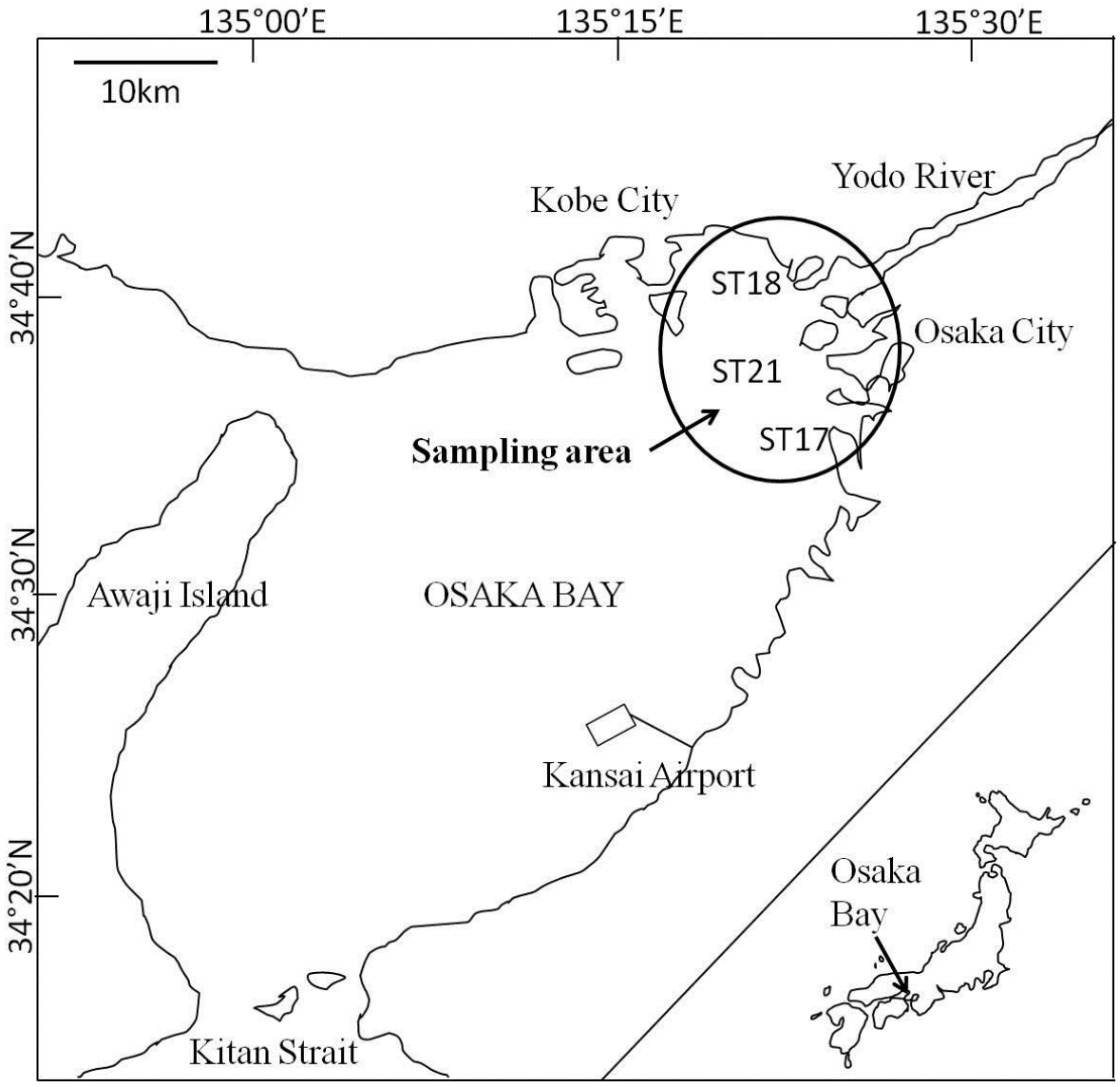


Figure 3.1 Polychaete and sediment sampling stations around Yodo river-mouth, Osaka Bay. Sediment samples were collected at ST17, ST18 and ST21 only.

Preparation of sodium acetate buffer

This buffer was used for EDCs analysis in sediment. 0.05 M sodium acetate buffer was prepared by adding 2.40 g of sodium acetate trihydrate into 300 mL mili-Q water, and 0.05 M acetic acid was prepared by mixing 0.30 g acetic acid into 100 mL mili-Q water. The pH of 0.05 M sodium acetate buffer was then adjusted to 7 by the

addition of 0.05 M acetic acid. This buffer was kept in refrigerator (18°C and below) and will last up to 3 days only.

EDCs analysis

1. Polychaete

Same as in chapter 2 – *see page* 29.

2. Sediment

Prior to analysis, *p*-n-NP-d4 was added to the sediment sample as an external standard. About 5 g of dry sediment were ultrasonically extracted twice with 20 mL of dichloromethane-methanol (3:1, v/v) for 20 min. After centrifugation at 760 x g for 10 min, the extract was concentrated to 0.5 mL by rotary evaporator and a gentle blow of N_2. Concentrated extracts were added with 4.5 mL of 0.05 M sodium acetate buffer before loading onto 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 mili-Q water in sequence. This was conducted on VacElut manifold. The target chemicals were eluted with 10 mL ethyl acetate after washing the cartridge with 4 mL 0.05 M sodium acetate buffer-methanol (9:1, v/v). The eluate was dried using N_2 gas and the residue added with 0.5 mL cyclohexane-ethyl acetate (9:1, v/v). The solution was loaded to Sep-pak silica cartridge (Waters Co., Massachusetts, USA) on VacElut manifold, which was 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 E2-16,16,17-d3, as internal standard was added to the eluate

followed by drying under gentle blow of N_2 gas. Finally, 200 μL acetonitrile was added to the dried residue.

External standard in this study is meant to check the accuracy of procedure and internal standard was added in order to correct for the loss of analyte during samples preparation.

Measurement using LC-MS/MS-ESI

Same as in chapter 2 – *see page* 30. The detection limit for each EDC is shown in Table 3.1.

Quality assurance

Same as in chapter 2 (Detection limit and recovery test) – *see page* 31.

Table 3.1 Precursor and daughter ions of EDCs and limit of detection by LC-MS/MS-ESI

EDCs	Precursor ions, m/z (amu)	Daughter ions, m/z (amu)	Limit of detection (ng/g)[a]
NP	219.0	132.9	0.3
OP	205.0	132.9	1.0
BP	227.1	211.8	0.4
E1	269.1	145.0	0.01
E2	271.1	144.9	0.05

[a]Detection limit using 0.6 g freeze-dried sample

RESULTS AND DISCUSSION

EDCs concentration in polychaete, Paraprionospio sp.

The concentrations of each EDC in *Paraprionospio* sp. (Figure 3.2) were at minimum of 1,460 and maximum of 4,410 ng/g w.w. (27,200–64,800 ng/g dry weight, d.w.) for NP; 18.9 and 45.4 ng/g w.w. (412–667 ng/g d.w.) for OP; 22.5 and 39.6 ng/g

w.w. (432–624 ng/g d.w.) for BP; 0.06 and 2.50 ng/g w.w. (1.0–8.0 ng/g d.w.) for E1 and 0.89 and 4.35 ng/g w.w. (13.0–48.0 ng/g d.w.) for E2. *Paraprionospio* sp. has accumulated NP in levels that were 100 times greater than the other four EDCs; while E1 and E2 were the least detected. In the previous study, Nurulnadia et al. (2013) did not detect natural estrogens (E1 and E2) in *Perinereis nuntia*, a kind of polychaete, suggesting that *Paraprionospio* sp. had accumulated both natural and synthetic estrogens (NP, OP and BP) from the sediment.

Seasonal changes of EDCs in Paraprionospio sp.

Paraprionospio sp. had accumulated highest NP and OP concentrations during July 2008 with 4,410 (64,800 ng/g d.w.) and 45.4 ng/g w.w. (667 ng/g d.w.), respectively. However, concentrations of BP, E1 and E2 in *Paraprionospio* sp. were not much changed within 3-year period of sampling. Isobe et al. (2001) also observed the highest concentration of alkylphenol compounds (i.e., NP and OP) in Tokyo Bay sediment between June to August (summer). As described by Renner (1997), alkyphenol polyethoxylates (APnEO) consist of 80% of NP polyethoxylates (NPnEO) and 20% of OP polyethoxylates (OPnEO). During high water temperatures in summer, significant bacterial production could be promoted in marine habitats (Hubas et al. 2007; Tanghe et al. 1998), which then facilitate greater degradation of APnEO to NP and OP in the benthic environment. The formation of shorter chain from EO degradation was also reported by Maruyama et al. (2000) to occur in Tokyo rivers during summer. Thus, high bacterial activities during summer probably contribute to higher concentration of alkylphenol (NP and OP) compounds in the sediment compared to other season which were then accumulated by *Paraprionospio* sp.

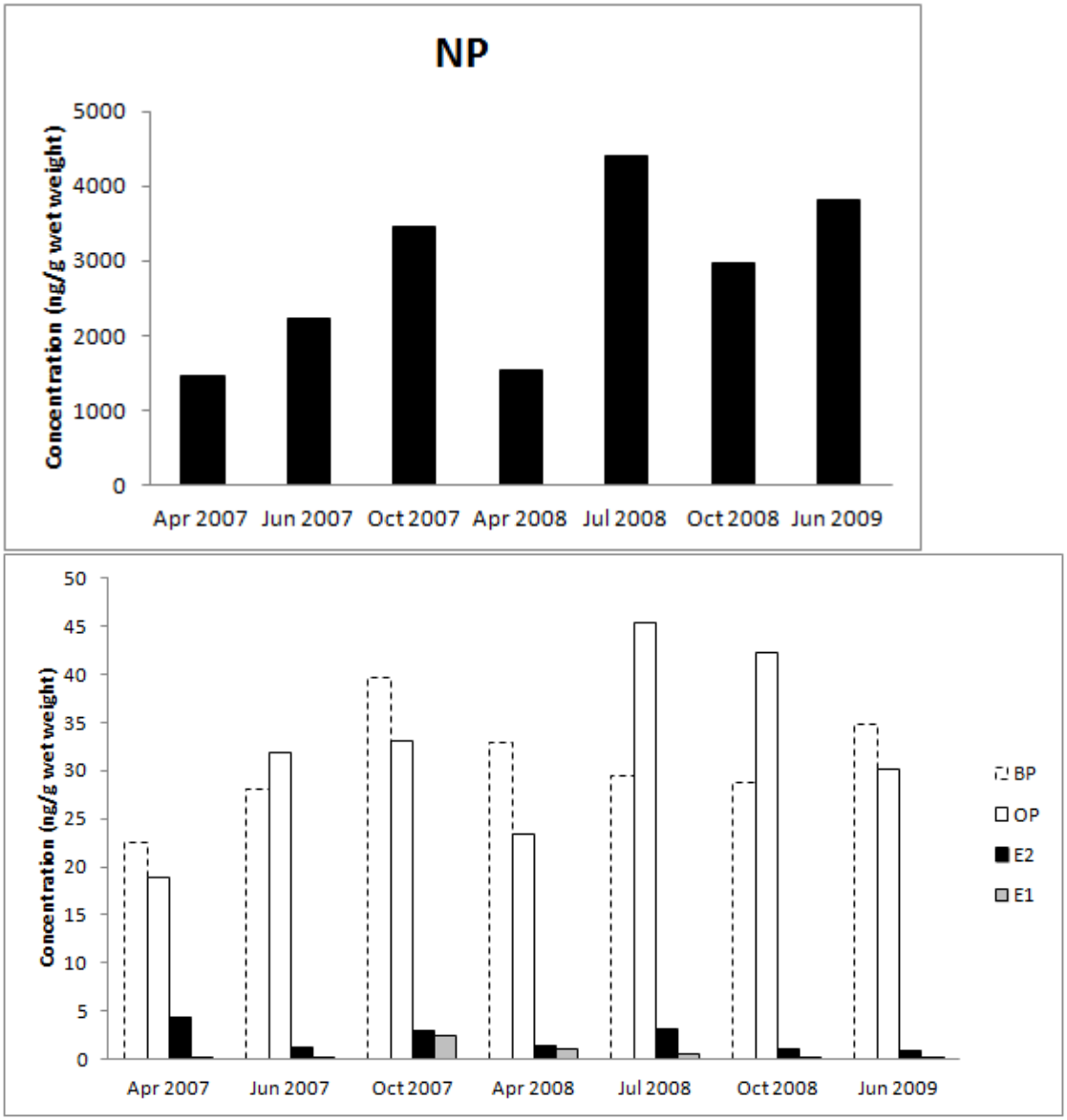


Figure 3.2 Mean concentrations of NP, OP, BP, E1 and E2 (ng/g wet weight) in *Paraprionospio* sp. (n=2)

Besides summer season, considerably high values of NP and OP in *Paraprionospio* sp. were observed in October. During month of October, the water temperature in Osaka Bay was reported to be similar to summer season (almost 25°C) (Tsujimoto et al. 2006; Yanagi 1987); thus *Paraprionospio* sp. still actively feed on particles (Yokoyama 1988), which could lead to high accumulation of NP and OP. The solubility of organic chemicals also affected by high water temperature and causes the

compound to move from sediment to pore water (Holmer and Kristensen 1996; Socha and Carpenter 1987; Woodburn et al. 1989). As sediment dwelling organisms, *Paraprionospio* sp. might have accumulated EDCs from the pore water. However, the concentration of BP, E1 and E2 were not affected by the seasonal changes as verified by small concentration ranges measured in this study.

EDCs concentration in other invertebrates

Although invertebrates represent 95% of the known species in the animal kingdom (deFur et al. 1999; Wilson 1999), only few studies concerning EDCs accumulation were reported in polychaetes. Comparison of EDC concentrations in various invertebrates can be done on three EDCs, which studied by several authors as shown in Table 3.2. Ferrara et al. (2008) and Zhang et al. (2011) reported high concentrations of NP in spottail mantis shrimp (7–1,220 ng/g w.w.) and short-necked clam (2,725 ng/g lipid weight), respectively. However, the highest concentration was observed in *Paraprionospio* sp. (1,460–4,410 ng/g w.w.) in the present study. Hence, as benthic organism, *Paraprionospio* sp. was probably polluted by high concentrations of NP from the sediment.

Apparently, OP values in invertebrates in previous reports (Ferrara et al. 2001, 2005, 2008) were very low, ranging from 0.80–18.6 ng/g w.w. However, the concentrations found in prawn, crab and white clam at 20.2, 20.4 and 44.9 ng/g w.w. (Basheer et al. 2004), respectively, were comparable to those of *Paraprionospio* sp. in this study with 18.9–45.4 ng/g w.w.

High concentrations of BP are shown in crab (213 ng/g w.w.) and squid (119 ng/g w.w.) (Table 3.2), which were sampled from the local supermarket in Singapore (Basheer et al. 2004). In the present study, BP values ranged from 22.5–39.6 ng/g w.w

lower than those levels by studies on cockle (56.5 ng/g w.w.) and white clam (27.4 ng/g w.w.) (Basheer et al. 2004). Despite the low concentration of BP noted in *Paraprionospio* sp., the data is a contribution to the sparse report on BP concentrations in invertebrates.

Studies on the natural estrogens of invertebrates are scarce. In the previous experiment, we have exposed polychaete, *Perineries nuntia* to both E1 and E2 through dietary exposure for 14 days. We observed that E1 was not detected, but *P. nuntia* had accumulated E2 in the concentration of 0.2–1.18 ng/g w.w., lower than concentration levels measured in the spiked diet (Nurulnadia et al. 2013). The value of E2 in the previous experiment was comparable to *Paraprionospio* sp. of Osaka Bay, which was detected from 0.89–4.35 ng/g w.w.

Table 3.2 shows that NP concentrations in those invertebrates were higher than the levels of other EDCs. Therefore, NP, the estrogenic EDC is well known to be ubiquitous in the environment. The production of APnEO in Japan was reported to be 50 thousand tons in 1997 (Nakamura 1998), which implies the massive use and discharge of these chemicals into the environment. The major usage also occurred in other countries such as China (Zhang et al. 2009) and Italy (Ferrara et al. 2005). This fact clarifies the reason of high levels of NP detected in this study compared to other EDCs.

Table 3.2 NP, OP and BP concentrations in various invertebrates

Invertebrate	EDCs concentration (ng/g)			Study area	Reference
	NP	OP	BP		
Squids[a]	389 – 696	3.9 – 18.6	n.a	Adriatic Sea, Italy	Ferrara et al. 2001
Cuttlefishes[a]	67 – 566	3.6 – 3.8	n.a		
Mussels[a]	254 – 265	4.4 – 4.9	n.a		
Clams[a]	243 – 252	2.7 – 2.8	n.a		
Spottail mantis shrimp[a]	118 – 254	2.7 – 3.4	n.a	Adriatic Sea, Italy	Ferrara et al. 2005
Norway lobster[a]	274 – 399	3.6 – 4.7	n.a		
Octopus[a]	6 – 58	0.80 – 14.4	n.a	Tyrrhenian Sea, Italy	Ferrara et al. 2008
Spottail mantis shrimp[a]	7 – 1220	1.4 – 15.7	n.a		
Crab[a]	103	20.2	213	Local supermarket, Singapore	Basheer et al. 2004
Squid[a]	64.8	10.2	119		
Prawn[a]	197	20.4	13.3		
Cockle[a]	54	6.7	56.5		
White clam[a]	46.6	44.9	27.4		
Bay scallop[b]	156	n.a	n.a	Bohai Bay, China	Hu et al. 2005
Crab[b]	154	n.a	n.a		
Burrowing shrimp[b]	174	n.a	n.a		
Short-necked clam[b]	170	n.a	n.a		
Veined rapa whelk[b]	143	n.a	n.a		
Short-necked clam[c]	2725	272	181	Yundang Lagoon, China	Zhang et al. 2011
Polychaete[a] (*Paraprionospio* sp.)	**1460 – 4410**	**18.9 – 45.4**	**22.5 – 39.6**	**Osaka Bay, Japan**	**Present study**

[a]wet weight; [b]lipid equivalent; [c]lipid weight; n.a – Not available

Table 3.3 EDCs concentration in sediment (ng/g w.w.) collected around Yodo River-mouth

Sampling station	NP	OP	BP	E1	E2
ST17	36.5	5.36	1.23	0.08	n.d (<0.05)
ST18	28.3	2.72	2.64	0.16	n.d
ST21	11.3	2.77	0.98	0.03	0.10

n.d – Not detected

Table 3.4 EDCs concentrations in sediment samples collected in Japan

Study area	Concentration in sediment (ng/g d.w.)					Reference
	NP	OP	BP	E1	E2	
Tokyo Bay	2.2 – 4560	n.a	0.11 – 48.0	<0.1 – 10.3	<0.1 – 4.8	Hashimoto et al. 2005b
	30 – 13000	3 – 670	n.a	n.a	n.a	Isobe et al. 2001
	n.a	n.a	n.a	0.05 – 3.60	n.d – 0.59	Isobe et al. 2006
Ishigaki Island	23 – 46	n.a	1.4 – 2.7	n.a	n.a	Kawahata et al. 2004
Okinawa Island	12 – 44	n.a	2.3 – 11	n.a	n.a	
Osaka Bay	220 - 680	10 – 41	<2.12	n.a	<0.01 – 0.04	Japanese Ministry of Environment, 2002
	n.a	n.a	n.a	0.10 – 1.30	0.17 – 2.33	Matsuoka et al. 2005
	9.80 - 119	0.36 – 15.6	<0.50 – 7.10	<0.10 – 0.90	<0.10 – 1.57	Koyama et al. 2013
Osaka Bay	**19.6 – 80.5**	**4.8 – 11.8**	**1.7 – 2.7**	**0.05 – 0.44**	**0.18**	**Present study**

n.a – Not available; n.d – Not detected

Table 3.5 EDCs concentrations in water samples collected in Japan

Study area	Concentration in water (μg/L)					Reference
	NP	OP	BP	E1	E2	
Tokyo Bay	0.03 – 0.10	n.a	0.02 – 0.03	0.0004 – 0.002	0.01 – 0.03	Hashimoto et al. 2005b
	0.05 – 1.08	0.01 – 0.18	n.a	n.a	n.a	Isobe et al. 2001
Ishigaki Island	n.d – 0.12	n.a	n.d – 0.08	n.a	n.a	Kawahata et al. 2004
Okinawa Island	n.d – 0.17	n.a	n.d – 0.06	n.a	n.a	
Osaka Bay	0.3 – 1.8	n.a	n.a	n.a	n.a	Sakai city, 2001

n.a – Not available; n.d – Not detected

Table 3.6 EDCs concentration in sediment and invertebrates

	Present study		Zhang et a. 2011	
EDCs	Sediment, ng/g w.w.	*Paraprionospio* sp., ng/g w.w.	Sediment, ng/g	Short-necked clam, ng/g
NP	11.3 – 36.5	1460 – 4410	1970 d.w. (906 w.w.)	2725 lipid wg. (13.9 w.w.)
OP	2.72 – 5.36	18.9 – 45.4	27.2 d.w. (12.5 w.w.)	272 lipid wg. (1.39 w.w)
BP	0.98 – 2.64	22.5 – 39.6	50.9 d.w. (23.4 w.w.)	181 lipid wg. (0.92 w.w.)
E1	0.03 – 0.16	0.06 – 2.50	11.2 d.w. (0.46 w.w.)	3.14 lipid wg. (0.02 w.w.)
E2	n.d – 0.10	0.89 – 4.35	3.21 d.w. (1.48 w.w.)	3.62 lipid wg. (0.02 w.w)

n.d – Not detected

EDCs concentration in sediment

EDCs concentration in the sediment of present study is shown in Table 3.3. The moisture content was computed to be within 42.4–63.3%. Among previous studies conducted at Osaka Bay, EDCs level in the sediment of present study were comparable to Koyama et al. (2013) and Matsuoka et al. (2005). However, NP and OP values were observed to be lower than the concentrations reported by Japanese Ministry of Environment (2002) as shown in Table 3.4.

Compared to other study area, EDCs concentration in this study were lower than those from Tokyo Bay (Hashimoto et al. 2005b; Isobe et al. 2001, 2006). However, study in Tokyo Bay has quantified lower EDCs values in water samples compared to sediment (Table 3.4 and 3.5). Similar findings were reported by other authors (Campbell et al. 2006; Kawahata et al. 2004; Zhang et al. 2009). Thus, polychaetes as deposit and suspended particles feeder can be assumed to have most probably accumulated EDCs from the sediment.

Comparison of EDCs concentrations in sediment and invertebrates

Besides EDCs concentration in short-necked clam, Zhang et al. (2011) also reported on the concentration levels in sediment. In order to make a comparison with the present study, concentrations in both short-necked clam and sediment were estimated to wet weight. EDC concentration in short-necked clam was converted to wet weight by considering the lipid percentage and the moisture content. The lipid percentage of short-necked clam was reported by Li et al. (2010), which was sampled at the same area with 3.4%. The percentage of moisture content in short-necked clam was reported by 85% by Wan et al. (2007, 2008).

The EDCs concentration in sediment was estimated to wet weight by considering the moisture percentage. Previous studies reported the moisture percentage of 49–59% (Liu et al. 2010) in an estuary of China and between 56 and 58% (Tanner and Leong 1997) in a bay of Hong Kong. Thus, median percentage of 54% was used in estimation.

The estimated wet weight values of EDCs in sediment and short-necked clam is shown in Table 3.6. Zhang et al. (2011) have concluded the study area as polluted by estrogenic compounds with EDCs concentrations in short-necked clam to be at lower levels than those in sediment, contrary to the finding in this study where higher concentration was measured in *Paraprionospio* sp. This suggests that *Paraprionospio* sp. could be a good bioindicator species for bioavailable EDCs in sediment and since it is fish prey (Livingston 1987; Labropoulou and Eleftheriou 1997), it could possibly transport EDCs from sediment into the food chain.

CONCLUSION

Published studies show NP as the ubiquitous EDC in the environment that should be continuously assessed in benthic invertebrates. Due to the hydrophobic characteristic of EDCs, they tend to bind to sediment and are most probably adsorbed through feeding by benthic invertebrate as shown in *Paraprionospio* sp. Furthermore, *Paraprionospio* sp. had accumulated higher concentrations of EDCs than found in sediment, indicating the ability of the polychaete to biomagnify these chemicals. Thus, biomagnification of EDCs through trophic transfer into fish should be investigated further.

CHAPTER 4

Bioaccumulation of dietary endocrine disrupting chemicals (EDCs) by the benthic fish, *Pleuronectes yokohamae*

ABSTRACT

In the previous chapter, *Paraprionospio* sp. (polychaete) in sediment of Osaka Bay was found to bioaccumulate relatively high concentration of EDCs. Since polychaete serves as food for benthivorous fish, it might transfers the sediment borne chemicals to higher trophic levels. Thus, this study aims to clarify the biomagnifications of EDCs through dietary route. *P. yokohamae*, a benthivorous fish, was exposed to the individual EDC spiked diet for 14 days. EDCs concentration in *P. yokohamae* had achieved the steady state at day 7 with BMFs of NP and OP ranges from 0.001–0.014 and 0.005–0.089, respectively. Three concentration groups were subjected but BMF values for all groups were below than 1 suggesting no biomagnifications. Thus, different concentration does not affect the BMF. BP, E1 and E2 peaks were not appeared after LC-MS/MS measurement and are speculate to be metabolized and/or not to be absorbed. Vitellogenin analysis also showed no induction due to dietary exposure.

INTRODUCTION

Endocrine disruption may be caused by elevated levels of natural and synthetic chemicals by the ability of compounds to mimic or antagonize the effects of endogenous hormones (through disruption of their synthesis and metabolism). Anthropogenic release of endocrine disrupting chemicals (EDCs) is a widespread concern. These chemicals may enter the aquatic environment via effluents of sewage

treatment plants, industry and agricultural land (Langston et al. 2005). Both field and laboratory studies provided abundant evidence that exposure to these chemicals can lead to abnormal modulation or disruption of development and reproduction in aquatic wildlife, especially fish (Routledge and Sumpter 1996; Harries et al. 1997; Jobling et al. 1998; Van den Belt et al. 2003).

Upon entering the water column, EDCs was demonstrated to be partitioned (Lai et al. 2000) which could lead to bioaccumulation, whether via dietary (food-borne accumulation/biomagnifications) and respiratory (bioconcentration). Study on the bioaccumulation of EDCs is crucial to determine the actual impact of EDCs in target organisms and to predict the potential for biomagnifications of these compounds through the food chain (Kristensen and Tyle 1990; Wang et al. 1996; Lai et al. 2000). Based on our previous study (Nurulnadia et al. 2014), EDCs concentration in the polychaete, *Paraprionospio* sp. was found to be higher than in the sediment. So, it is expected that polychaete had bioaccumulated those compounds from the sediment through dietary uptake. It becomes a great concern that these compounds could probably be transferred into benthic fish through the food chain. In order to verify this assumption, *Pleuronectes yokohamae* was chosen as the test fish.

P. yokohamae occurs in Japan 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. This species inhabits sandy-mud bottoms less than 100m depth (Sakamoto 1984) and commercially important (Park 1988). The spawning season of flounder in Japan occurs during winter. Both male and female reach maturity at an age of 1 year (Park and Simizu 1990). *P. yokohamae* prey mainly on benthic animals is known to accumulate organic compounds such as tributyltin (TBT) (Takayama et al. 1995), DDE, HCB and PCBs (Kammann et al. 1993; Loizeau and Menesguen 1993) in the liver.

Thus, *P. yokohamae* provides a good indicator of EDCs accumulation. This study aims to determine the biomagnifications factors of EDCs in *P. yokohamae* through dietary exposure.

MATERIALS AND METHODS

Test organism (P. yokohamae)

P. yokohamae were purchased from Regional Government Aquaculture Center in Yamaguchi, Japan. They were acclimatized in the laboratory condition for six months to the desired body size and fed with commercial diet. *P. yokohamae* have been kept in the water temperature below 25°C as it will die in warm water.

Preparation of EDCs spiked diet

In this experiment, EDC standards were prepared by the individual compound and test group as in Table 4.1. These EDCs were dissolved in diethyl ether and spiked to the 100 g of commercial diet in stainless tray to aid drying process. Spiking was done by pasteur pipette while mixing well the solvent and diet. The spiked diet was left overnight prior to use with gentle mixing for every 3-4 hours.

Table 4.1 Nominal concentrations of individual EDC spiked onto the diet

EDC	Concentration (ng/g)		
	Low	Medium	High
NP	1,000	5,000	25,000
OP	8	40	200
BP	8	40	200
E1	2	10	50
E2	0.8	4	20

Preparation of 0.02 M Tris-HCl, pH 8.0, containing 2% NaCl & 0.1% NaN_3 and 1000 KIU aprotinin/mL Tris-HCl

This buffer was prepared as the working solution for aprotinin, the protein inhibitor. The solution contains 0.61 g tris(2-amino-2hydroxymethyl)-1,3-propanediol, 5.0 g NaCl and 0.25 g NaN_3 which was dissolved in 250 mL mili-Q water. The pH of buffer was adjusted to 8 by hydrochloric acid (precaution: drop by drop while mixing).

1000 KIU aprotinin/mL Tris-HCl was added in the blood sample in order to inhibit enzyme activities which degrade the protein. To prepare this solution, 3 mg of aprotinin was dissolved into 20 mL 0.02 M Tris-HCl, pH 8.0 containing 2% NaCl and 0.1% NaN_3.

Exposure scheme

Sixteen tanks of 35 x 48 x 18 cm were prepared for the exposure test of individual EDC (Figure 4.1). Twenty-four of *P. yokohamae* were placed in each tank including the control and maintained in a water-flow-through system. Test water was completely replaced in 30 L/60 times/day with natural sand-filtered seawater and aerated well. At day 0, three of *P. yokohamae* were sampled for the initial data.

Figure 4.1 Tanks set-up for individual EDC exposure

P. yokohamae were exposed to the individual EDC by dietary route using commercial fish diet (Nippon Formula Feed Manufacturing Co., Japan) spiked with individual EDC dissolved in diethyl ether. *P. yokohamae* were fed twice daily with a total feeding rate of 2% of their body weight. The exposure groups were fed with EDCs spiked fish diet for 14 days. The sampling was done at day 0, 2, 4, 7 and 14. The average body weight of individual *P. yokohamae* at the initial of exposure was 2.95 g and 3.57 g, for control and exposure groups, respectively. Non-consumed food residues were removed daily. During sampling, *P. yokohamae* were anaesthetized by 0.05% 2-phenoxyethanol. After measuring the body length and weight, blood samples were collected from caudal vessels. 5 µL of aprotinin was added into each 0.2 mL volume of blood sample and allowed to clot at 4°C for several hours. The serum was then separated by centrifugation at 1500 x g for 15 min and stored at -80°C for experimental use. The fishes were kept in freezer (-18°C) until analysis. Water parameters such temperature, dissolved oxygen, pH and salinity were measured periodically.

Pre-treatment of samples

After thawing, each benthic fish was weighed into a glass bottle to record the wet weight. The fishes were then freeze dried at -48°C and 7.8 Pa for 7 days. Dry weights of fishes were recorded right after the process.

EDCs analysis

1. Benthic fish and Diet

The freeze-dried benthic fish (weights of homogenate sample ranged from 1.30–4.10 g) and diet samples were analyzed the same way as in chapter 2 – *see page* 29.

2. Seawater

Same as in chapter 2 – *see page* 30.

Measurement using LC-MS/MS-ESI

Same as in chapter 2 – *see page* 30.

Table 4.2 Precursor and daughter ions of EDCs and limit of detection by LC-MS/MS-ESI

EDCs	Precursor ions, m/z (amu)	Daughter ions, m/z (amu)	Limit of detection (ng/g)[a]	Recovery (%)
NP	219.0	132.9	3.24	25.0
OP	205.0	132.9	1.53	40.3
BP	227.1	211.8	1.76	92.7
E1	269.1	145.0	1.67	102
E2	271.1	144.9	0.36	92.0

[a]Detection limit using 0.3 g diet sample

Detection limit and recovery test

The detection limit was determined as the minimum concentrations corresponding to those of signal to noise ratio of 3 on the chromatogram of actual sample. The ratio between intensity of signal of each compound was obtained under selected ion monitoring (precursor and daughter ions, m/z) conditions and intensity of noise. The limit of detection and recovery percentage of each EDC is shown in Table 4.2.

The recovery test was performed using fish diet spiked with 100 ng/g of each EDC standards and internal standard (E2-16,16,17-d3). The EDCs standard extraction and measurement was done as above procedures. Mean percent recovery, as determined from an estimate of the amount of added and recovered, were between 25.0% and 102%. Analysis was conducted in duplicate (n = 2).

Data analysis

In this experiment, BMFs were calculated as the ratio of concentration in organism to diet at steady state (Kobayashi et al. 2011) as follows: BMF = (EDCs concentration in *P. yokohamae* at steady state)/(EDCs concentration in diet).

Vitellogenin (Vg) analysis

Vitellogenin was analyzed by our collaborator, Dr. Amano as described in Amano et al. (2007). Vitellogenin data was used in this study with her permission.

RESULTS AND DISCUSSION

Water parameters and control diet

Mean water temperature, pH, salinity and dissolve oxygen were 26.7±09°C, 8.28±0.1, 28.0±0.2 and 6.67±0.4 mg/L, respectively. None of EDCs were detected in the water sample. The initial concentrations of each EDC in the spiked diet are given in Table 4.3. NP, OP and BP levels in the control diet were 28.3 ng/g, 3.02 ng/g and 2.68 ng/g, respectively. Pellet of commercial diet generally consist of 36–52% fish meal from wild catches (Olsen 2011). Thus, it was expected that low concentration of EDCs would be detected in the control diet.

Table 4.3 Mean EDCs concentration, ng/g dry weight, in control and spiked diet, n=2

EDCs	Control	Low	Medium	High
NP	28.3	652	2870	14,000
OP	3.02	3.20	16.3	153
BP	2.68	5.00	34.8	128
E1	n.d	1.70	4.85	35.2
E2	n.d	n.d	3.59	12.3

n.d – Not detected

NP and OP concentrations in P. yokohamae

In this study, the moisture content of *P. yokohamae* was calculated in the range of 65.5–73.4%. The concentration of NP and OP in *P. yokohamae* is shown in Figure 4.2. However, BP, E1 and E2 were not detected. These compounds were probably degraded and biotransformed into polar form to facilitate elimination (Marx 1974; Klaassen 2001). In fish most of the organic xenobiotics biotransformed into glucuronide conjugation (Melancon and Lech 1976; Varanasi and Gmur 1981). As biotransformation of EDCs is one of the prime factors determining their distribution in fish (Lech 1974), the presence of polar compound would prevent the bioaccumulation

(van den Berg et al. 2003). This point will be investigated further in the next experiment.

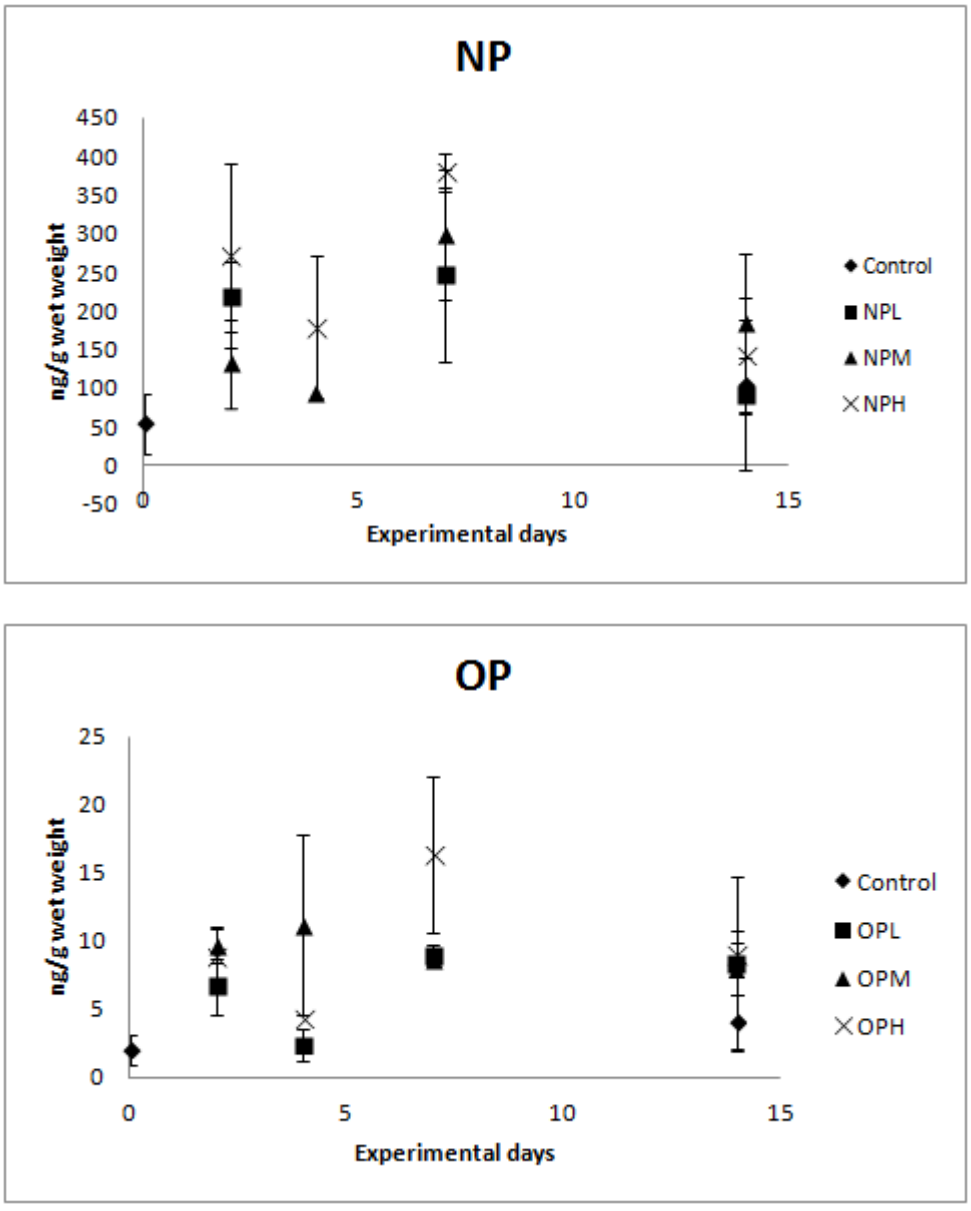


Figure 4.2 Accumulation of NP and OP (ng/g wet weight) in *P. yokohamae* in control, medium, low and high exposure groups. Error bars indicate the standard deviation (n=3).

On day 0, NP and OP were detected in *P. yokohamae* at 53.6 ng/g w.w. and 2.05 ng/g w.w. As expected, NP and OP were detected in control fish as *P. yokohamae* accumulate very low concentration of respective compound from the control diet during the acclimatization period. The concentration of NP and OP increased rapidly from day 0 and the highest concentration was achieved on day 7 for most of the exposure groups (Figure 4.2). The maximum concentrations of NP in low, medium and high exposure groups were 248 ng/g w.w., 299 ng/g w.w., and 380 ng/g w.w. respectively, whereas maximum concentration of OP in low, medium and high exposure groups were 9.02 ng/g w.w., 11.3 ng/g w.w., and 16.3 ng/g w.w., respectively. Thus, day 7 was decided as the point which steady state was reached for both NP and OP and these concentrations were used for the BMF computation.

Biomagnification factor (BMF)

BMFs were calculated from the concentration of NP and OP at day 7 of each group relative to measured concentration of the respective compound in the spiked diet (Table 4.4). BMF values of both EDCs in each group were less than 1 suggesting that these compounds were not biomagnified by *P. yokohamae* from the diet (Berntssen et al. 2007). Nonetheless, NP and OP were assimilated by *P. yokohamae* as showed by the increasing concentrations during the exposure experiment (Figure 4.2). *P. yokohamae* was subjected to three concentration groups of EDCs but BMF values do not seem to affect by the differences.

In a food web analyses conducted in Tokyo Bay, BMF of 0.196 and 0.277 have been measured for respective NP and OP (Takeuchi et al. 2009) by the ratio of predator to prey. Extensive study of NP biomagnifications was also determined based on specific tissue such as liver by Diehl et al. (2012) in Table 4.5. The most related BMF

value to be compared to this study is the goby liver (predator) and benthic invertebrates (prey) with BMF of 0.3, slightly higher than 0.007 of the present study. The reason could be NP tends to accumulate in fish liver compared to homogenate fish tissues of the present study (Shao et al. 2005; Tsuda et al. 2000b).

BMF values of NP and OP of previous studies shown in Table 4.5 were below than 1, indicate that biomagnifications does not occur. However, five of the BMF values were greater than 1. These values were reported from field studies by comparing the concentrations in predator to prey. Considering BMF computed from field sample, the values could be attributed from bioconcentration (water) and biomagnifications (prey). Therefore, the apparent BMF values of NP and OP become greater than 1. This could be the reason of low BMF values computed in this study since dietary is the only route of exposure.

In a study conducted using algae exposed to NP as the diet, they found that biomagnification does not occurred in the fish despite the elevated concentration measured in the diet (Ahel et al. 1993). This finding was confirmed by Hu et al. (2005) after computed the BMF from the herring gull and various fish. Flounder (*Platichthys flesus*) had been feed orally to high concentration (10 to 100 mg/kg) of OP but the highest accumulation recorded was 9% (Madsen et al. 2003). In another experiment, only 0.6% of OP was measured from 1.0 mg OP/kg feed to *P. flesus*, similar to the present study that only 0.1% of OP concentration measured in *P. yokohame* after feed with 0.15mg OP/kg. This would suggest that regardless any levels of concentration of NP or OP exposed to the fish, the accumulation percentage was small. This finding was also observed in rainbow trout (Pedersen et al. 2003).

Table 4.4 BMF in low, medium and high exposure groups

EDCs	Low	Medium	High
NP	0.014	0.005	0.001
OP	0.089	0.021	0.005

Table 4.5 BMF of NP and OP measured from ratio of predator to prey

Predator	Prey	BMF		Location	Reference
		NP	OP		
Food web analysis		0.196 [a]	0.277 [a]	Tokyo Bay, Japan	Takeuchi et al. 2009
Ghost shrimp	Benthic invertebrates	0.2[b]	n.a	California estuaries, USA	Diehl et al. 2012
Goby liver	Benthic invertebrates	0.3[b]	n.a	California estuaries, USA	
Sculpin liver	Goby liver	2.7[b]	n.a	California estuaries, USA	
Seabird liver	Sanddab or sculpin liver	0.1[b]	n.a	California estuaries, USA	
Seabird liver	Goby liver	0.3[b]	n.a	California estuaries, USA	
Porpoise liver	Sanddab or sculpin liver	0.1[b]	n.a	California estuaries, USA	
Snail	Oyster	1.3[c]	2.0[c]	West coast of Taiwan	Cheng et al. 2006
Herring gull	Various fish	1.02[d]	n.a	Bohai Bay, China	Hu et al. 2005
Not mention		n.a	1	Reagent properties	Neamtu et al. 2009
Polychaete	**Diet**	**0.001[e]**	**0.005[e]**	**Exposure test**	**Nurulnadia et al. 2013**
P. yokohamae	**Diet**	**0.007[e]**	**0.038[e]**	**Exposure test**	**This study**

n.a – Not available; [a]stable isotope $\delta^{15}N$; [b]lipid weight; [c]dry weight; [d]lipid weight; [e]wet weight

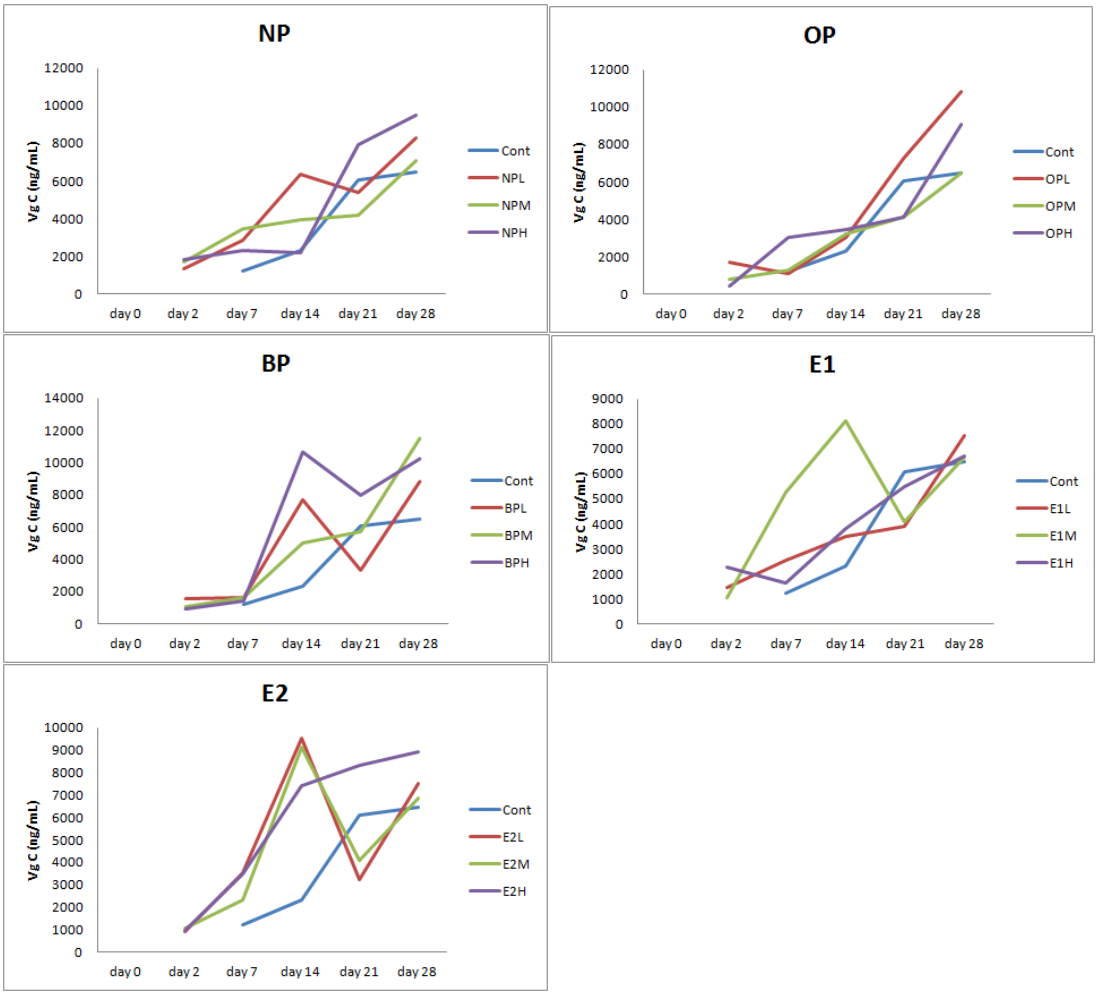


Figure 4.3 Induction of vitellogenin C in *P. yokohamae* at day 0, 2, 7, 14, 21 and 28 in control and exposed groups

Induction of vitellogenin (Vg) is another important biomarker which was analyzed in this study by our collaborator. The following characteristics make Vg a functional biomarker for exposure of fishes to EDCs in aquatic environments (Palmer and Selcer 1996): (1) induction of vitellogenesis is a specific physiological response of fishes to estrogen or estrogenic chemicals; (2) Vg appears naturally in maturing females but not in males or immature fish, although vitellogenesis is induces in males and juveniles exposed to estrogen or estrogenic EDCs. As described in Hiramatsu et al. (2002, 2005), members of advanced teleost taxa generally express three types of Vg

which are VgA, B and C. VgA and B consist of a complete suite of yolk protein (lipovitellin, phosvitin, β'-component) domains, whereas VgC is distinctive in that it lacks phosvitin and β'-component domains and low molecular weight (Hiramatsu et al. 2005, 2006).

In the present study, extremely high induction of VgC was measured compared to A and B. The measurement was done starting from day 0 to day 28 of experimental period as shown in Figure 4.3. The results described VgC induction (ng/mL) in *P. yokohamae* in the control and exposed fishes. Generally, the amount of VgC induction was increased until the end of experiment in both control and exposed fish. Thus, induction of VgC probably occurred due to natural factors of exposure scheme rather than affected by EDCs. In addition, induction of VgC does not fulfill the concentration–response trend regardless of three concentration groups applied. So, this study demonstrated no induction of Vg through dietary exposure of EDCs to *P. yokohamae*, hence verified low biomagnification values of each compound and/or low potential of EDCs biomaginification due to possible metabolism. However, VgC has been proven as a good bioindicator for natural and estrogenic chemicals in *P. yokohamae* through waterborne exposure (unpublished result).

At the end of exposure, NP and OP concentrations in *P. yokohamae* in exposure groups are higher than control. So, although low concentration of administered dose retained in the homogenate tissues, both compounds were absorbed by *P. yokohame* (Figure 4.2). There is possibility that EDCs were assimilated by *P. yokohamae* but the compound had been biotransformed and excreted as metabolite. Before conducting the identification of EDCs metabolite, we will investigate the assimilation rate of respective compound by *P. yokohamae* as suggested by Wan et al. (2007). Pedersen et al. (2003) had analyzed OP concentration in the fish feces in order to explain the

assimilation efficiencies by comparing the concentration in feces to the diet. This is a good measure to indicate the assimilation of organic compounds in aquatic organisms such fish. This point will be examined in the future experiment.

CONCLUSION

In this study, five EDCs were spiked to the diet but only NP and OP detected in the homogenate tissues of *P. yokohamae*. BP, E1 and E2 most probably were biotransformed and degraded into a polar form to ease the excretion process but further analysis will be done in order to verify this speculation. BMF values of NP and OP were very low compared to other studies and the results were verified by no induction of Vg. However, the concentrations of both compounds were higher compared to control. Thus, an assimilation efficiencies experiment must be carried out in order to determine the assimilation rate of respective EDC in the *P. yokohamae*.

CHAPTER 5

Assimilation efficiencies of dietary endocrine disrupting chemicals (EDCs) by the benthic fish, *Pleuronectes yokohamae*

ABSTRACT

To verify the low bioaccumulation values in the previous exposure experiments, this study was performed to determine the assimilation efficiencies of target EDCs (NP, OP, BP, E1 and E2) in *P. yokohamae*. By introducing the inert marker of Cr(III) to diet with EDCs, the assimilation efficiencies was computed by the absorbance of Cr (VI) in diet and feces to the concentration of EDCs in diet and feces. The assimilation percentages of each EDC were 49.6 for NP, 87.9 for OP, 93.9 for BP, 92.4 for E1 and 96.4 for E2. This result indicates that the target EDCs was assimilated efficiently in *P. yokohamae* except for NP. Thus, low bioaccumulation of EDCs in the previous experiment was probably due to intensive metabolism which could not be recognized during the LC-MS/MS measurement. The metabolism of EDCs in *P. yokohamae* would be the objective to be achieved in the next experiment.

INTRODUCTION

Assimilation efficiency (AE) is one of the important parameter to determine the bioaccumulation/biomagnifications of chemicals from dietary exposure (Landrum et al. 1992; Wang and Fisher 1999). AE is the fraction of ingested compounds that is incorporated into biological tissues (Jones et al. 2001; Olsen and Olsen 2008), whereas absorption efficiency is the fraction of material that is taken up across the gut lining (Penry 1998). Thus, assimilation is equal to absorption minus excretion in the digestive

tract (feces). Both AE and absorption efficiency terms have been used interchangeably in toxicological studies (Wang and Fisher 1999).

The dietary exposure of EDCs to *Pleuronectes yokohamae* in the previous chapter demonstrated that these compounds were not bioaccumulated but seems to be transferred. The possible reasons for no bioaccumulation could be: (1) EDCs were not taken up/assimilated in *P. yokohamae* (Thomann 1981); or (2) the compounds have been metabolised and biotransformed into polar form/glucuronidated (Varanasi and Gmur 1981). So, this study aims to determine the first reason which is to examine the assimilation efficiencies of each EDC in *P. yokohamae* by the concentration ratio in feces to the spiked diet.

MATERIALS AND METHODS

Test organism (P. yokohamae)

Same as experiment 4 – *see page* 58.

Preparation of 0.5% chromium trivalent oxide (Cr (III) oxide) in diet

To prepare the 0.5% Cr (III) oxide in diet, 100 g of commercial fish diet was ground by pestle and mortar and mixed with 0.5 g of Cr (III) oxide. After addition of water (10 mL per addition), the mixture was mixed very well. This step is very crucial to make sure the Cr (III) homogenate with the diet. The additional of water was stopped when the texture of diet can be pelletized by syringe on stainless tray (Figure 5.1). The diet was dried under sunlight for 2 days and cut into appropriate size for feeding (referring to the size of benthic fish mouth).

Figure 5.1 Cr (III) oxide mixed with ground diet and pelletized

Preparation of EDCs spiked diet

The mixture of EDCs was prepared according to the nominal concentration as shown in Table 5.1. EDCs were dissolved in diethyl ether and spiked to 50 g commercial diet (Nippon Formula Feed Manufacturing Co., Japan) containing 0.5% Cr (III) oxide in stainless tray to aid drying process. Spiking was done by pasteur pipette while mixing well the solvent and diet. The spiked diet was left overnight prior to use with gentle mixing for every 3-4 hours.

Table 5.1 Nominal concentration of mix EDCs spiked to the diet

EDC	Concentration (ng/g)
NP	2,000
OP	500
BP	500
E1	500
E2	500

Exposure scheme

P. yokohamae was exposed to dietary EDCs and Cr (III) oxide in a glass aquarium of 15 x 25 x 18 cm which maintained in a semi-static-system and aerated

well. The seawater was replaced once daily. The aquariums were placed in a water bath to maintain 20°C of water temperature (Figure 5.2). In this experiment, a benthic fish was introduced in each aquarium and starved for 2 days to make the gut empty.

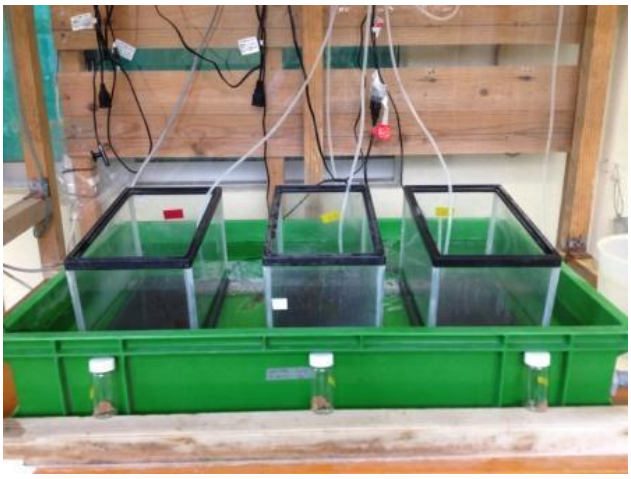


Figure 5.2 Glass aquariums in water bath set-up for assimilation rate experiment

After starving, they were fed with diet containing Cr (III) oxide for 3 days to replace their digestive tract with a total feeding rate 5% of the body weight. Feeding was done twice daily and diet that remained uneaten after 30 minutes was removed immediately. Their diet was changed to EDCs spiked diet (contains Cr (III) oxide) at day 4–6 which feces collection was done just before the next session of feeding. The feces were then centrifuged at 760 x g for 10 min. After removing the supernatant, the feces were kept in freezer (-18°C) until analysis. This flow of exposure scheme was repeated for another two times in order to make into 3 replicate of samples. A session of exposure (containing 3 fishes) represents a replicate and the feces were combined prior to analysis. The body weight of individual *P. yokohamae* at the initial of exposure ranged from 17.0 g to 26.0 g. Water parameters such temperature, dissolved oxygen, pH and salinity were measured periodically.

Pre-treatment of samples

After thawing, the feces samples (a sample per session) were weighed into a glass bottle to record the wet weight. The samples were then freeze dried at -48°C and 7.8 Pa for 4 days. Dry weights of feces were recorded right after the process.

Chromium hexavalent, Cr (VI) analysis

Spiked diet and feces

The whole Cr (VI) analysis was performed in draft chamber. For the digestion of Cr (III) to Cr (VI), each of spiked diet (0.3 g) and feces (ranged of weight from 0.01–0.017 g) samples was added with 5 mL concentrated nitric acid (HNO_3 for poisonous metal analysis) in a beaker, covered with a watch glass and kept for overnight. Next day, the sample was refluxed on a hotplate with a gradual increasing temperature from 60–220°C with watch glass cover. The watch glass cover was removed if the brown fumes generated indicating the oxidation of samples by HNO_3. After precipitation to 1 mL and cooled down at room temperature, 3mL HNO_3 was added and heated again. This step was repeated over and over until no brown fumes were given off by the sample. The sample was then let to cool down before the addition of 3 mL perchloric acid ($HClO_4$ for poisonous metal analysis). The sample was heated on a hotplate in a gradual increasing temperature from 220–330°C with watch glass cover for 40 min. The watch glass cover was removed if the white fumes generated and this step was repeated over and over until no white fumes were given off by the sample. After the sample solution turning to yellow colour, the watch glass was removed to precipitate the sample to 1 mL and finally diluted with 20 mL mili-Q water. The absorbances of samples were measured by UV-visible Spectrophotometer.

Measurement using spectrophotometer

Since the data analysis required only the absorbance of Cr (VI), the standards were not prepared in this measurement. Cuvettes with a path length 1 cm were used and the blank absorbance due to mili-Q water was subtracted. The intensity of Cr (VI) in the digested sample was measured by UV-visible spectrophotometer (Shimadzu UV-1600, Japan) with a wavelength of 350 nm. The measurement was repeated 3 times and the average values were taken.

EDCs analysis

1. Feces and diet (Benthic fish – improved procedure)

After addition of external standard of *p*-n-NP-d4, target chemicals in the freeze-dried benthic fish (weights of homogenate sample ranged from 0.78-3.05 g) were ultrasonically extracted twice with 20 mL of methanol for 15 min. After centrifugation (4°C, 760 x g, 10 min), the organic layer was collected and transferred into a separating funnel, followed by the additional of 3 mL mili-Q water and 3 mL hexane to make it saturated. The lipid was separated from methanol by 5 min shaking into 20 mL hexane for three times. After shaking, hexane layer was discarded while methanol (denser than hexane) was collected and combined. Methanol was put into the separating funnel again and mixed with 50 mL of 5% NaCl and 0.2 mL concentrated hydrochloric acid. The mixture of solvents was extracted twice with 20 mL DCM with 10 min shaking and concentrated to 0.5 mL using a rotary evaporator and gentle stream of N_2. The concentrated extract was then replaced with 3 mL hexane and concentrated again to 0.5 mL by N_2. This step was repeated for three times. Clean up was performed in a 5 mL glass pipette (6 mm i.d. x 17 cm) filled with florisil (containing 3% water) and prewashed with 30 mL hexane. The target chemicals were eluted with 50 mL hexane-

isopropyl alcohol (9:1, v/v). The eluate was then concentrated to dryness using a rotary evaporator and gentle stream of N_2. Finally, internal standard (E2-16,16,17-d3) and acetonitrile were added to make the final volume of 0.2 mL before LC-MS/MS measurement.

Measurement using LC-MS/MS-ESI

Same as in chapter 2 – *see page* 30. The detection limit for each EDC is shown in Table 5.2.

Table 5.2 Precursor and daughter ions of EDCs and limit of detection by LC-MS/MS-ESI

EDCs	Precursor ions, m/z (amu)	Daughter ions, m/z (amu)	Limit of detection (ng/g)[a]	Recovery (%)
NP	219.0	132.9	1.89	57.1
OP	205.0	132.9	1.17	59.0
BP	227.1	211.8	2.08	102
E1	269.1	145.0	1.13	104
E2	271.1	144.9	0.22	92.3

[a]Detection limit using 0.3 g diet sample

Detection limit and recovery test

The detection limit was determined as the minimum concentrations corresponding to those of signal to noise ratio of 3 on the chromatogram of actual sample. The ratio between intensity of signal of each compound was obtained under selected ion monitoring (precursor and daughter ions, m/z) conditions and intensity of noise. The limit of detection of each EDC is shown in Table 5.2.

The recovery test was performed using fish diet spiked with 100 ng/g of each EDC standards and internal standard (E2-16,16,17-d3). The EDCs standard extraction and measurement was done as above procedures. Mean percent recovery, as determined

from an estimate of the amount of added and recovered, were between 57.1% and 104%. Analysis was conducted in duplicate (n = 2). The recovery percentage of each EDC was increased after the improvement of procedure as shown in Table 5.2. In addition, lipid content in the final eluate was reduced indicating by the disappearance of yellowish suspended liquid in the bottom of vial bottle.

Data analysis

Assimilation efficiencies (%) was computed by 100 – [100 x (Absorbance of Cr in diet/Absorbance of Cr in feces) x (EDC in feces/EDC in diet)]; where Cr is chromium absorbance/g in diet or feces, and EDC is EDC concentration.

RESULTS AND DISCUSSION

Water parameters and EDCs spiked diet

Mean water temperature and dissolved oxygen concentration were 21.7±0.9°C and 5.86±1.27 mg/L, respectively, whereas pH and salinity were 7.80±0.2 and 33.3±0.2, respectively. The concentration of respective EDC spiked to diet is shown in Table 5.3.

Table 5.3 Mean concentration of EDCs, ng/g dry weight (d.w.) in spiked diet, n=3

EDCs	Mean	*SD
NP	1,200	±190
OP	479	±110
BP	424	±21
E1	136	±8.7
E2	552	±5.0

*SD standard deviation

Cr (III) oxide was used as the inert marker in this study for the estimation of digestability because it homogenously incorporated into the feed, accurately analyzed at low concentrations, indigestible and does not affect the metabolism of animal and pass through the gastro-intestinal tract as the dietary nutrients (Austreng et al. 2000). The absorbance of Cr (VI) was measured by UV-visible Spectrophotometer to be 2.65±0.2/g in spiked diet and 12.0±2.7/g in feces sample. The concentration of each EDC was measured in feces as shown in Table 5.4.

Table 5.4 Mean concentration of EDCs, ng/g d.w. in feces, n=3

EDCs	Mean	*SD
NP	2,786	±1,120
OP	253	±44
BP	105	±73
E1	42	±26
E2	78	±65

*SD standard deviation

Assimilation efficiencies (AE)

In the present study, *P. yokohamae* had assimilated high percentage of EDCs from the administered concentration. The assimilation efficiency of each EDC is shown in Table 5.5. *P. yokohamae* assimilated the lowest of NP and the highest of E2 with 49.6 and 96.4%, respectively. Percentage of AE for BP, E1 and E2 were overmthan 87 indicate that *P. yokohamae* efficiently assimilated the target EDCs except for NP.

Table 5.5 Assimilation efficiency of EDCs by *P. yokohamae*, n=3

EDCs	Percentage (%)	*SD
NP	49.6	±9.4
OP	87.9	±3.5
BP	93.9	±5.4
E1	92.4	±6.2
E2	96.4	±3.6

*SD standard deviation

Table 5.6 Assimilation efficiency of EDCs (%) in various test subjects

Study	AE of EDCs (%)					Exposure route	Test subject
	NP	OP	BP	E1	E2		
Pickford et al. 2003	L[a]	n.a	n.a	n.a	n.a	diet	Fathead minnows
Müller et al. 1998	95	n.a	n.a	n.a	n.a	oral & intravenous	Human
Pedersen et al. 2003	n.a	99.8	n.a	n.a	n.a	diet	Rainbow trout
Knaak and Sullivan 1966	n.a	n.a	65	n.a	n.a	oral & intravenous	Rat
Pottenger et al. 2000	n.a	n.a	7–14	n.a	n.a	oral, intraperitoneal & subcutaneous	Rat
Christiansen et al. 2002	n.a	n.a	n.a	85	85	oral	Women
This study	**49.6**	**87.9**	**93.9**	**92.4**	**96.4**	**diet**	**Marbled flounder**

[a]very low of parent compound in feces; n.a – Not available

Study on AE of EDCs through dietary uptake by fish is rarely reported by researchers. Most of the studies focused on the accumulation of contaminant by the fish tissues and neglected this point. Thus, the gathered data from aquatic organisms is not sufficient for the comparison purposes, hence the comparison was done on broad test subject including human with various exposure route (Table 5.6). In spite of this, these studies have reported the percentage of parent compound that was measured in the feces which was used to estimate the AE.

After exposing fathead minnows to dietary NP, Pickford et al. (2003) found very low percentage of NP in the feces indicating absorption from the gastrointestinal tract was almost complete. However, *P. yokohamae* in this study seemed to moderately assimilated NP (49.6%). This probably suggests the assimilation of NP is differs between species. The percentage of OP in rainbow trout in feces was computed as low as 0.2% (≈ 500 times lower), hence estimated AE would approximately be 99.8% (Pedersen et al. 2003). This value is similar to AE of OP in this study (87.9%), indicates both rainbow trout and *P. yokohamae* can efficiently assimilated OP. Instead, no description found about the assimilation of BP by aquatic organism. However, AE of BP was determined in rat with 65% (Knaak and Sullivan 1966) and 7–14% (Pottenger et al. 2000) lower than the present study. Both of the studies had applied similar exposure route but the results was quite different. This result suggests *P. yokohamae* can assimilate higher BP than rat. For natural estrogen, assimilation of external E1 and E2 only reported by human test subject which almost as high as *P. yokohamae* (Christiansen et al. 2002).

The AE percentages propose that *P. yokohamae* efficiently assimilated the administered EDC through dietary exposure. Thus, low bioaccumulation of EDCs in homogenate fish tissues in previous experiment was probably due to intensive

metabolism (Pickford et al, 2003; Müller et al. 1998; Pottenger et al. 2000). Gastrointestinal tract was shown to play role in metabolizing organic contaminants by fish which can substantially reduce their bioaccumulation (Kleinow and James 2001; Nichols et al. 2004). In order to prove this speculation, an experiment examining the metabolism of EDCs in *P. yokohamae* intestine will be carried out.

CONCLUSION

Assimilation efficiencies percentage indicate that *P. yokohamae* efficiently assimilate the respective EDCs except for NP. This result is not consistent with the finding of low concentration of EDCs in the fish tissues. Thus, *P. yokohamae* successfully assimilate the administered EDCs but the compound probably went through intensive metabolism and could not be recognized during the detection. The metabolism of EDCs in *P. yokohamae* would be the next objective to be achieved in the next experiment.

CHAPTER 6

Glucuronidation activity of bisphenol A (BP) in *Pleuronectes yokohamae* intestine and liver

ABSTRACT

Due to the contradictive finding of high assimilation efficiency but low biomagnification of EDCs in *Pleuronectes yokohamae*, these compounds were speculated to be metabolized by glucuronidation. As first-pass metabolism organ, UDP-glucuronosyltransferase (UGT) activity of bisphenol A in intestine was analyzed in its microsomal with value of 11.44±2.5 nmol/min/mg. High microsomal UGT activity in *P. yokohamae* indicated the efficient metabolizing of BP in their body. Thus, glucuronidation mediated by UDP-glucuronosyltransferases (UGT) is a significant metabolic pathway that facilitates elimination EDCs from *P. yokohamae*. The other target EDCs were assumed to be glucuronidated as demonstrated by previous studies. Consequently, UGT activity has justified that low bioaccumulation of EDCs through dietary exposure was attributed by the formation of EDCs glucuronide to ease the excretion.

INTRODUCTION

After assimilation into fish, the nature of many persistent organic contaminants predisposes to accumulation, whereas certain compounds may be appreciably modulated by metabolism (Coldham et al. 1998). Glucuronidation is one of the major metabolic pathways involved in the elimination of endogenous and exogenous compounds in fish (Dutton 1980; Stageman et al. 2010; Yokota et al. 2002). This phase is catalyzed by UDP-glucuronosyltransferase (James 2011) through conjugation with

glucuronic acid to produce strongly acidic compound which is more water-soluble than the precursor. The conjugation then facilitates excretion of glucuronide metabolite through the excretory routes such as hepatic, renal, bile and intestinal (Brodie and Hogben 1957). Therefore, UDP-glucuronosyltransferases (UGTs) are an important phase II enzymes for detoxification of exogenous compounds. UGTs are induced by a variety of natural and estrogenic compounds and play a key role in catalyzing the glucuronidation and potential excretion of different xenoestrogen in fish (Clarke et al. 1992). Thus, the bioaccumulation/biomagnification of natural and estrogenic chemicals might be reduced by glucuronidation.

The dietary exposure of EDCs to *Pleuronectes yokohamae* in the chapter 4 demonstrated that these compounds were not bioaccumulated. These findings were contradicted to high assimilation efficiencies of EDCs which was examined in the following chapter 5. The possible reason could be due to compounds have been metabolised and biotransformed into polar form/glucuronidated as reported by previous studies (e.g James 2011, Thibaut et al. 1998, Yokota et al. 2002). After glucuronidation formed in intestine following assimilation, the metabolite will be transferred to liver and excreted into bile (Simon and Boudou 2001). Since EDCs accumulation through dietary uptake is concern, two excretory routes (intestine and liver) that directly involved in the production and transferred of glucuronidated compounds were chosen in this analysis. So, this study aims to examine the glucuronidation/UGT activity of BP in the intestine and liver of *Pleuronectes yokohamae*. The glucuronidation activity was determined only in BP due to availability of the glucuronide standard for analysis.

MATERIALS AND METHODS

Test organism (P. yokohamae)

Same as experiment 4 – *see page* 58.

Preparation of buffer 0.1 mM Tris-HCl containing 5mM $MgCl_2$ (pH 7.4)

0.1 mM Tris-HCl buffer was prepared from 0.02 M Tris- HCl (pH 8.0) as described in Chapter 4 (*see page* 59) and was added with 5mM $MgCl_2$. The pH was then adjusted to pH 7.4.

Preparation of test organism

Prior to analysis, benthic fish was starved for one day to empty the digestive tracts. This step is crucial in order to eliminate the effects of EDCs from the commercial diet which was fed during the acclimatization term.

Preparation of intestine and liver microsomes from P. yokohamae

After anaesthetized by 0.05% 2-phenoxyethanol, the body length (mean of 8.94 cm) and weight (mean of 8.19 g) of *P. yokohamae* were recorded followed by dissecting to collect the whole intestine and liver tissues. The average of intestine and liver tissues weight are 0.343 and 0.247 g, respectively. Each tissue was homogenized using an automatic Heidolph homogenizer, in 4 volume solution containing 0.15 M KCl and 1mM EDTA.2Na in ice. The homogenate was centrifuged at 10,000 x g at 4°C for 10 min to obtain crude microsomes. Rapid preparation of the microsomal fraction was important to prevent the loss of UGT activities. The microsomes were activated by the incubation with 0.01% CHAPs ((3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate, detergent of solubilizing membrane proteins: Dojindo Molecular

Technologies, Inc., Japan) for 30 minutes at 0°C. The protein concentration of crude microsome was determined by Bio-Rad Protein Assay using bovine serum albumin as a standard and measured by the microplate reader (MTP-32, Corona Electric Co. Ibaraki, Japan) at a wavelength of 630 nm.

Enzyme analysis

Enzyme analysis was done according to Yokota et al. (2002) with modifications. UDP-glucuronosyltransferase activity toward BP was assayed in 400 µL volume from the combination of 100 µL crude microsomes, 250 µL of buffer 0.1 mM Tris-HCl containing 5mM $MgCl_2$ (pH 7.4), BP (substrate) and 40 µL of 5 mM UDPGA trisodium salt (uridine 5'-diphosphoglucuronic acid trisodium salt, Sigma-Aldrich, Japan) at 30°C for 30 minutes incubation period. In this study, target concentration of 5 µg/mL was used for substrate due to the sensitivity of instrument (U-HPLC). The enzyme activity was stopped by boiling in water for 5 minutes and the reaction products were filtered using a disposable disk (Whatman syringe filter pore size 0.2 µm, Sigma-Aldrich, Japan) and analyzed by a U-HPLC system. Substrate-glucuronides peaks were estimated using authentic bisphenol A mono-β-D-glucuronide (Sigma-Aldrich, Japan) standards. The incubation temperature was decided at 30°C based on previous analysis done by Yokota et al. (2002).

Measurement using U-HPLC

A Merck Hitachi LaChromUltra U-HPLC system (Hitachi Tokyo, Japan) equipped with 2 unit pumps (L-2160U), autosampler (L-2200U), diod array detector (L-2455U), fluorescence detector (L-2485U) and column ovens (L-2300 and L-2350) which maintained column temperature at 40°C was used. The filtered samples were

injected and eluted with 100% acetonitrile (mobile phase A) and 10 mM ammonium acetate (mobile phase B). Separation was performed on an Inertsil ODS-3 column (2.1 mm i.d. x 100 mm, 3.0 μm of particle size, GL Sciences Inc., Japan). Sample injection was 10 μL, at constant flow rate of 0.6 mL/min which was eluted by the following gradient methods for mobile phase A and B ratio set on the system; for the initial 10 min at 5 and 95%, next 10 to 16 min at 50 and 50%, and the last 16.1 to 22 min at 5 and 95% again. Fluorescence excitation and emission wavelengths were 275 and 308 nm, respectively.

RESULTS AND DISCUSSION

Water parameters during acclimatization period

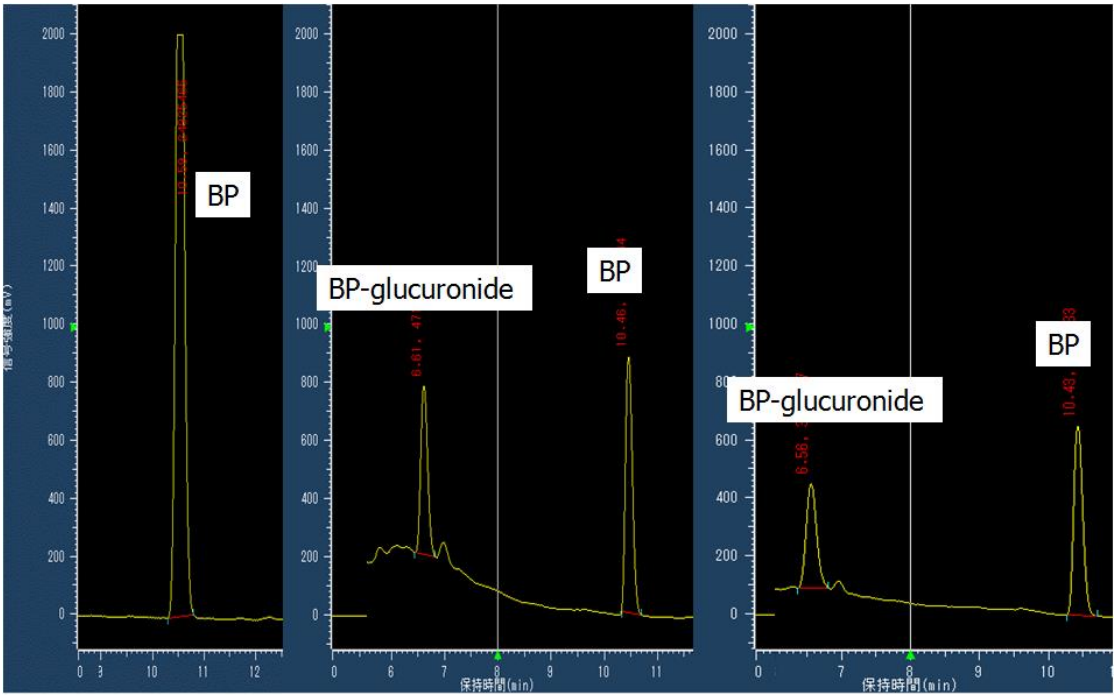
Mean water temperature and dissolved oxygen concentration were 19.7±0.4°C and 5.20±0.21 mg/L, respectively, whereas pH and salinity were 8.10±0.2 and 33.2±0.1, respectively.

Bisphenol A glucuronidation in P. yokohamae intestine and liver

The chromatogram of U-HPLC analysis of BP obtained *in vitro* from *P. yokohamae* intestine and liver microsomes are shown in Figure 6.1. Unconjugated BP in the absence of UDP-glucuronic acid was eluted at 10.5 min, which is the same elution time for standard of BP. The retention of BP glucuronide was identified by referring to the standard of bisphenol A mono-β-D-glucuronide which eluted at 6.6 min.

This study showed BP was glucuronidated by microsomes prepared from intestine and liver of *P. yokohamae* after activation with UDP-glucuronic acid and incubation. UGT activities were analyzed to be higher in liver with 26.53±18.1

nmol/min/mg than 11.44±2.5 nmol/min/mg in intestine (Table 6.1). However, statistical analysis has proven no significant difference ($p<0.05$) of UGT activities between these microsomes.



(a) (b) (c)

Figure 6.1 Chromatograms of BP in control sample (liver/ intestine) without activation by UDP-glucuronic acid and incubation (a), liver sample with activation by UDP-glucuronic acid and incubation (b), and intestine sample with activation by UDP-glucuronic acid and incubation (c)

Table 6.1 Mean microsomal UGT activities (nmol/min/mg) toward BP by *P. yokohamae* intestine and liver (n=3)

Microsome	nmol/min/mg	*SD
Intestine	11.44	2.5
Liver	26.53	18.1

*SD standard deviation

As first-pass metabolism organ, intestine plays an important role for metabolism of compounds after dietary uptake (Van Veld et al. 1988). Thus, UGT activity in intestine was considered for the evaluation of glucuronidation in *P. yokohamae*. UGT activities toward BP in intestine of carp and *P. yokohamae* are shown in Figure 6.2. High microsomal UGT activity in *P. yokohamae* verified the efficient metabolizing of BP in their body. Higher UGT activity was reported in intestine of *P. yokohamae* compared to carp (Daidoji et al. 2006; Yokota et al. 2002) could be due to metabolism levels of BP varying with fish species (Lindholst et al. 2001, 2003).

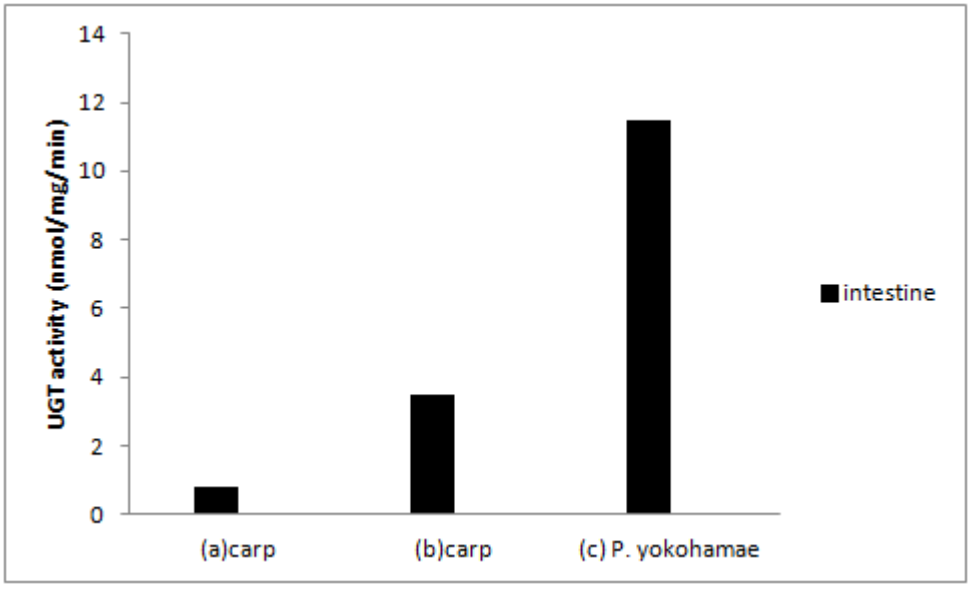


Figure 6.2 Microsomal UGT activities toward BP by intestine of carp and *P. yokohamae*; (a) Daidoji et al. 2006, (b) Yokota et al. 2002, (c) this study.

After glucuronidation in intestine, the glucuronidated BP is transferred to liver and then excreted into bile. The bile content was then transferred back to intestine, where it can be excreted in the feces (Oliveira Ribeiro et al. 1999). Thus, high UGT activity measured in the *P. yokohamae* intestine seems to favor the elimination of BP

and lead to no bioaccumulation of this compound in the benthic fish. Substrates such EDCs can form active glucuronides at certain position for instance at 3-OH and 17-OH of 17β-estradiol, E2 (Ritter 2000). Similar form of active glucuronides (-OH) were observed for nonylphenol (NP), octylphenol (OP) and estrone (E1). Thus, the other target EDCs were assumed to be glucuronidated like BP. In addition, glucuronidation of NP (Thibaut et al. 1998), OP (Ferreira-Leach et al. 2001; Pedersen and Hill 2000) and E2 (James 2011; Jurgella et al. 2006) in fishes had been demonstrated by other authors.

CONCLUSION

UGT activities toward BP in the intestine and liver of *P. yokohamae* have verified the presence of BP glucuronide in the test organism. High UGT activity in the microsomes of intestine suggesting efficient metabolism and elimination of BP form the *P. yokohamae* body. Therefore, this compound was not bioaccumulated/biomagnified in the previous dietary exposure. The other target EDCs were assumed to be glucuronidated as verified by previous studies conducted in fishes by Jurgella et al. (2006), Ferreira-Leach et al. (2001) and Thibaut et al. (1998).

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

Endocrine Disrupting Chemicals (EDCs) can disturb development of the endocrine system and of the organs that respond to endocrine signals in organisms. Consequently, this situation has lead to intersex, altered mating behaviour, reduced testicular growth and disrupts reproductive output in fishes. Target EDCs in this study consist of natural (E1 and E2) and estrogenic chemicals (NP, OP and BP) which were reported as potentially estrogenic to aquatic organisms. The concentration levels have been measured in water, biota and extremely high concentration was found in the sediment. Thus, benthic organisms probably might accumulate these compounds through dietary uptake and possibly transfers them into food chain. For that reason, EDCs bioaccumulation through food chain was examined in plychaete (*P. nuntia* and *Paraprionospio* sp.) and benthivorous fish (*P. yokohamae*).

From the initial exposure experiment, EDCs were found not biomagnified in polychaete, *P. nuntia* through dietary exposure, but in the field experiment, EDCs concentration in polychaete, *Paraprionospio* sp. was unexpectedly high (4,410 ng/g w.w. for NP) compared to the sediment. Thus, in certain cases, bioaccumulation of EDCs under laboratory condition might not represent the natural environment. Given the relatively high concentration of EDCs measured in *Paraprionospio* sp. than sediment, *Paraprionospio* sp. probably has biomagnified these chemicals from the sediment and might transfer the compounds into their predator such benthic fish. For that reason, bioaccumulation of EDCs was examined in benthic fish; *P. yokohamae* through dietary route. However, EDCs were not biomagnified in benthic fish, *P. yokohamae* indicated by low BMF values. This finding was verified by no induction of

Vg, a sensitive biomarker for estrogens exposure (Donohoe and Curtis 1996; Kime et al. 1999; Folmar et al. 2000). Besides that, three of the target EDCs (BP, E1 and E2) was not detected in fish by LC-MS/MS. The reason that EDCs were not bioaccumulated in *P. yokohamae* probably due to low uptake and assimilation, but the accumulation trend indicated the increasing concentration during the exposure scheme. Therefore, assimilation efficiency of EDCs was examined in *P. yokohamae* as it is an important part of bioaccumulation study (Landrum et al. 1992; Wang and Fisher 1999). Assimilation efficiencies study showed *P. yokohamae* efficiently assimilated the target EDCs with percentage of over 88% except for NP. This result was contradicted to low bioaccumulation values observed in polychaete and benthic fish. Thus, low bioaccumulation of EDCs in homogenate fish tissues and presence of compounds concentration below detection limit (BP, E1 and E2) in this study were probably due to intensive metabolism as been reported by several of recent studies (Melancon and Lech 1976; Varanasi and Gmur 1981; George 1994; Yokota et al. 2002). In order to prove this speculation, glucuronidation activity of BP was analyzed in the intestine and liver of *P. yokohamae*. High glucuronidation activities in these microsomes verified the presence of BP glucuronidation in the test organism which favors the elimination from fish body (Van Veld et al. 1988). For that reason, low bioaccumulation of EDCs through dietary exposure was attributed by the formation of EDCs glucuronide to ease the excretion after assimilation.

As the conclusion, EDCs in this study were not bioaccumulated through the food chain. This finding has been verified by high glucuronidation activities in intestine (first-pass metabolism organ) of *P. yokohamae*. No induction of vitellogenin as indicator for estrogen exposure suggests glucuronidated EDCs have almost no potential to induce vitellogenin.

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