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Preliminary Ecological Risk Assessment of Butylparaben and Benzylparaben —1. Removal Efficiency in Wastewater Treatment, Acute/Chronic Toxicity for Aquatic Organisms, and Effects on Medaka Gene Expression

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Butylparaben and benzylparaben, used as preservatives mainly in cosmetic products, have recently been found to be weakly estrogenic. Batch activated-sludge treatment and batch chlorination were carried out to roughly determine the removal efficiency of a wastewater treatment plant. Combining the removal efficiency with the estimated annual consumption and the unaltered excretion ratio, the maximum predicted environmental concentration (PEC) was estimated. Conventional acute/chronic toxicity tests were conducted using Japanese medaka (*Oryzias latipes*), daphnia (*Daphnia magna*), and green algae (*Pseudokirchneriella subcapitata*) for *n*-butylparaben, *i*-butylparaben, and benzylparaben. Medaka vitellogenin assays were also conducted for the three compounds and DNA microarray analysis was carried out to examine the effects of benzylparaben on gene expression. The plasma vitellogenin concentration of male medaka increased for concentrations of 200, 100, and 100 μg L^{-1} *n*-butylparaben, *i*-butylparaben, and benzylparaben for 14 days, respectively, while the expression levels of genes encoding proteins such as p53, cytochrome P450 3A40, and choriogenin-L increased for concentrations higher than 4 μg L^{-1} of benzylparaben. Furthermore, the predicted no-effect concentration (PNEC) was calculated using the lethal or effect concentration 50 (LC_{50} or EC_{50}) values and no-effect concentrations (NOECs) obtained in the toxicity tests for these compounds. The maximum concentrations found in the aquatic environment or sewage effluent (MEC_{eff}) were used to carry out preliminary environmental risk assessment. The calculated MEC/PNEC ratio suggests the necessity of further study such as a more detailed large-scale monitoring and chronic toxicity tests including reproduction inhibition and endocrine disruption.

1. Introduction

Parabens, formally alkyl esters of p-hydroxybenzoic acid, are a class of antimicrobials particularly useful against molds and yeasts. These compounds meet several criteria of ideal preservatives, which include a broad spectrum of antimicrobial activities and weak toxicity for humans/mammals. Some parabens are used at concentrations below 1% in cosmetics such as creams, lipsticks, skin lotions, hair sprays, hair dyes, shampoos, and bubble bath powders.[1] The paraben species found at the highest concentrations in various cosmetic products are methylparaben followed by ethylparaben, *n*-propylparaben, *n*-butylparaben, and benzylparabens,[2] although the antimicrobial activity of parabens increases as the chain length of the ester increases.

A number of studies have suggested the estrogenic activity of parabens. Methylparaben, ethylpraben, *n*-propylparaben and *n*-butylparaben were all found to be weakly estrogenic by a rat estrogen receptor binding assay and a yeast-based estrogen assay, while *n*-butylparaben showed estrogenic activity in a rat uterotrophic assay.[3] Darbre *et al.*[4] also found an increase in rat uterine weight induced by benzylparaben exposure. Okubo *et al.*'s results[5] using *in vitro* tests suggested that parabens with longer linear chains are more potent than those with shorter chains, and that those with branched chains are more potent than those with linear chains. The estrogenic activity of benzylparaben was stronger than those of linear alkyl parabens and the highest potency was found for *i*-butylparaben followed by *n*-butylparaben among seven parabens using the human estrogen receptor binding assay and yeast two-hybrid assay.[6] Watanabe *et al.* also found that benzylparaben has estrogenic activity using the yeast two-hybrid assay and that benzylparaben has higher activity than bisphenol A.[7] As far as aquatic organisms are concerned, *n*-propylparaben was found to induce increases in plasma vitellogenin (VTG) concentration and up-regulate the gene expression of VTG-1, VTG-2, choriogenin (CHG)-L and CHG-H in male medaka.[8] Intraperitoneal injections of ethylparaben, *n*-propylparaben and *n*-butylparaben in rainbow trout cause estrogenic responses such as significant VTG induction at doses of 100–300 mg kg^{-1}.[9] The exposure of rainbow trout to 20l μg L^{-1} *n*-butylparaben for 12 days also increased the plasma VTG concentration of the fish.[10]

Despite several reports on the estrogenic activities and wide use of parabens in cosmetic, pharmaceutical, and food products, few studies have been conducted to determine the fate of these compounds in aquatic environments, and the environmental/ecological risk of this class of chemicals has remained unclear. Ethylparaben was detected in Japanese river sediments from two sampling sites at concentrations as high as 3.3 μg kg^{-1} but methylparaben, propylparaben, *n*-butylparaben and *i*-butylparaben were under the detection limit for the other river water and sediment samples.[11] Benzylparaben, ethylparaben and *n*-propylparaben were detected at concentrations as high as 1 μg L^{-1} in effluent from Swedish wastewater treatment plants (WWTPs).[12] *n*-Butylparaben was detected at 0.01–0.26 μg L^{-1} in effluent from Canadian[13] and Spanish[14] WWTPs. In a comparison of the concentrations of parabens in the influent and effluent from WWTPs, the removal efficiencies in conventional WWTPs were as high as 96, 99, 96, and 99% for *n*-butylparaben, propylparaben, ethylparaben and methylparaben, respectively.[13] As for chlorination, 1.60 mg L^{-1} chlorine addition as sodium hypochlorite into butylparaben-containing water resulted in a half-life ($t_{1/2}$) of 5 min for *n*-butylparaben.[14]

In this study, we selected three parabens with relatively high estrogenic potencies, namely, *n*-butylparaben, *i*-butylparaben and benzylparaben, and attempted to clarify the environmental/ecological risk of these compounds using the same method used in our previous study for eight human pharmaceuticals[15] with slight modifications and additions. Therefore, the objectives of this first part of a series of consecutive works were set to reveal (1) the removal efficiency in wastewater treatment plant by batch activated-sludge treatment and batch chlorination, (2) the conventional acute/chronic toxicities for aquatic organisms using Japanese medaka, daphnia and

green algae, (3) the estrogenic activities and effects of parabens on the gene expression of fish using the medaka vitellogenin test and medaka DNA microarray, and (4) to conduct preliminary ecological risk assessment by determining the maximum predicted environmental concentration (PEC) or measured environmental concentration (MEC) and the predicted no-effect concentration (PNEC) for the three parabens.

2. Materials and Methods

2.1 *Materials*

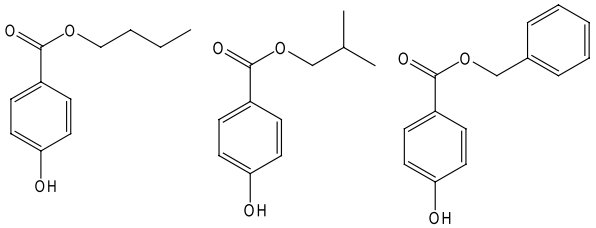
n-Butyl-*p*-hydroxybenzoate (*n*-butylparaben) of at least 98% purity was purchased from Wako Pure Chemical Industries (Osaka, Japan). *i*-Butyl-*p*-hydroxybenzoate (*i*-butylparaben) of at least 99% purity was purchased from Tokyo Kasei Co. (Tokyo, Japan). Benzyl-*p*-hydroxybenzoate (benzylparaben) of at least 99% purity and sodium hypochlorite with at least 5% effective chlorine was purchased from Wako Pure Chemical Industries (Osaka, Japan). 17β-Estradiol of at least 97% purity was purchased from Sigma-Aldrich Chemical (Milawaukee, WI, USA).

The chemical structures, acidity constant (pK_a) values and octanol-water distribution constants (log D_{ow}) of the parabens selected in this study are shown in Table 1. The excretion ratios of the unaltered forms were taken from a report of Ye *et al.*[16] The log D_{ow} and pK_a values were estimated using the ACD software LogD Suite. The annual consumption of the compounds in Japan was estimated as shown in Section 2.6.1.

2.2 *Procedure of batch activated-sludge treatment and chlorination*

Preliminary batch activated-sludge treatment test was conducted as shown in our previous study.[15] Briefly, activated-sludge was sampled from a WWTP using the conventional activated-sludge process and used for the batch experiments immediately after sampling. The concentration of the mixed liquor suspended solid (MLSS) was set at 2,000 mg L^{-1}, and the initial concentration of each paraben was set at 100 μg L^{-1} in a 100-mL Erlenmeyer flask. The mixed liquor was aerated with an

Table 1
Parabens selected in this study.

Parabens	*n*-Butylparaben	*i*-Butylparaben	Benzylparaben
Estimated domestic consumption as cosmetic products or topical medicine[a)]	168 t	3.0 t	25 t
Estimated domestic consumption as food products[a)]	12 t	1.5 t	none
Excretion ratio of unaltered form[b)]	17%	unknown	< 1%
Chemical structure			
log D_{ow}[c)] (at pH 7)	3.43	3.27	3.61
pK_a[c)]	8.22	8.17	8.18

[a)]The procedure used for estimation is described in Section 2.6.1.; [b)]Cited from attachment of each pharmaceutical compound (oral intake); [c)]Predicted by log D Suite (ACD software).

aeration pump in the dark at 25°C for 6 h. After the reaction, the mixed liquor was centrifuged, the supernatant was filtered through a 0.2-μm-pore-size membrane filter (OMNIPORE membrane, Millipore Co., Billerica, MA) and analyzed by high-performance liquid chromatography (HPLC), as described below. For the sludge phase, the supernatant was carefully decanted and acetonitrile was added. After sonication, the extract was filtered through the membrane filter, and analyzed by HPLC.

The batch chlorination test was conducted according to the methods used by Tyrell *et al.*[17] Effective chlorine (2.0 mg L^{-1}) was added as sodium hypochlorite in the secondary effluent of the wastewater treatment plant. The initial concentration of each paraben was set at 100 μg L^{-1} in a 100-mL Erlenmeyer flask. After 15 min of contact, sodium thiosulfate was added to stop the reaction, and the concentration of the parabens in the filtrate of the 0.2-μm membrane filter was determined by HPLC as shown below.

The HPLC was an LC-10AD VP series (Shimadzu, Kyoto, Japan) equipped with a 3-μm ODS column (Cadenza CD-C18, Imtakt, Kyoto, Japan) and a UV/visible absorbance (SPD-10A VP, Shimadzu, Kyoto, Japan) detector. In order to avoid preventive peaks and noise originated from activated sludge, sludge blanks with no parabens were prepared for all the HPLC analytical conditions and no preventive peak/noise near the retention time of the selected parabens was confirmed.

2.3 *Procedures of acute/chronic toxicity tests*

2.3.1 *Fish acute toxicity test*

Fish acute toxicity tests were conducted using Japanese medaka (*Oryzias latipes*) bred in the National Institute for Environmental Studies (Tsukuba, Japan) and acclimated in a laboratory of The University of Tokushima for at least two months. Tests were conducted in conformity with "OECD Test Guideline for Testing of Chemicals No. 203."[18] Briefly, approximately ten ten-day-old fish were exposed to at least six different concentrations of the parabens in a 100-mL beaker. A half of the paraben solution was replaced every 24 h (semistatic test), and lethal concentration 50 (LC_{50}) value was determined using the probit conversion analysis.

2.3.2 *Daphnia acute immobilization test*

Daphnia magna provided by the National Institute for Environmental Studies (Tsukuba, Japan) were used for the acute immobilization tests after at least a two-month acclimation period in the laboratory of The University of Tokushima. Tests were conducted in conformity with "OECD Test Guideline for Testing of Chemicals No. 202."[19] Briefly, 20 larvae, less than 24 hours old, (five larvae per beaker) of daphnids were exposed to at least six different concentrations of the parabens in 50-mL beakers. The number of immobilized bodies was counted after 48 h of exposure and the effect concentration 50 (EC_{50}) value was determined using the probit conversion analysis.

2.3.3 *Daphnia reproduction test*

Similarly, a daphnia reproduction test was conducted in conformity with "OECD Test Guideline for Chemicals 211."[20] Briefly, ten larvae, less than 24 hours old, (one larva per test tube) of daphnids were exposed to at least six different concentrations of the parabens in 20-mL test tubes. Test waters were replaced every 48 h and the fecundity of these daphnia was examined by counting the cumulative number of broods. The no-effect concentration (NOEC) of both the cumulative number of broods and immobilized bodies was determined in 21 days.

2.3.4 *Algal growth inhibition test*

Pseudokirchneriella subcapitata was purchased from the National Institute for Environmental Studies (Tsukuba, Japan) and was acclimated for at least one month in a laboratory of The University of Tokushima before the exposure tests. Tests

were conducted in conformity with "OECD Test Guideline for Testing of Chemicals No. 201."[21] Briefly, a preincubated algal suspension was exposed to at least five different concentrations of the parabens in 100-mL Erlenmeyer flasks in AAP medium[22] at 24°C with illumination controlled at 5,000 lux. The number of algae was measured every 24 h during the 96-h exposure period using a UV/Visible spectrophotometer at 450 nm after calibration with known algal counts. The 50% growth inhibition concentration (EC_{50}) was calculated by linear correlation using a log-normalized plot. NOEC was also determined.

2.4 *Procedures of medaka vitellogenin test and DNA microarray test*

2.4.1 *Medaka vitellogenin test*

The medaka vitellogenin (VTG) tests were conducted using 2.5-month-old male medaka. The fish were exposed to the three parabens individually for 14 days in a custom-made flow-through system with a 2.3-liter glass tank and a tube pump at 5.6 mL min^{-1}. The nominal concentrations of parabens were set at 8, 40, 200, and 1,000 μg L^{-1} for *n*-butylparaben and 4, 20, 100, and 500 μg L^{-1} for *i*-butylparaben and benzylparaben referring to the conventional research[10] and our preliminary experiments. The measured concentrations were at least 80% of the nominal ones. At least 5–8 samples were prepared for each exposure concentration. The negative control without paraben exposure and the positive control with exposure to 0.1 and 1 μg L^{-1} 17β-estradiol were prepared for a comparison in addition to the female medaka without paraben exposure. No solvent was used to prepare the paraben or 17β-estradiol solution.

Artemia salina was fed twice a day during the exposure period of 14 days. The light-dark cycle was set at 16-h light and 8-h dark, and the laboratory was maintained at 25°C. After the exposure, blood was collected using a capillary soaked in sodium heparin over ice. The collected blood was diluted with an assay buffer and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant (plasma) was further diluted with the assay buffer, and the plasma VTG concentration was measured using the EnBio VTG analysis kit (Amersham Biosciences Co. Tokyo, Japan).

2.4.2 *Medaka cDNA microarray gene expression analysis*

Male medaka exposed to benzylparaben in the VTG test, as a representative of the three parabens because of its hydrophobicity and strong estrogenic activity, were used for the microarray analysis in addition to male and female controls. These fish were dissected in RNA*later* solution (Ambion Inc., Austin, TX) to prevent the degradation of total RNA. Then, liver samples were collected and stored in RNA*later* solution at –20°C until total RNA extraction was carried out. The cDNA microarray used in this study was the Medaka 750 EG microarray manufactured by Ecogenomics, Inc. (Fukuoka, Japan), and it contained 833 medaka cDNA gene probes (300±25 bases).[23] Each probe was spotted three times on the microarray. The hybridization of the Cy5-labeled target antisense RNA (aRNA) samples and the cDNA probes on the microarray was carried out for 16 h at 42°C in 50% formamide, 5x SSC, and 0.5% SDS solution.

For a statistical analysis of the microarray data, the expression signal data collected from each of the 833 gene probes were normalized by the expression signals of acidic ribosomal phosphoprotein PO. Then, they were statistically analyzed using the ArrayStat z-test (Imaging Research/GE Healthcare, Piscataway, NJ) with offset correction, outlier detection, and $p < 0.05$ (significance cutoff) to obtain an expression level ratio of the benzylparaben-exposed group to the unexposed group for each of the genes on the microarray.

2.5 *Procedure of measuring liposome/water partition coefficient*

The partition coefficient of the parabens between liposomes and water was determined using equilibrium dialysis developed by Escher and Schwarzenbach,[24] later

modified by Yamamoto and Liljestrand.[25] The final aqueous concentration of the parabens was determined by HPLC as described above, and the partition coefficient was determined by the initial and final paraben concentrations, and the concentration of liposome. The total organic carbon (TOC) concentration of the membrane vesicle suspension was measured using a TOC analyzer (TOC-5000, Shimadzu, Kyoto, Japan).

2.6 *Procedure of initial ecological risk assessment*

2.6.1 *Estimation of annual consumption in Japan*

Neither the production nor the consumption of any of the paraben species in Japan has been officially announced. *n*-Butylparaben is the paraben used most frequently in food products, mainly in soy sauce, and the estimated annual consumption is reported to be 12 tons, and that of total parabens is reported to be 18 tons.[26] Since no information is available on the other parabens in food products, namely, ethylparaben, *n*-propylparaben, *i*-propylparaben, and *i*-butylparaben, these compounds are assumed to be equally consumed (i.e., 1.5 tons annually). The only paraben used in pharmaceuticals is n-butylparaben and the total is 50 tons per year according to "Survey of Pharmaceutical Industry Productions, Fiscal Year 2003."[27]

Parabens in cosmetic products are more complicated. The Japan Ministry of Economy, Trade, and Industry officially announces "Statistics of Chemical Industry,"[28,29] which includes information on the monthly production/shipment of cosmetics, identifying each product, for example, shampoo and cleansing oil. Since the content of each paraben species in cosmetic products is reported by several researchers,[2,30–37] the arithmetic mean of paraben content for each cosmetic product is used for an estimation and is multiplied by the annual consumption in fiscal year 2006. For cosmetic products with no paraben content data available such as hair dye and foundation, similarly used products such as shampoo and eye makeup, respectively, are used for a rough estimation.

2.6.2 *Estimation of PNEC and PEC*

PNEC was calculated on the basis of experimental EC_{50} and LC_{50} values for three acute tests and NOEC for algal and daphnia chronic tests, similarly to our previous study.[15] PNEC was determined by dividing these experimental EC_{50}/LC_{50} or NOEC values by an assessment factor as follows:

$$\text{PNEC} = \text{minimum of } \frac{\text{minimum of } EC_{50}/LC_{50} \text{ in three acute tests}}{AF_{\text{acute-3}}} \quad \text{and } \frac{\text{minimum of algal and daphnia NOECs}}{AF_{\text{chronic-2}}} \tag{1}$$

where $AF_{\text{acute-3}}$ is the assessment factor for at least one acute EC_{50}/LC_{50} from each of the three trophic levels of the base set (fish, daphnia, and algae), and $AF_{\text{chronic-2}}$ is that for two chronic NOECs. In the present study, an assessment factor of 100 was used for both the minimum of the acute results ($AF_{\text{acute-3}}$) and the minimum of the daphnia reproduction and algal growth inhibition NOECs ($AF_{\text{chronic-2}}$) based on the "Initial Ecological Risk Assessment Guideline for Chemicals"[38] directed by the Japan Ministry of the Environment.

Predicted environmental concentration (PEC) for WWTP effluent (PEC_{eff}) and that for surface water (PEC_{sw}) were calculated from the estimated domestic consumption in Japan, the excretion ratio of the unaltered form, the total volume of domestic wastewater in Japan, and the removal rate in the activated sludge treatment/chlorination (assumed as 1% for those with less than 1%) obtained in this study as follows:

$$PEC = \frac{A \times U/100 \times (100-R)/100}{V \times D} \quad (2)$$

where A is the estimated annual consumption of the paraben in Japan (kg year^{-1}), U is the unaltered excretion ratio (%), R is the measured removal efficiency in WWTP (activated sludge treatment and chlorination are combined; an efficiency larger than 99% was simply assumed to be 99%) (%), V is the volume of wastewater per year in Japan (m^3 year^{-1}), and D is the dilution factor. No dilution factor was used for WWTP effluent (PEC_{eff}) and 10 was used for surface water (PEC_{sw}), which was recommended by EMEA[39] and is widely accepted as the relationship between the environmental standard and the effluent standard in Japan. Since the parabens used in cosmetics or topical medicines are mostly washed out and released into household effluents, the unaltered excretion ratio U was assumed to be 99%. For the food products, the ratio shown in Table 1 was used, while the ratio for *i*-butylparaben was assumed to be identical to that for *n*-butylparaben. Since a significant amount of parabens was used as cosmetics, these fractions might be released into aquatic environments without any treatment in the areas with no sewage (or integrated septic tank) service coverage, which accounts for approximately 20% of the population in Japan. Therefore, the PEC for the untreated water (i.e., R = 0) released into surface water (i.e., D = 10) was also calculated as PEC_{utsw}. Maximum measured concentration in WWTP effluent or surface water was also searched and used as maximum measured environmental concentration (MEC_{eff} or MEC_{sw}).

PEC/PNEC or MEC/PNEC ratios were used to assess the ecological risk of the parabens. According to the Japan Ministry of the Environment's "Initial Ecological Risk Assessment Guideline for Chemicals,"[38] if the PEC/PNEC or MEC/PNEC ratio is larger than 0.1, further environmental risk assessment is necessary. As far as bioaccumulation is concerned, those compounds with a bioconcentration factor larger than 1000 are considered to be "highly bioaccumulative," which is used as criteria in the Chemical Substances Control Law for newly registered chemicals other than pesticides and pharmaceuticals in Japan.[40]

3. Results

3.1 *Removal efficiency in batch activated-sludge treatment and chlorination*

Results of 6-h preliminary batch activated-sludge treatment and 15-min batch chlorination are summarized and shown in Table 2. The removal efficiency equaled the summation of the sludge phase (i.e., sorbed fraction) and the unknown (i.e., transformed or unextractable).

As can be seen in Table 2, the removal efficiencies of all the selected parabens by activated sludge were significantly high and were approximately 99%. The sludge phase was under the detection limit for *i*-butylparaben and benzylparaben, and the unknown fraction was higher than 99%, which are possibly biologically transformed. In contrast, the removal efficiencies in the 15-min chlorination were at most 72%.

Table 2
Results of batch activated-sludge treatment and batch chlorination.

	Activated sludge (6 h)				Chlorine (15 min) removal efficiency (%)
	Aqueous phase (%)	Sludge phase (%)	Unknown (%)	Removal efficiency (%)	
n-Butylparaben	1.0	6.7	92.3	99.0	67
i-Butylparaben	0.1	<0.1	99.8<	99.9	72
Benzylparaben	<0.1	<0.5	99.4	99.9<	71

3.2 *Determination of PEC*

By using the combined efficiency of removal, three different PEC values were calculated and are shown in Table 3. Since the combination of activated sludge and chlorination becomes larger than 99% for all three parabens, the combined efficiencies of removal for them were all assumed to be 99%. As can be seen from Table 3, the highest values were the PEC_{utsw} values and they were 10-fold higher than the PEC_{eff} values because of the combination of 99% removal (or 1% residual) in the wastewater treatment and the dilution factor of 10 for the surface water.

3.3 *Acute/chronic toxicity tests for aquatic organisms*

Results of toxicity tests using medaka (*Oryzias latipes*), daphnia (*Daphnia magna*), and green algae (*Pseudokirchneriella subcapitata*) are shown in Table 4. The EC_{50}, LC_{50}, and NOEC values ranged from 0.52 to 9.5 mg L^{-1}. When comparing the toxicities of the selected parabens, the toxicity for benzylparaben was found to be relatively higher than those of the other two.

The toxicities of these three parabens were also estimated using ECOSAR v0.99h,[41] and the predicted values are also shown in Table 4. As can be seen, two predictions were output by treating the parabens as different chemicals, one as esters and the other as phenols. The predicted daphnia EC_{50} and fish LC_{50} values were lower for those treated as phenols while the predicted algal EC_{50} or ChV (i.e., arithmetic mean of NOEC and LOEC) values were lower for those treated as esters. The measured and predicted values are similar and the factor of predicted/measured was no more than 10 although slight underestimations were found for the fish LC_{50} of benzylparaben and a few others.

3.4 *Medaka plasma VTG assays*

Results of medaka plasma VTG tests are summarized in Fig. 1. As can be seen from Fig. 1, the plasma VTG concentration in male fish started to increase after ex-

Table 3
Predicted environmental concentrations (PECs) and maximum measured environmental concentrations (MECs) of selected parabens (μg L^{-1}).

	WWTP effluent (PEC_{eff})	Surface water		Maximum measured environmental concentration (MEC)
		WWTP treated (PEC_{sw})	Untreated (PEC_{utsw})	
n-Butylparaben	0.11	0.011	1.1	0.01 (WWTP effluent in Canada)[13] < 0.027 (Japan Ministry of Environment)[11]
i-Butylparaben	0.0020	0.00020	0.020	< 0.023 (Japan Ministry of Environment)[11]
Benzylparaben	0.015	0.0015	0.15	1 (WWTP effluent in Sweden)[12]

Table 4
Results of measured acute/chronic toxicity for fish, daphnia, and green algae (mg L^{-1}).

	Fish [*Oryzias latipes*]		Daphnia [*Daphnia magna*]				Green Algae [*Pseudokirchneriella subcapitata*]			
Endpoint	Measured 96-h LC_{50}	Predicted 96-h LC_{50}	Measured 48-h EC_{50}	Predicted 48-h LC_{50}	Measured 21-d NOEC	Predicted 21-d ChV	Measured 96-h EC_{50}	Predicted 96-h EC_{50}	Measured 96-h NOEC	Predicted 96-h ChV
n-Butylparaben	2.9	3.5 / 4.8	1.9	2.6 / 7.1	0.80	0.38 / NA	9.5	4.8 / 0.40	0.80	1.1 / 0.32
i-Butylparaben	4.6	3.9 / 5.2	3.3	2.8 / 8.2	0.64	0.42 / NA	4.0	5.5 / 0.44	0.60	1.2 / 0.35
Benzylparaben	0.73	3.0 / 4.3	2.1	2.5 / 5.1	0.84	0.33 / NA	1.2	3.5 / 0.36	0.52	0.95 / 0.29

The 95% confidence interval for measured values are shown within the parentheses. ChV is the arithmetic mean of NOEC and LOEC. The left of the two predicted values was assumed for phenols and the right was assumed for esters.

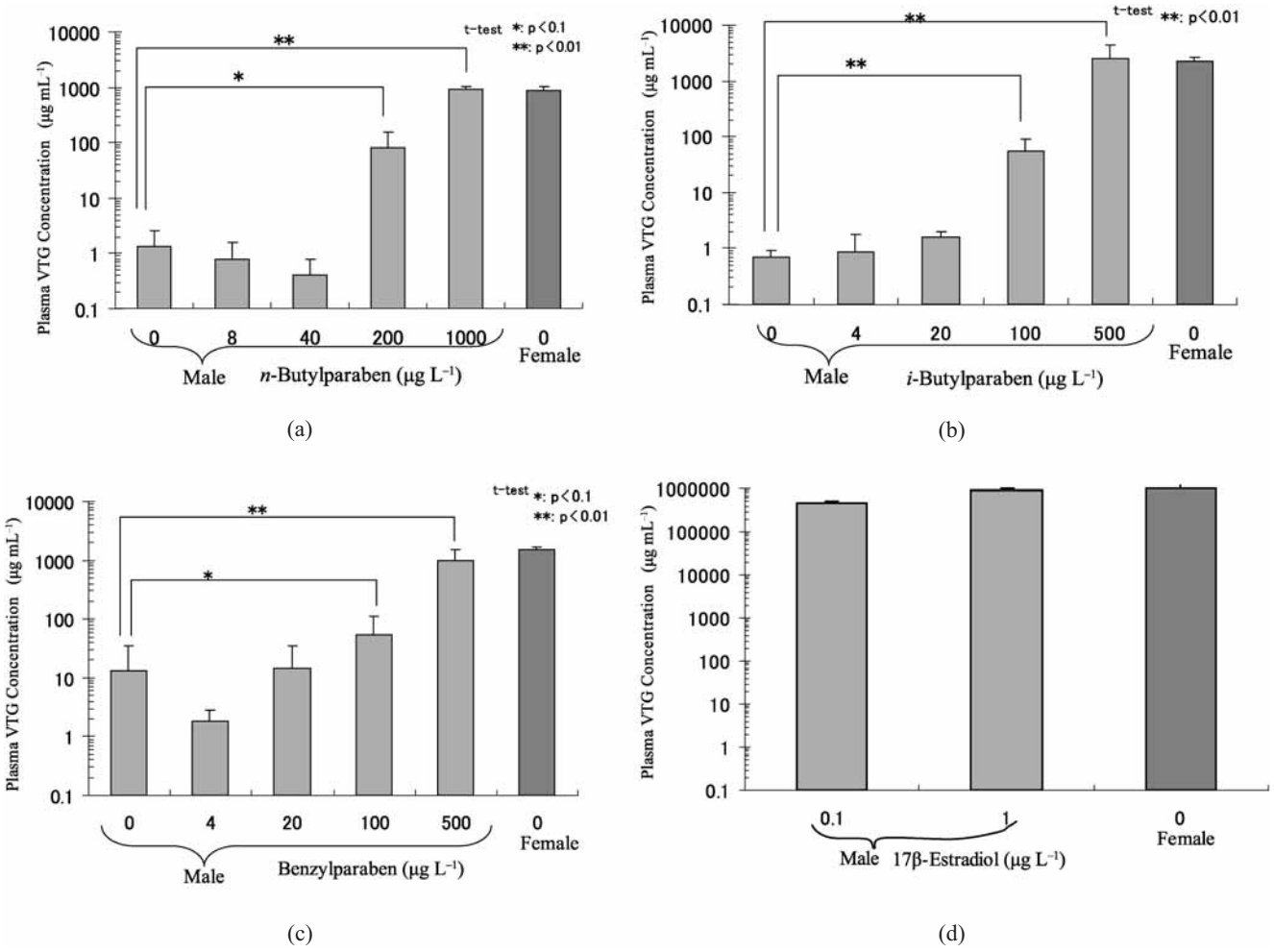


Fig. 1. Plasma vitellogenin concentrations in medaka exposed to (a) *n*-butylparaben, (b) *i*-butylparaben, (c) benzylparaben, and (d) 17β-estradiol.

posure to 200, 100, and 100 μg L^{-1} *n*-butyl, *i*-butyl, and benzylparaben, respectively. Thus, the apparent NOEC values were 40, 20, and 20 μg L^{-1} for *n*-butyl, *i*-butyl, and benzylparaben, respectively. The plasma VTG concentration in exposed male medaka became similar to that in female blank medaka at 1,000, 500, and 500 μg L^{-1} *n*-butyl, *i*-butyl, and benzylparaben, respectively.

3.5 *Medaka DNA microarray analysis*

The comparison of male and female blank samples suggests 41 genes showed sexually differentiated expression. Eleven out of the 41 genes were highly regulated for female, which included VTG-I, VTG-II, CHG-L, CHG-H and estrogen receptor alpha. As results of the DNA microarray analysis for benzylparaben-exposed medaka, we found that the numbers of up-regulated (i.e., exposed/blank > 2) genes were 41, 35, 21 and 6 for 500, 100, 20 and 4 μg L^{-1}, respectively, and those of down-regulated (i.e., exposed/blank > 0.5) genes were 21, 3, 13, and 2 for 500, 100, 20 and 4 μg L^{-1}, respectively. The summarized lists of up-regulated and down-regulated genes for a comparison of benzylparaben-exposed fish and blanks are shown in Table 5. The list includes genes up- or down-regulated at multiple concentration sections of benzylparaben or those genes up-regulated at 500 μg L^{-1} and down-regulated at blank female on the basis of blank male fish (i.e., male/female ratio was less than 0.5). As can be seen in Table 5, genes such as CHGs, VTGs, globins, annexins, p53,

Table 5
List of significantly up-regulated or down-regulated genes for benzylparaben-exposed medaka.

Significantly affected genes[a)]	Accession Number	500 μg L^{-1} mean FD (Exposed/Blank)	100 μg L^{-1} mean FD (Exposed/Blank)	20 μg L^{-1} mean FD (Exposed/Blank)	4 μg L^{-1} mean FD (Exposed/Blank)
Vitellogenin I	AB064320	8781	ND	ND	ND
Vitellogenin II	AB074891	1927	419	ND (5.93)	ND (5.03)
Choriogenin H minor	AB025967	96.0	19.9	3.88	ND (2.04)
Choriogenin L	AF500194	46.7	11.4	ND	2.85
Choriogenin H	D89609	22.6	11.1	3.11	ND
HOXD4B	AB026957	16.8	4.29	ND (2.82)	ND
Estrogen receptor alpha	AB033491	14.5	ND (2.90)	ND	NS
Cytochrome P450 1A	AY297923	13.4	5.53	NS	NS
cDNA similar to probable thioredoxin peroxidase (EC 1.11.1.-) PAGA - human	ECO013193	6.73	5.82	NS	NS
Cytochrome P450 3A40	AF251272	5.90	3.33	2.58	2.27
p53	U57306	3.82	3.05	2.07	2.40
Annexin max4	Y11255	3.62	2.93	2.01	NS
HOXA9B	AB026971	2.52	2.90	3.02	NS
Embryonic beta-type globin	AB080118	2.37	2.28	2.24	NS
Adult beta-type globin	AB080120	2.29	2.43	2.25	NS
Annexin max1	Y11252	2.28	2.40	NS	NS
Me-FKH1, forkhead transcription factor-1 (play critical roles in the maintenance of immune homeostasis)	AB001573	2.28	2.73	NS	NS
Metallothionein (heavy metal responsible gene)	AY466516	2.23	NS	3.16	NS
cDNA similar to retinol-binding protein, cellular - human (specific transport protein to deliver to tissues)	AU243133	NS	2.14	2.07	2.52
cDNA similar to hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5) (chicken), sequence different from AV668802	AV668802	0.01	0.09	ND	0.06
cDNA similar to translation elongation factor eEF-2 - rat	AU241319	0.05	0.07	ND (0.08)	NS

[a)]List of genes up- (Exposed/Blank > 2) or down-regulated (Exposed/Blank < 0.5) at multiple concentration sections of benzylparaben or those genes up-regulated at 500 μg L^{-1} and (blank male)/(blank female) ratio was less than 0.5. ND: not determined because of large statistical variability; NS: not significantly up- or down-regulated (0.5 < Exposed/Blank < 2).

and cytochrome P450 family members were up-regulated. The transcription of some of these genes was induced by benzylparaben at 4 μg L^{-1} and was considered as the $LOEC_{DNA}$, which is much lower than the apparent LOEC found for VTG assays, as shown in Fig. 1.

3.6 *Liposome/water partitioning coefficient*

The liposome/water distribution coefficients (log D_{lipw}) of the three parabens are shown in Table 6. D_{lipw} values larger than 7,000 (log D_{lipw} > 3.83) were found for all three selected parabens. BCF values for medaka fish are also estimated on the basis of the lipid percentage of the medaka body in Table 6.

3.7 *Determination of PNEC*

The PNEC values were calculated for the three parabens from the results of the conventional acute/chronic toxicity tests (Table 3) and are shown with the estimated NOEC values for the medaka VTG assay ($NOEC_{VTG}$) and LOEC values for the medaka DNA microarray test ($LOEC_{DNA}$) in Table 7. Either the algal or daphnia chronic NOEC was used to determine PNEC. The $NOEC_{VTG}$ values were significantly lower than the algal or daphnia NOEC values but were larger than the PNEC values

Table 6
Results of liposome/water partition tests and estimated medaka BCF values.

	log D_{lipw}	Estimated medaka BCF
n-Butylparaben	3.83	440
i-Butylparaben	3.83	440
Benzylparaben	4.06	750

Table 7
Predicted no-effect concentrations of selected parabens calculated from conventional acute/chronic toxicity tests and estimated NOEC for medaka VTG and LOEC for DNA microarray test (μg L^{-1}).

	PNEC	NOEC (medaka VTG)	LOEC (medaka DNA Microarray)
n-Butylparaben	8.0	40	NA
i-Butylparaben	6.0	20	NA
Benzylparaben	5.2	20	≤ 4[a)]

[a)]Effective at 4 μg L^{-1} but not examined at lower concentrations.

because of the assessment factor of 100. The $LOEC_{DNA}$ for benzylparaben was also significantly lower than the algal and daphnia NOEC values and was even lower than the PNEC, which suggests the extremely high sensitivity of the gene expression analysis using the medaka DNA microarray.[23] Although the usage of biomakers such as VTG and microarray for ecological risk assessment is still controversial, we tentatively use them as predictors of PNEC in a subsequent section.

4. Discussion

The removal efficiencies for butylparaben and benzylparaben obtained in this study based on the combination of activated-sludge treatment and chlorination agree with the 96% removal of *n*-butylparaben reported by Lee *et al.*,[13] who apparently had difficulties in measuring *n*-butylparaben in the wastewater effluent near the detection limit. A slightly worse removal of *i*-butylparaben owing to its branched chain was suggested, but the difference was negligible or contrary in this study. The removal efficiency of *n*-butylparaben for chlorination also agrees with those reported by Canosa *et al.*,[14] despite slight differences in chlorine dose and reaction time. Overall, the removal efficiencies for the three selected parabens in WWTPs or septic tanks aremore than 20% of the population in Japan is covered by neither the sewage system nor septic tank coverage, and most of this household effluent is released into the environment without any treatment.

The levels of both *n*-butylparaben and *i*-butylparaben reported by the Japan Ministry of Environment[11] in a monitoring carried out all accross Japan for 33 sampling sites were below the detection limit. Since the PEC_{sw} values were also much lower than the detection limit and the sampling sites are mostly large rivers or bays in urban areas with sewage service coverage, the absence of detection was reasonable. The accuracy of the PEC could be examined by measuring these parabens in WWTP effluents and small urban rivers with no sewage coverage, and such studies should be conducted. No data is available for benzylparaben in Japan. A few researchers outside of Japan have reported the measured values of *n*-butylparaben and benzylparaben, as shown in Table 6. The maximum concentration of *n*-butylparaben in WWTP effluent in Canada was 0.01 μg L^{-1}, much lower than the PEC_{eff}; this large overestimation suggests either the difference in consumption in Japan and Canada or an overestimation of PEC_{eff} mainly attributed to the large unaltered excretion ratio for cosmetics of 99%. The only reported concentration of benzylparaben in a Swedish WWTP was extremely high compared with the PEC values, which again suggests

either the difference in consumption in Japan and Sweden or possible other pathways of benzylparaben pollution such as color developer[7] or industrial use as an intermediate compound not shown in our estimation.

No comparable results for conventional acute/chronic toxicity tests are available except for the ECOSAR predictions shown in Table 4. Compared with the human pharmaceuticals examined by the authors previously, the levels of acute toxicity of the selected parabens are similar to propranolol[15] and fluoxetine;[42,43] both are examples of the most toxic pharmaceuticals.

As far as plasma VTG concentrations are concerned, our results of LOEC and NOEC for *n*-butylparaben are similar to those obtained by Alslev *et al.*[10] who found no significant effect for rainbow trout exposed to 35 μg L^{-1} but found a significant increase for those exposed to 201 μg L^{-1}. Pedersen *et al.*[9] only administered ethylparaben, *n*-propylparaben, and *n*-butylparaben orally and not at comparable doses. Inui *et al.*[8] also investigated plasma VTG concentration in medaka but the lowest concentration of propylparaben was 0.055 μM (= 9.2 mg L^{-1}) and was much higher than ours (i.e., the highest concentration was 1 mg L^{-1}). The slight variance of VTG concentration found in male blanks (Fig. 1) in this study might be attributable to the slight difference in the growing of each fish in each test.

The PEC or MEC/PNEC ratios were calculated, as shown in Fig. 2. As can be seen from Fig. 2, the highest values were found for the MEC/PNEC ratio for benzylparaben followed by PEC_{utsw}/PNEC for *n*-butylparaben; both were above 0.1 as "Necessary to collect further information" as indicated by the Japan Ministry of Environment.[38] For the other PEC or MEC/PNEC analyses, the ratios were below zero and at the level of "No more investigation is necessary." However, monitoring data of aquatic environments are limited and the production/consumption data are possibly inaccurate; accurate data need to be collected vigorously. The highest value of 0.19 for benzylparaben was apparently attributed to the unrealistically high concentration detected from WWTP effluent in Sweden of 1 μg L^{-1}.[12] As presented above, the relatively large PEC_{utsw}/PNEC for *n*-butylparaben was mainly because of the relatively high production/consumption volume of this compound (i.e., nearly 200 t) in Japan. Further investigation, especially that using fish chronic tests such as the early life stage test of OECD Test guideline No. 210, which decreases the assessment factor by 10, is necessary to more accurately determine PNEC.

$NOEC_{VTG}$ and $LOEC_{DNA}$ were tentatively used as predictors of PNEC and the values are shown in Fig. 3. Again, the highest value was found for the MEC/PNEC ratio for benzylparaben of 0.25, which combines with 0.19 determined using conventional acute/chronic tests and becomes in the level of "Necessary to collect further information." Further investigation is necessary, including a large-scale monitoring of the three parabens, a more detailed endocrine disruption analysis, and gene expression analysis such as using a medaka DNA microarray for the two butylparabens that were not analyzed with respect to gene expression in this study.

The measured liposome/water partitioning coefficients shown in Table 5 are similar or slightly higher than those obtained for 17β-estradiol and 17α-ethynylestradiol.[25] The estimated BCF values are all below 1,000, the standard of high bioaccumulation indicated by the Chemical Substances Control Law in Japan,[40] and are considered as "not highly bioaccumulative." Additionally, the estimated BCF values in this study are larger than the real BCF values because of metabolic pathways in aquatic organisms. Alslev *et al.*[10] measured *n*-butylparaben in the plasma of rainbow trout exposed to 35 and 201 μg L^{-1} and found that they were 9 and 183 μg L^{-1}, respectively. The estimated BCF values are 0.2 and 0.9, and these values are much lower than the estimated BCF in this study; however, the concentrations of *n*-butylparaben in the liver of orally administered fish were larger than 1 μg g^{-1} and could be much higher than that in plasma. The measurement of BCF using medaka could be a future work if relatively high MEC/PNEC values are determined after a large-scale monitoring.

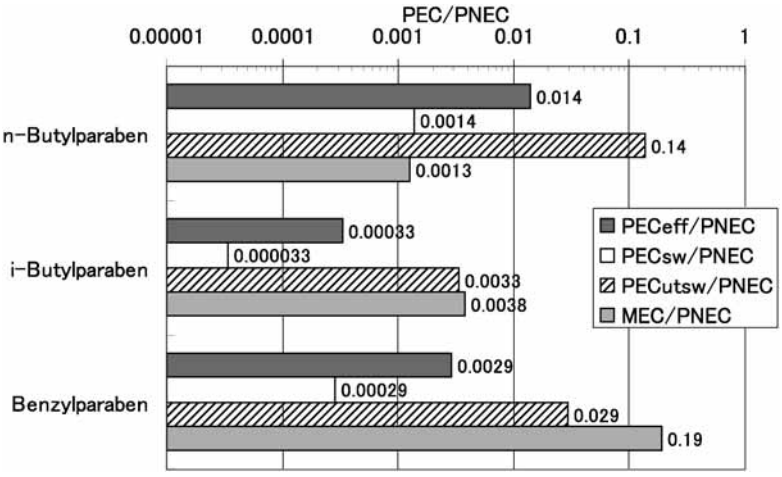


Fig. 2. PEC/PNEC and MEC/PNEC ratios for conventional acute/chronic toxicity tests.

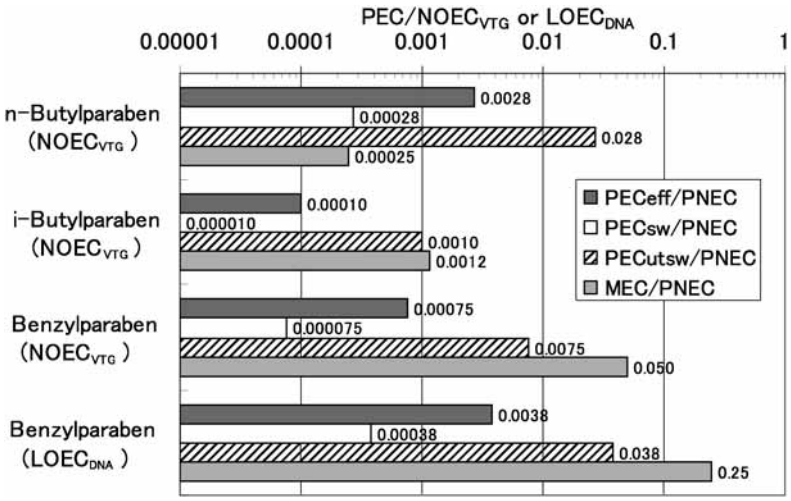


Fig. 3. PEC/PNEC and MEC/PNEC ratios for NOEC$_{VTG}$ and LOEC$_{DNA}$.

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