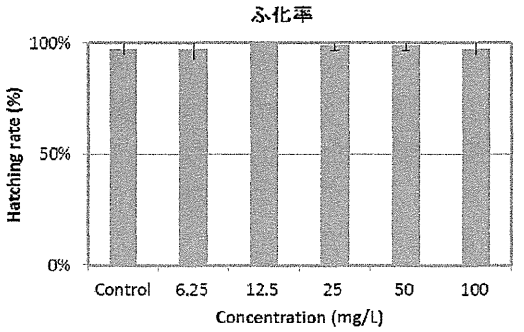
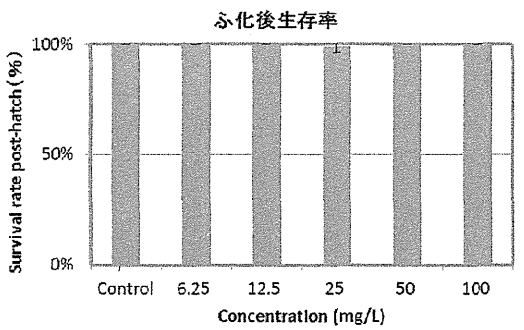
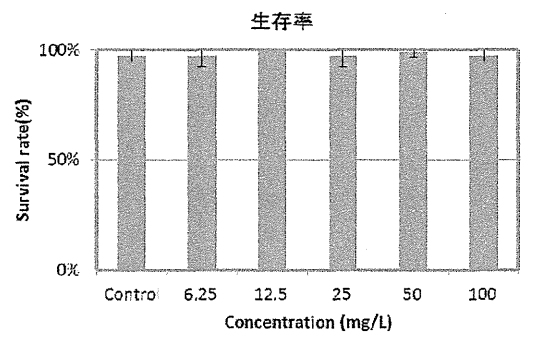






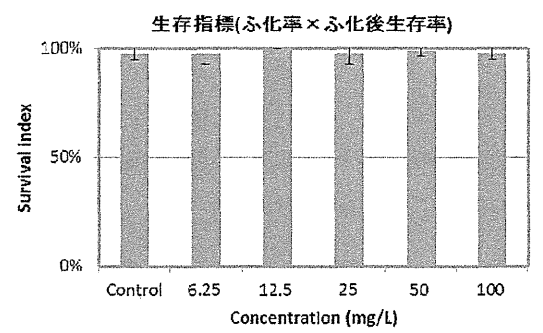
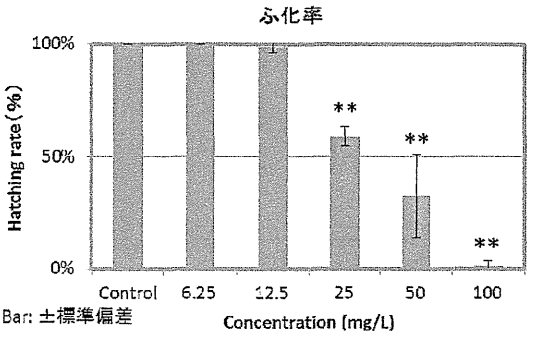
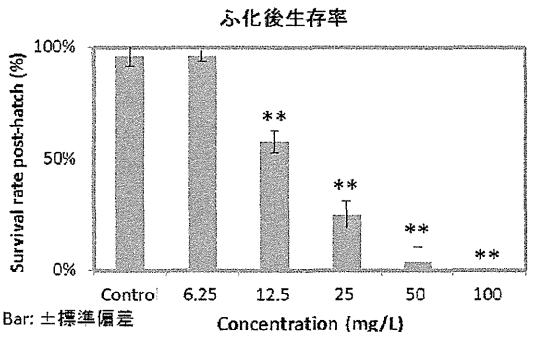
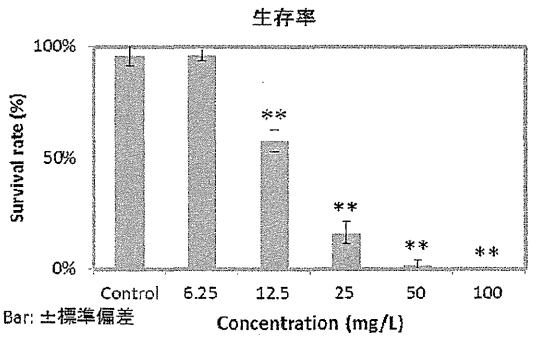


図-5　魚類のふ化率・ふ化後生存率・生存率・生存指標：　ベザフィブラート（Mean±SD　(n=4)







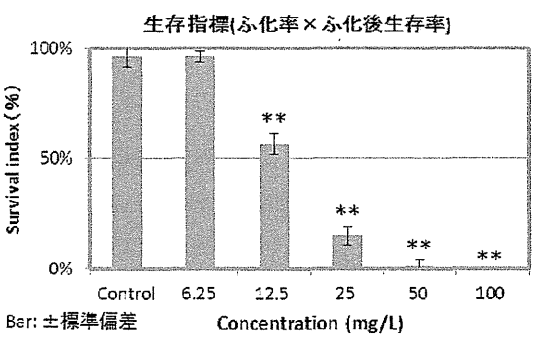


図-6　魚類のふ化率・ふ化後生存率・生存率・生存指標：　ケトプロフェン（Mean±SD (n=4), **: p<0.01 で対照区との有意差あり）

表 **C-1**　ベザフィブラートおよびケトプロフェンの **NOEC** と **EC50**

医薬品名	藻類		甲殻類		魚類 (生存指標)	
	NOEC	EC50	NOEC	EC50	NOEC	EC50
ベザフィブラート	>100mg/L	NA	25 mg/L	49 mg/L	>100mg/L	NA
ケトプロフェン	37.5μg/L	150 μg/L	25 mg/L	32 mg/L	6.25 mg/L	17 mg/L

の異常に伴うと思われる遊泳障害を除き、顕著な行動異常は認められなかった。

ケトプロフェンばく露時はふ化遅延およびふ化後生存への影響が見られ、ふ化率のNOEC は 12.5 mg/L、ふ化後生存率・生存率・生存指標の NOEC は 6.25 mg/L であった。外見異常として、一般に浸透圧調節の障害に起因するとされる腹部浮腫の他、対照区では認められない頭部や総排泄腔部,尾部等における水疱状の皮疹が、12.5mg/L～100mg/L のばく露区で観察された。発生の割合は，何れの試験区でも生存個体の約半数であった。

以上の 3 つの生物試験結果をまとめて表 C-1 に示した。ベザフィブラートは甲殻類に対して繁殖影響、魚類に対して外見異常を示したが、ふ化阻害や致死影響は見られなかった。ケトプロフェンは 3 生物すべてに影響を示し、藻類に対し、μg/L レベルで生長阻害影響を示し、その影響は標準培地に継代してもほとんど回復しないことが分かった。

２．多摩川河川水（下水処理水）の慢性影響に対する医薬品の寄与評価

２．１河川水（下水処理水）の慢性影響

藻類および魚類は St.0~6 のすべての地点において、最高濃度 80%においても影響（藻類：生長阻害、魚類：致死、ふ化遅延等）は見られなかった。ミジンコに対しては、St.2 の多摩川上流水再生センターの放流水に対して、初め 40%，80%の 2 濃度区で試験を行ったところ、40%において半数の試験個体が死亡し、対照区に対して有意な産仔阻害（阻害率 42%）が見られた。NOEC が算出できなかった（40%未満）ため、40%濃度区から 5 濃度区で再試験を行ったところ、40%濃度区における産仔阻害率は 19%と前回よりやや低減し、対照区に対して有意な産仔阻害は見られなかったため、NOEC は 40%となった。しかし、40%濃度区において 80%の試験個体が産仔後に死亡しており、LC50 は 30%と算出された。したがって、St.2 の処理水中には、繁殖阻害

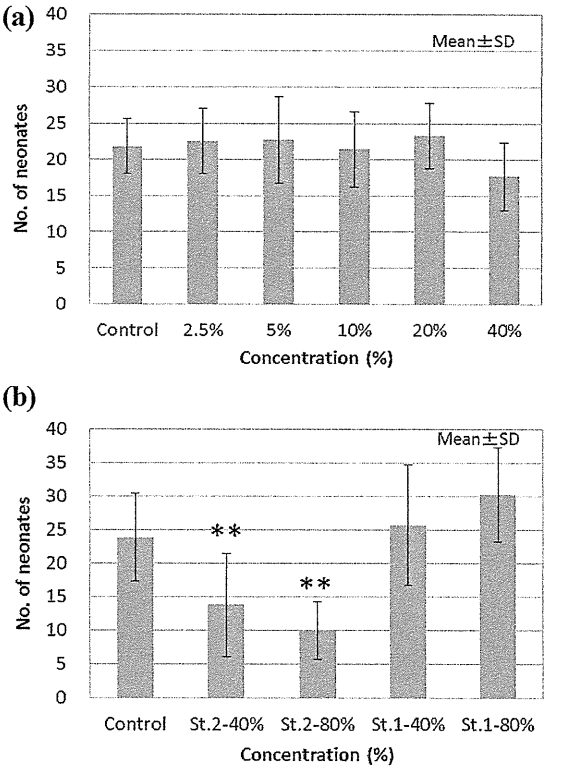


図-7　下水処理水（St.1, 2）ばく露試験における甲殻類の産仔数：(a) St.1, 2 のスクリーニング試験、(b) St.2 の再試験

影響は小さいが、成熟後に大きな致死影響を及ぼす毒性物質が存在すると考えられる。

２．２．河川水中の医薬品濃度

医薬品測定結果は研究協力者の鈴木氏による研究報告書の表 2 に示されたものに同じである（A~F がそれぞれ St.0~6 に当たる）。検出濃度は 0.001～1 μg/L であり、クライスロマイシンが St.1 において最も高濃度で検出された。詳細は氏の報告書を参照されたい。

表 C-2　各生物試験における下水処理水の最大無影響濃度

NOEC (%)	St.0 羽村堰	St.1 八王子	St.2 多摩川上流	St.3 浅川	St.4 北多摩2号	St.5 南多摩	St.6 北多摩一号
藻類	80%	80%	80%	80%	80%	80%	80%
甲殻類	80%	80%	40%	80%	80%	80%	80%
魚類	80%	80%	80%	80%	80%	80%	80%

表 C-3　下水処理水中の各医薬品の NOEC および MEC/NOEC（上から藻類・甲殻類・魚類）

Pharmaceuticals	NOEC (ug/L) for Algae	MEC/NOEC for Algae					
		St.1	St.2	St.3	St.4	St.5	St.6
sulpiride	50000	1.E-05	5.E-06	1.E-05	2.E-05	1.E-05	1.E-05
epinastine	2300	3.E-05	6.E-05	4.E-05	5.E-05	4.E-05	7.E-05
carbamazepine	4300	1.E-05	3.E-05	2.E-05	2.E-05	2.E-05	2.E-05
crotamiton	5200	5.E-05	1.E-04	7.E-05	8.E-05	6.E-05	9.E-05
diclofenac	25000	5.E-06	7.E-06	7.E-06	1.E-05	5.E-06	9.E-06
mefenamic acid	600	4.E-06	4.E-06	3.E-05	9.E-06	1.E-05	1.E-05
fenofibrate	200	ND	ND	ND	ND	ND	ND
SUM		0.0001	0.0002	0.0002	0.0002	0.0002	0.0002

Pharmaceuticals	NOEC (ug/L) for Daphnids	MEC/NOEC for Daphnids					
		St.1	St.2	St.3	St.4	St.5	St.6
sulpiride	100000	5.E-06	2.E-06	7.E-06	8.E-06	7.E-06	7.E-06
epinastine	2800	2.E-05	5.E-05	3.E-05	4.E-05	4.E-05	6.E-05
carbamazepine	10000	5.E-06	1.E-05	6.E-06	9.E-06	8.E-06	9.E-06
crotamiton	6220	4.E-05	8.E-05	6.E-05	6.E-05	5.E-05	7.E-05
diclofenac	2000	7.E-05	9.E-05	8.E-05	1.E-04	7.E-05	1.E-04
mefenamic acid	3900	6.E-07	6.E-07	4.E-06	1.E-06	2.E-06	2.E-06
fenofibrate	200	ND	ND	ND	ND	ND	ND
SUM		0.0001	0.0002	0.0002	0.0002	0.0002	0.0003

Pharmaceuticals	NOEC (ug/L) for Fish	MEC/NOEC for Fish					
		St.1	St.2	St.3	St.4	St.5	St.6
sulpiride	100000	5.E-06	2.E-06	7.E-06	8.E-06	7.E-06	7.E-06
epinastine	48200	1.E-06	3.E-06	2.E-06	2.E-06	2.E-06	3.E-06
carbamazepine	50000	1.E-06	3.E-06	1.E-06	2.E-06	2.E-06	2.E-06
crotamiton	25100	1.E-05	2.E-05	1.E-05	2.E-05	1.E-05	2.E-05
diclofenac	2500	5.E-05	7.E-05	7.E-05	1.E-04	5.E-05	9.E-05
mefenamic acid	1300	2.E-06	2.E-06	1.E-05	4.E-06	5.E-06	6.E-06
fenofibrate	200	ND	ND	ND	ND	ND	ND
SUM		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

2．3．下水処理水（河川水）の慢性影響に対する医薬品の寄与評価

既に慢性影響データがあり、今回測定対象となった医薬品類「スルピリド、エピナスチン塩酸塩、カルバマゼピン、クロタミトン、ジクロフェナクナトリウム、メフェナム酸、フェノフィブラート」の７種について、各測定濃度（MEC）を NOEC で除した値を算出したところ、すべての地点の試料、各生物種に対して 10^{-4}～10^{-6} と極めて低

かった（表 C-3）。その総和をとっても、0.0001~0.0002 と極めて低いため、St.2 においてミジンコに対して観測された影響は、未検出の化学物質（医薬品以外も含む）に因ると推定された。あるいは医薬品類の相乗作用が考えられるが、昨年、同上の医薬品を含む 14 種を、河川水中検出最高濃度の 1000 倍で混合しても影響が見られなかったことから、当該医薬品による相乗影響の可能性は低いと考えられる。2-3 腹目まで産仔してから供試個体が死亡した様子から、Ni による影響の特徴が見られたため、試料を重金属分析に供したところ、ミジンコに対する毒性要因として Ni が関与している可能性が示された。

D．考察

これまでの調査で 14 種の医薬品類の慢性毒性データが取得されたが、生物影響を示す濃度レベルは最も低いケトプロフェン（藻類）でも数十 μg/L であり、下水処理水中検出濃度レベル 0.001～1 μg/L より一桁以上高くなっている。したがって環境中において個別の医薬品が生態リスクを及ぼす可能性は低いと考えられるが、複合影響についてはまだ明らかになっていない部分が多い。慢性毒性試験を用いることで、下水処理水などの複数化学物質を含む試料の総体的な影響を直接評価することができるが、その影響に対する各化学物質群の寄与を定量的に推測するには、物質間の相加作用を仮定した、Σ MEC/NOEC による推定方法に限られている。今回、多摩川流域の下水処理水がミジンコに対して示した影響は、化学分析データや産仔後に供試個体が死亡した様子から、医薬品類ではなく重金属類であると推測されたが、このように毒性要因に関する情報が明らかではない場合に、MEC/NOEC の総和をとる以外に、どのように医薬品類による寄与の有無を推測するべきなのか、新たなアプローチが必要とされる。

E．結論

多摩川流域から検出された医薬品 2 種について、藻類・甲殻類・魚類を用いた短期慢性毒性試験を実施したところ、ベザフィブラートは甲殻類に対して繁殖影響、魚類に対して外見異常を示したが、ふ化阻害や致死影響は見られなかった。ケトプロフェンは 3 生物すべてに影響を示し、藻類に対し、μg/L レベルで生長阻害影響を示し、その影響は標準培地に継代してもほとんど回復しないことが分かった。

さらに、多摩川流域下水道の下水処理水を同様の短期慢性毒性試験に供して総体毒性を評価し、これに対する医薬品類の寄与を、既存データのある 7 種について MEC/NOEC から推定した。MEC/NOEC の総和は 0.0001～0.0002 であり、したがって医薬品類の寄与は極めて小さいと推測されるが、相乗作用などの複合影響を考慮に入れるためには Σ MEC/NOEC に代わる新たなアプローチが必要である。

F．健康危機情報

なし

G．研究発表

1．論文発表

1) Tamura, I., Kagota, K. I., Yasuda, Y., Yoneda, S., Morita, J., Nakada, N., Kameda, Y., Kimura, K., Tatarazako, N., Yamamoto, H.: Ecotoxicity and screening level ecotoxicological risk assessment of five antimicrobial agents: triclosan, triclocarban, resorcinol, phenoxyethanol and p-thymol. J Appl Toxicol. 2012 Jul 13.

2) Yamamoto, H., Tamura, I., Hirata, Y., Kato, J., Kagota, K., Katsuki, S., Yamamoto, A., Kagami, Y., Tatarazako, N.. Aquatic toxicity

and ecological risk assessment of seven parabens: Individual and additive approach. Sci Total Environ. (2011) 410-411, 102-11.

2．学会発表

なし

H．知的財産権の出願・登録状況

（予定を含む）

1．特許取得

なし

2．実用新案特許

なし

3．その他

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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Ⅳ. 研究成果の刊行物・別刷

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Aquatic toxicity and ecological risk assessment of seven parabens: Individual and additive approach

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ABSTRACT

In the present study, aquatic concentrations of seven parabens were determined in urban streams highly affected by treated or untreated domestic sewage in Tokushima and Osaka, Japan. The detected highest concentrations were 670, 207, and 163 ng l^{-1} for methylparaben, *n*-propylparaben, and *n*-butylparaben, respectively in sampling sites with watershed area of no sewer system in Tokushima. Conventional acute/chronic toxicity tests were conducted using medaka (*Oryzias latipes*), *Daphnia magna*, and *Psuedokirchneriella subcapitata* for four parabens, which was consistent with our previous study on three parabens, *n*-butylparaben, *i*-butylparaben, and benzylparaben. The aquatic toxicity on fish, daphnia, and algae was weaker for the parabens with a shorter alkyl chain than those with a longer alkyl chain as predicted by their hydrophobicity. Medaka vitellogenin assays and DNA microarray analysis were carried out for methylparaben and found induction of significant vitellogenin in male medaka at 630 μg l^{-1} of methylparaben, while the expression levels of genes encoding proteins such as choriogenin and vitellogenin increased for concentrations at 10 μg l^{-1} of methylparaben. Measured environmental concentrations (MECs) of seven parabens in Tokushima and Osaka were divided by predicted no effect concentrations (PNECs) and hazard quotient (MEC/PNEC) was determined for individual parabens. The MEC/PNEC was highest for *n*-propylparaben and was 0.010 followed by *n*-butylparaben (max. of 0.0086) and methylparaben (max. of 0.0042). The sum of the MEC/PNEC for the seven parabens was 0.0049. Equivalence factors were assigned for each paraben on the basis of the toxicity of *n*-butylparaben for each species, and *n*-butylparaben equivalence was calculated for the measured environmental concentrations. The MEC/PNEC approach was also conducted for the *n*-butylparaben-based equivalence values. The maximum MEC/PNEC was 0.018, which is lower than the trigger level for further detailed study such as large-scale monitoring for chronic toxicity tests including full-life cycle tests for fish.

1. Introduction

Alkyl esters of *p*-hydroxybenzoic acid, frequently called "parabens" are a class of preservatives widely used in cosmetics such as creams, skin lotions, and shampoos (e.g., Rastogi et al., 1995; Wang and Chang, 1998; Melo and Queiroz, 2010). Some of these compounds are also used in food products (e.g., Zhang et al., 2005) and pharmaceuticals (e.g., Huang et al., 2003). Among these paraben species, the highest concentrations found in various cosmetic products is methylparaben followed by *n*-propylparaben, ethylparaben, and *n*-butylparaben (e.g., Wang and Chang, 1998; Melo and Queiroz, 2010).

Numerous studies have suggested the estrogenic activities of parabens in vitro (e.g., Routledge et al., 1998; Okubo et al., 2001; Morohoshi et al., 2005) and in vivo (e.g., Oishi, 2001; Darbre et al., 2003) for mammalian species, and health aspects of these compounds have long been focused (e.g., Soni et al., 2005). As for aquatic organisms, intraperitoneal injections of ethylparaben, *n*-propylparaben and *n*-butylparaben in rainbow trout were found to cause estrogenic responses such as significant vitellogenin (VTG) induction in the male fish (Pedersen et al., 2000). *n*-Propylparaben was found to induce increases in plasma VTG concentration and up-regulate the expression of VTG and choriogenin (CHG) relevant genes in male medaka (Inui et al., 2003). The exposure of male rainbow trout to *n*-butylparaben also increased the plasma VTG concentration of the fish (Alslev et al., 2005). In our previous study, we found significant VTG induction in male medaka by *n*-butylparaben, *i*-butylparaben, and benzylparaben at 200, 100, and 100 μg l^{-1}, respectively. We also

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conducted DNA microarray analysis for male medaka exposed to benzylparaben and found up-regulations of CHG and VTG-responsive genes at the lowest concentration of 4 $\mu g\,l^{-1}$ (Yamamoto et al., 2007a).

In spite of numerous reports on the estrogenic activities and wide use of parabens, few studies were conducted to determine conventional ecotoxicity on aquatic organisms until the project by Danish Environmental Protection Agency (Madsen et al., 2001), which included acute toxicity of methylparaben, ethylparaben, and *n*-propylparaben on *Daphnia magna* and a green alga *Pseudokirchneriella subcapitata*. Kamaya et al. (2005) also conducted extensive studies on substituted benzoic acids including methylparaben using *D. magna*. The lowest median effect concentration (EC_{50}) and lethal concentration (LC_{50}) of the report was 11.2 $mg\,l^{-1}$. In our previous study (Yamamoto et al., 2007a), we also assessed the ecological risk of *n*-butylparaben, *i*-butylparaben, and benzylparaben based on acute toxicity, VTG induction, and gene expression in medaka (*Oryzias latipes*), and acute/chronic toxicity on *D. magna* and *P. subcapitata*. EC_{50} and no observed effect concentration (NOEC) values for the green alga were as low as 0.52 $mg\,l^{-1}$. On the basis of these toxicity values and the predicted environmental concentration (PEC), we found that the hazard quotient was 0.14 and 0.25 for *n*-butylparaben and benzylparaben, respectively, which suggests the necessity of further detailed investigation. More recently, Terasaki et al. (2008) found much stronger acute toxicity for chlorinated by-products of parabens than parental compounds using *D. magna* and *Vibrio fischeri*. Dobbins et al. (2009) conducted a probabilistic ecological hazard assessment based on acute/subchronic toxicity on daphnia and fathead minnow, and they derived a hazard quotient using the NOEC for the most sensitive assay and found at most 2.3×10^{-4} for benzylparaben, which suggests negligible ecological risk although they warned the potential endocrine effects and impacts from manufacturing facilities in developing countries (Larsson et al., 2007).

Both the toxicity and environmental concentrations of personal care products including parabens were reviewed by Brausch and Rand (2011). The aqueous concentration of parabens ranged from the order of 0.01 to that of 1 $\mu g\,l^{-1}$. For example, benzylparaben, *n*-propylparaben, ethylparaben, and methylparaben were detected at concentrations 1 $\mu g\,l^{-1}$ in effluent from Swedish wastewater treatment plants (WWTPs) (Paxeus, 1996). Lee et al. (2005) analyzed *n*-butylparaben, *n*-propylparaben, ethylparaben, and methylparaben in influent and effluent of Canadian WWTPs and detected at as high as 2.43 and 0.04 $\mu g\,l^{-1}$, respectively. Canosa et al. (2006a, b) also measured the four parabens, methylparaben, ethylparaben, *n*-propylparaben, and *n*-butylparaben, in hospital sewer, WWTP influent and effluent in Spain and the concentration was as high as 2.4, 2.92, and 0.064 $\mu g\,l^{-1}$, respectively. These studies suggest the significantly high removal efficiencies up to 99% of parabens in WWTPs, which agreed to our laboratory experiments (Yamamoto et al., 2007a).

When the parabens are released into the aquatic environment, they are predicted to be slightly accumulative in sediment and moderately persistent against sunlight and microbes according to our previous study in a laboratory (Yamamoto et al., 2007b). Some parabens were detected in river water up to 0.15 $\mu g\,l^{-1}$ in a river in South Wales (Kasprzyk-Hordern et al., 2008) while the concentration is less than 0.017 $\mu g\,l^{-1}$ in a Swiss river (Jonkers et al., 2009). However, the concentration could be higher in effluent-dominated streams (Brooks et al., 2006) and could cause physiological response in aquatic organisms (Ankley et al., 2007), especially in a residential area without sewer system (Tamura et al., 2010).

In the present study, we selected seven parabens and attempted to clarify the ecological risk of these compounds using the similar method used in our previous studies for eight human pharmaceuticals (Yamamoto et al., 2007c) and for three parabens (Yamamoto et al., 2007a) with slight modifications and additions. The objectives of this study were to reveal (1) the aqueous concentrations of parabens in effluent-dominated streams with and without sewer system, (2) the conventional acute/chronic toxicities of the parabens 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 assessments for the seven parabens individually and together.

2. Materials and methods

2.1. Materials

Methyl-*p*-hydroxybenzoate (methylparaben), ethyl-*p*-hydroxybenzoate (ethylparaben), *n*-propyl-*p*-hydroxybenzoate (*n*-propylparaben), *i*-propyl-*p*-hydroxybenzoate (*i*-propylparaben), *n*-butyl-*p*-hydroxybenzoate (*n*-butylparaben), and benzyl-*p*-hydroxybenzoate (benzylparaben) of at least 99, 99, 95, 98, 98, and 99% purity, respectively, were 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). d_4-Methylparaben of at least 98% purity was purchased from Kanto Chemicals Co. (Tokyo, Japan), while $^{13}C_6$-*n*-butylparaben of at least 99% purity was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).17β-estradiol of at least 97% purity was purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA).

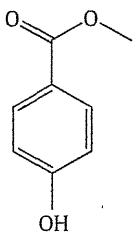
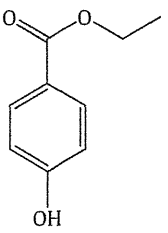
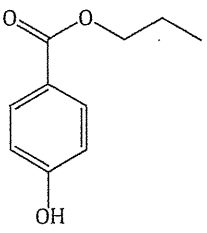
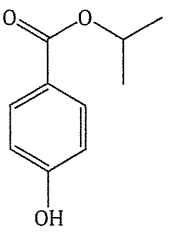
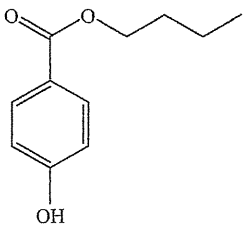
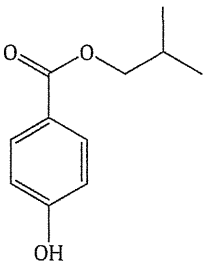
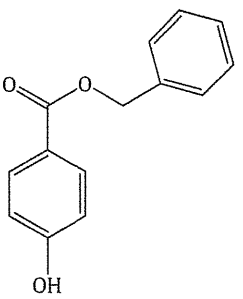
The chemical structures, acidity constant (pK_a) values and octanol–water distribution constants (log D_{ow}) of the parabens selected in this study are shown in Table 1. The log D_{ow}, pK_a and aqueous solubility values were estimated using the ACD software log D suite (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada).

2.2. Determination of aqueous concentrations in urban streams

River water samples were collected from six sites in a Tokushima suburb and six sites in Osaka city, both western part of Japan as shown in Fig. 1. The Tokushima sites include Imagire River (St. 1), Tamiya Creek (St. 2), Tsumeta Creek (St. 3), Utebi Creek (St. 4), Yoshino River (St. 5), and Shinmachi River (St. 6) while the those of Osaka city include Daini-neya River (St. 7), Hirano River (St. 8), Sakuranomiya Bridge of Okawa River (St. 9), Neya River (St. 10), Kyobashi Bridge of Okawa River (St. 11), and Tenpozan wharf (St. 12). The watershed areas of Tokushima sites (St. 1 to 6) are with no or little sewer system while those of Osaka sites (St. 7 to 12) are with sewer system. One liter of water samples were collected at the selected sites twice, a day in January 2010 and February 2010, known amount of surrogate standards, d_4-methylparaben and $^{13}C_6$-*n*-butylparaben, were added, and stored at 4 °C until pretreatment.

Water samples were filtered through a glass-fiber filter (GF/B, Whatman, Maidstone, ME, USA), and 500 ml of the filtrates (duplicates) were extracted using an Oasis HLB 6 cc extraction cartridge purchased from Waters Co. (Milford, MA, USA). Methanol was used for the elution from Oasis HLB. The extract was concentrated to dryness to remove methanol, dissolved in hexane. The clean-up was conducted using Sep-Pak Florisil cartridge (Waters Co, Milford, MA, USA) with hexane, and acetone/hexane (20:80 v/v) was used for the elution. The extract was completely dried under nitrogen purge and then dissolved in methanol for the analysis using an LC/MS/MS (API2000, Applied Biosystems/Life Technologies, Tokyo, Japan). Five microliters of the sample were injected with negative ion modes. An ODS column of 150 mm, 20 mm i.d., and 3 μm diameter (L-Column2 ODS, Chemical Evaluation and Research Institute, Tokyo, Japan) was used, with mixture of acetic acid and acetonitrile for a mobile phase. MRM modes were used for the quantification, and the Q1/Q3 ions are listed in Table 2. Detailed information about the analytical condition is presented in Supplement Information (Table S1).

Table 1
Chemical structure, log D_{ow}, pK_a, and aqueous solubility of the selected parabens.

	Methylparaben	Ethylparaben	*n*-propylparaben	*i*-propylparaben
Chemical structure				
log D_{ow} (at pH 7)	1.86	2.37	2.88	2.73
pKa	8.31	8.31	8.23	8.40
Aqueous solubility	5600 mg l^{-1}	2500 mg l^{-1}	1200 mg l^{-1}	1300 mg l^{-1}
	n-butylparaben	*i*-butylparaben	benzylparaben	
Chemical structure				
log D_{ow}	3.38	3.23	3.54	
pKa	8.22	8.17	8.18	
Aqueous solubility	540 mg l^{-1}	600 mg l^{-1}	160 mg l^{-1}	

log D_{ow}, pKa, and aqueous solubility was estimated using ACD Software log D Suite.

2.3. Acute/chronic toxicity tests

Fish acute toxicity tests were conducted using Japanese medaka (*O. latipes*) provided by the National Institute for Environmental Studies (NIES, Tsukuba, Japan) and acclimated in a laboratory of the University of Tokushima for more than four years. Tests were conducted in conformity with the OECD Guideline for Testing of Chemicals No. 203 (OECD, 1992). Ten fish (10 d-old) were exposed to at least five different concentrations of the parabens in a 100-ml beaker at 25 ± 1 °C. The chemicals were diluted with dechlorinated tap water (approximately 40 mg $CaCO_3$ l^{-1} hardness, pH 7.5 ± 0.2). Half of the paraben solution was replaced every 24 h (static renewal test), and dissolved oxygen (DO) and pH were monitored to check if they are over 80% of saturation and in the range between 6.5 and 8.5. The photoperiod was a 16/8 h light/dark cycle by room light. The median lethal concentration (LC_{50}) was determined based on the measured concentration using Ecotox-Statistics software ver. 2.6 provided by the Japanese Society of Environmental Toxicology to conduct probit or logit conversion analysis.

D. magna were also provided by NIES (Tsukuba, Japan) and used for the daphnia immobilization tests after at least a two-month acclimation period in the laboratory. Tests were conducted in conformity with the OECD Guideline for Testing of Chemicals No. 202 (OECD, 2004). Briefly, 20 neonates of daphnids (less than 24-h-old, five neonates per beaker), were exposed to at least five different concentrations of the parabens in 50-ml beakers. The chemicals were diluted with M4 medium (hardness: 250 mg $CaCO_3$ l^{-1}, pH 8.0). The number of immobilized bodies was counted after 48 h of exposure at 20 ± 1 °C. The photoperiod was a 16/8 h light/dark cycle by room light. The median effective concentration (EC_{50}) was

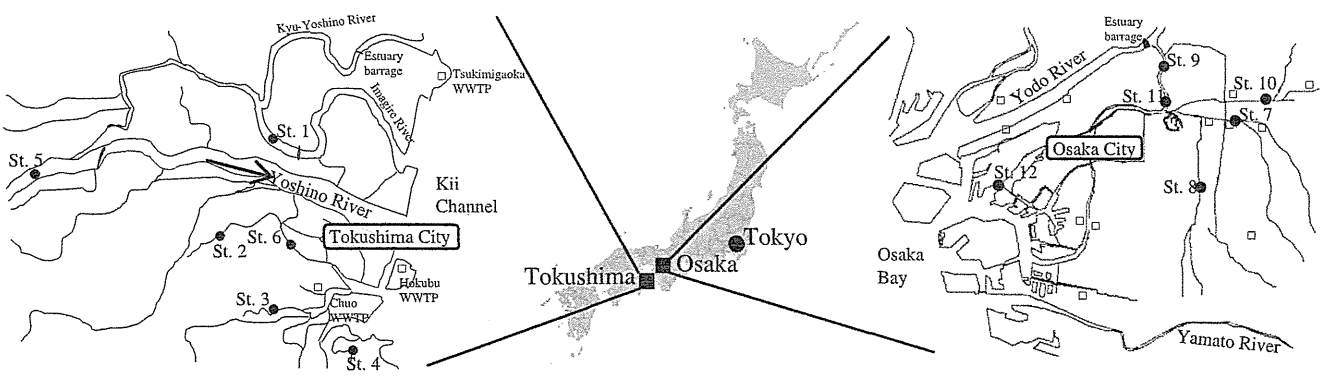


Fig. 1. Map of the sampling sites in Tokushima and Osaka, Japan. □ (white square) represents a WWTP; ● (black circle) represents a sampling site.

Table 2
Monitoring ions, detection/quantification limit, and recovery of the selected parabens in the measurement of aqueous concentration using an LC/MS/MS.

	methylparaben	ethylparaben	*n*-propylparaben	*i*-propylparaben	*n*-butylparaben	*i*-butylparaben	benzylparaben
Q1/Q3 (m/z)	151.01/91.81	165.01/91.90	179.01/91.90	178.95/92.82	193.00/91.89	193.00/91.89	227.15/91.82
LOD/LOQ (ng l^{-1})	1.8/6.1	1.3/4.4	0.8/2.7	1.6/5.3	0.6/2.1	1.2/3.9	0.2/0.7
Recovery	100 ± 18%	102 ± 3%	88 ± 8%	75 ± 10%	91 ± 7%	107 ± 2%	81 ± 27%

determined on the basis of the measured concentrations by probit or logit conversion analysis using Ecotox-Statistics software as with the medaka acute test described above.

Similarly, a daphnia reproduction test was conducted for methylparaben in reference to the OECD Guideline for Chemicals No. 211 (OECD, 2008). Briefly, ten neonates (less than 24 h old, one neonate per test tube) of daphnids were exposed to at least five different concentrations of methylparaben in 20-ml test tubes, which are smaller than the chamber size recommended in the guideline (i.e., 50 to 100 mL). Thus, the DO was carefully checked every 48 h at the replacement of test water but the data should be regarded as preliminary. The other testing conditions were similar to OECD test guideline No. 202 described above. The fecundity of the daphnia was examined by counting the cumulative number of neonates. ANOVA analysis was conducted, followed by Dunnett's test using Ecotox-Statistics software to determine the lowest effect concentration (LOEC). The maximum no-effect concentration (NOEC) in 21 days was determined as the next lower concentration of the LOEC.

Unicellular green algae (*P. subcapitata*) were also obtained from NIES (nies-35, Tsukuba, Japan) and acclimated for at least three months in the laboratory before the tests. Tests were conducted in conformity with the OECD Guideline for Testing of Chemicals No. 201 (OECD, 2006). Briefly, a preincubated algal suspension was exposed to at least five different concentrations of the parabens in 100-ml Erlenmeyer flasks containing OECD medium, at 24 °C with illumination controlled at 5000 lx. The number of algae was measured every 24 h during the 72-h exposure period using a UV/visible spectrophotometer at 450 nm, after calibration with known algal counts. Three replicates were prepared for both blank and all the tested concentrations. The growth rate was determined between 24 h and 72 h. The 50% growth inhibition concentration (EC_{50}) in 72 h was calculated by linear correlation using a log-normalized plot. The 72-h NOEC was also determined using Ecotox-Statistics software (Japanese Society of Environmental Toxicology), as similar to the *Daphnia* chronic toxicity data described above.

2.4. Medaka vitellogenin test and DNA microarray analysis

The medaka vitellogenin (VTG) tests were conducted using three month-old male medaka. The fish were individually exposed to methylparaben for 14 days in a custom-made flow-through system with a 2.3-liter glass tank (two to three replacements per day). The nominal concentrations of methylparaben were set at 40, 200, 1000, 5000, and 25,000 μg l^{-1}. The concentrations were selected based on conventional research (Inui et al., 2003; Alslev et al., 2005; Bjerregaard et al., 2008) and our previous study (Yamamoto et al., 2007a). At least five replicates (n = 5) were prepared for each exposure concentration. The negative control without methylparaben exposure and the positive control with exposure to 0.1 μg l^{-1} 17β-estradiol were prepared for comparison, in addition to female medaka without methylparaben exposure. No solvent was used to prepare methylparaben or 17β-estradiol solution. *Artemia salina* was fed twice a day during the exposure period of 14 d. The light–dark cycle was set at 16-h light and 8-h dark, and the laboratory was maintained at 25 ± 1 °C. After 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 an EnBio VTG analysis kit (Amersham Biosciences Co., Tokyo, Japan).

The DNA microarray analysis was conducted as shown in our previous paper (Yamamoto et al., 2007a). Briefly, male medaka fish exposed to methylparaben were used for DNA microarray analysis in addition to the unexposed male and female controls. These fish were dissected in RNA*later*® solution (Ambion Inc., Austin, TX, USA) to prevent the degradation of total RNA. Then, liver samples were collected and stored in RNA*later*® solution at −20 °C until the total RNA extraction processes from all of the samples were completed. The DNA microarray used in this study was the Medaka EG1000 oligo DNA microarray (its 4×2K platform was provided by CombiMatrix Corporation, Mukilteo, WA, USA) manufactured by Ecogenomics, Inc. (Kurume, Fukuoka, Japan), on which each of the 1000 oligo DNA probes (35–40-mer oligo nucleotides) was spotted twice. The hybridization of the Cy5-labeled target antisense RNA (aRNA) samples and the oligo DNA probes on the microarray was carried out for 16 h at 45 °C in the hybridization solution (25% formamide, 6xSSPE, 0.05%Tween-20, 20 mM EDTA, and 0.04%SDS) recommended by CombiMatrix. After the hybridization, the microarrays were washed and scanned according to the CombiMatrix's hybridization manual, and expression signals were quantified from each of the gene probes. For data analysis, the signal strength data were then normalized by the expression signal of a reference gene (acidic ribosomal phosphoprotein P0) within each of the microarrays, and they were statistically analyzed using arraySTAT™ z-test software (Imaging Research/GE Healthcare, Piscataway, NJ) with offset correction, outlier detection, and $p<0.05$ (significance cutoff) to obtain expression ratio of the methylparaben-exposed group to the unexposed group for each of the genes on the microarray.

2.5. Individual and summational ecological risk assessment

For the individual parabens, predicted no-effect concentration (PNEC) was calculated based on the EC_{50} and LC_{50} values for three acute tests, the NOEC values for chronic toxicity tests, similarly to our previous study (Yamamoto et al., 2007a). $PNEC_{acute}$ and $PNEC_{chronic}$ were determined by dividing the experimental EC_{50} (or LC_{50}) and NOEC values, respectively, by an assessment factor as defined in the followings.

$$PNEC_{acute} = (EC_{50}/LC_{50}\text{of three acute toxicity tests})/AF_{acute} \quad (1)$$

and

$$PNEC_{chronic} = (\text{NOEC in chronic tests})/AF_{chronic} \quad (2)$$

where AF_{acute} is the assessment factor for acute EC_{50} (or LC_{50}) from each of the three trophic levels of the base set (fish, daphnia, and algae). $AF_{chronic}$ is the assessment factor for chronic NOECs. In the present study, an assessment factor of 100 was used for the acute results, and an assessment factor of 10 was used for the chronic NOECs for daphnia and algae based on the strategy of ecological risk assessments conducted by Japan Ministry of Environment (2011). No assessment factor was used for NOEC determined for medaka VTG induction because this biomarker is not directly considered as an adverse effect but is apparently linked to reproductive impairment (Miller et al., 2007). As for the chronic data for daphnia and fish VTG induction, linear

Table 3
Detected concentrations of seven parabens in urban streams in Tokushima and Osaka, Japan.

		Methyl-paraben	Ethyl-paraben	*n*-propyl-paraben	*i*-propyl-paraben	*n*-butyl-paraben	*i*-butyl-paraben	benzyl-paraben
Tokushima sites								
January	Imagire River (St. 1)	185	2.8	24	<1.6	17	2.8	2.1
	Tamiya Creek (St. 2)	191	64	150	2.6	34	4.9	0.7
	Tsumeta Creek (St. 3)	89	15	23	3.4	10	5.4	<0.2
	Utebi Creek (St. 4)	676	59	207	5.7	27	8.6	1.6
February	Imagire River (St. 1)	266	55	164	46	54	13	2.1
	Tamiya Creek (St. 2)	108	21	47	3.2	13	3.8	1.7
	Tsumeta Creek (St. 3)	106	6.3	40	2.6	21	2.1	<0.2
	Utebi Creek (St. 4)	49	7.0	7.5	<1.6	55	1.4	<0.2
	Yoshino River (St. 5)	93	41	58	<1.6	29	3.9	<0.2
	Shinmachi River (St. 6)	171	64	146	<1.6	163	8.5	2.3
Osaka sites								
January	Daini-neya River (St. 7)	199	<1.3	3.1	<1.6	0.9	<1.2	<0.2
	Hirano River (St. 8)	80	<1.3	19	<1.6	0.7	<1.2	<0.2
	Sakuranomiya Bridge (St. 9)	38	<1.3	2.7	<1.6	1.0	<1.2	<0.2
	Neya River (St.10)	41	<1.3	20	<1.6	2.6	<1.2	<0.2
February	Daini-neya River (St. 7)	60	10	1.9	<1.6	<0.6	<1.2	<0.2
	Hirano River (St. 8)	118	12	13	<1.6	1.5	<1.2	<0.2
	Sakuranomiya Bridge (St. 9)	31	<1.3	<0.8	<1.6	<0.6	<1.2	<0.2
	Kyobashi Bridge (St. 11)	25	<1.3	2.6	<1.6	<0.6	<1.2	<0.2
	Tenpozan Harbor (St. 12)	26	<1.3	<0.8	<1.6	<0.6	<1.2	<0.2

Unit: ng l^{-1}

relationship between the logarithm of toxicity values and log D_{ow} (Dobbins et al., 2009) was assumed to fill the lack of the data for ethyl-paraben, *n*-propylparaben, and *i*-propylparaben. Finally, the hazard quotient was determined for a wide variety of measured environmental concentrations (MECs) divided by $PNEC_{acute}$ or $PNEC_{chronic}$.

As for the additivity evaluation, two approaches were used in this study assuming the additive effects of the seven parabens with similar chemical structure and presumably similar mode of action. The one is the simple summation of the hazard quotients for the seven individual parabens. The other was the *n*-butylparaben equivalence approach. EC_{50}, LC_{50} or NOEC values of *n*-butylparaben was divided by those of each paraben to estimate the factor of *n*-butylparaben-equivalence for each paraben. This approach can be considered as concentration addition approach often applicable for endocrine disruptors (Kortenkamp, 2007). The total *n*-butylparaben-equivalence for the MEC was determined using the following equation.

$$\text{(Total } n\text{-butylparaben equivalence)} = \sum(\text{MEC}_i) \times \text{(equivalence factor of paraben specie } i) \quad (3)$$

where MEC_i is the measured environmental concentration of the

Table 4
Results of acute toxicity tests for seven parabens using medaka, *Daphnia magna*, and green alagae.

	Algae (72 h-EC_{50})		Daphnia (48 h-EC_{50})		Fish (96 h-LC_{50})	
	Our results	Literature	Our results	Literature	Our results	Literature
Methyl-paraben	80,000 (ND[b])	91,000[a]	34,000 (30,000–39,000)	11,200[a] 41,100[c] 62,000[e] 24,600[d]	63,000 (50,000–93,000)	<160,000[d]
Ethyl-paraben	52,000 (ND[b])	18,000[a]	7400 (6200–8900)	20,000–50,000[a] 32,000[e] 18,700[d]	14,000 (10,000–19,000)	34,300[d]
n-propyl-paraben	36,000 (ND[b])	15,000[a]	2000 (770–2900)	15,400[a] 23,000[e] 12,300[d]	4900 (3600–6700)	9700[d]
i-propyl-paraben	48,000 (33,000–69,000)		3500 (3100–4200)	30,000[e] 8500[d]	4500 (3100–6800)	17,500[d]
n-butyl-paraben	9500[f]		1900[b] (1700–2600)	9200[e] 5300[d]	3100[b] (2500–8200)	4200[d]
i-butyl-paraben	4000[f]		3300[f]	9800[e] 7600[d]	4600[f]	6900[d]
Benzyl-paraben	1200[f]		2100[f]	6600[e] 4000[d]	730[f]	3300[d]

Unit: μg l^{-1}
Range of 95% confidence level was determined and presented within the parentheses for the acute data.
[a] From Madsen et al. (2001).
[b] Not determined due to the high jump in inhibition ratio.
[c] From Kamaya et al. (2005).
[d] From Dobbins et al. (2009).
[e] From Terasaki et al. (2008).
[f] From Yamamoto et al. (2007a).

Table 5
Results of chronic toxicity tests, medaka vitellogenin assay, and DNA microarray analysis for the seven parabens.

	Algae	Daphnia		Fish			
	Our results (72 h-NOEC)	Our results (21 d-NOEC)	Literature (10 d- LOEC growth/reproduction)	Our results (14 d-NOEC VTG)	Literature (LOEC VTG)	Our results (14 d-LOEC microarray)	Literature (7 d-LOEC growth)
Methyl-paraben	21,000	2400	1500[a]	160		<10	25,000[a]
Ethyl-paraben	18,000	[1600]	2300[a]	[80]			17,000[a]
n-propyl-paraben	7400	[1100]	400[a]	[40]			2500[a]
i-propyl-paraben	11,000	[1200]	2000[a]	[50]	<9200[b]		9000[a]
n-butyl-paraben	800[c]	*800*[c]	300[a]	30[c]	210[d] 134[e]		1000[a]
i-butyl-paraben	600[c]	*640*[c]	200[a]	*20*[c]			3500[a]
Benzyl-paraben	520[c]	*840*[c]	100[a]	*20*[c]		*<4.0*[c]	1700[a]

Unit: μg l^{-1} Predicted values are presented within the parenthesis; Nominal values are italicized.
[a] From Dobbins et al. (2009).
[b] From Inui et al. (2003).
[c] From Yamamoto et al. (2007a).
[d] From Alslev et al. (2005).
[e] From Bjerregaard et al. (2008).

paraben specie *i*. Similarly, the additive ecological risk was assessed for dividing the total *n*-butylparaben equivalence by the toxicity value for *n*-butylparaben.

3. Results

3.1. Measured aqueous concentrations of seven parabens

The lower limit of detection (LOD) and the lower limit of quantification (LOQ) for each paraben are summarized in Table 2. The measured concentrations of parabens in Tokushima and Osaka sites are presented in Table 3. As can be seen, the highest concentration was found for methylparaben followed by *n*-propylparaben and ethylparaben or *n*-butylparaben for most of the sampling sites both in Tokushima and Osaka. Methylparaben was detected in all sampling sites and events, and the concentration ranged from 25 to 676 ng l^{-1} while *n*-propylparaben was detected from 17 out of 19 samples and the concentration ranged from <0.8 to 207 ng l^{-1}. Overall, the detected concentrations were higher in Tokushima sites than Osaka sites. *i*-Propylparaben, *i*-butylparaben and benzylparaben were only detectable in some or all Tokushima sites whereas these parabens were under the detection limit in all the Osaka sites.

3.2. Acute/chronic toxicities for aquatic organisms

Results of acute toxicity tests using medaka, daphnia, and green algae based on analytical concentrations are shown in Table 4. The EC_{50} and LC_{50} values ranged from 730 to 80,000 μg l^{-1}. The EC_{50} or LC_{50} values were lowest for daphnia except for benzylparaben among three aquatic organisms. When comparing the toxicities of the seven parabens, the toxicity of the most hydrophobic paraben, benzylparaben, was the strongest followed by *n*-butylparaben or *i*-butylparaben except for daphnia, which was similarly sensitive against benzylparaben, two butylparabens, and two propylparabens. Methylparaben was least toxic for all three species and the EC_{50} and LC_{50} values ranged from 34,000 to 80,000 μg l^{-1}.

Preliminary acute tests for the mixture toxicity were conducted for methylparaben and *n*-butylparaben using daphnia and medaka, and these results are shown in Figure S1. As can be seen, mixture toxicity values (EC_{50} or LC_{50}) with the unit of *n*-butylparaben-equivalence μg l^{-1} for both species are within the range of 95% confidence level of *n*-butylparaben's EC_{50} or LC_{50} values.

Results of chronic tests (daphnia and algae) are presented in Table 5. As can be seen, the chronic toxicity of methylparaben was much weaker than butylparaben and benzylparaben for both species. Whereas the NOEC values for algae and daphnia were similar each other for benzylparaben and the two butylparabens, the NOEC value for daphnia was

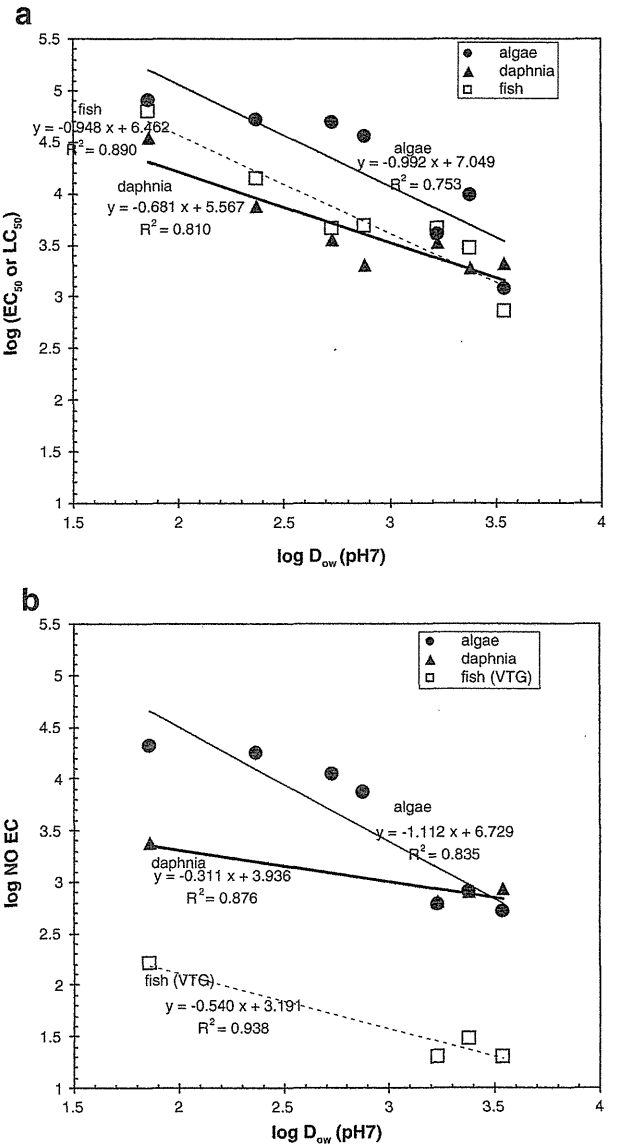


Fig. 2. Correlation between the log D_{ow} of the seven parabens and logarithm of their (a) acute toxicity values (EC_{50} or LC_{50}) or (b) chronic toxicity values (NOEC).

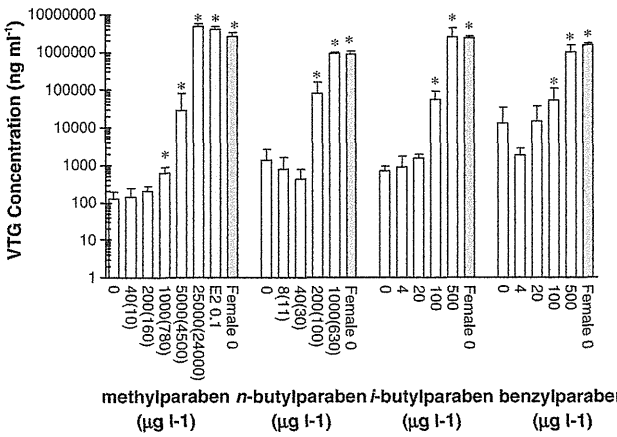


Fig. 3. Results of the medaka vitellogenin (VTG) tests for the methylparaben, butylparaben, and benzylparaben. *$p<0.05$ compared with the male control. Values in parentheses are geometrical means of analytically measured methylparaben or *n*-butylparaben concentrations.

almost one order of magnitude lower than that for algae. The relationship between log D_{ow} values and acute/chronic toxicity values are plotted in Fig. 2 and the chronic toxicity value of ethyl, *n*-propyl, and *i*-propylparaben were predicted and shown in Table 5 within the parenthesis. This plot is further discussed in Discussion section.

3.3. Medaka plasma VTG assays and DNA microarray analysis

Results of medaka plasma VTG tests are presented in Fig. 3 and the summary was shown in Table 5 along with the other chronic results. As can be seen, the plasma VTG concentration in male fish started to increase after exposure to 780 μg l^{-1} of methylparaben. The apparent NOEC values for methylparaben was 160 μg l^{-1} (200 μg l^{-1} as nominal), which was significantly higher than 30 (40 μg l^{-1} as nominal), 20, and 20 μg l^{-1} for *n*-butyl, *i*-butyl, and benzylparaben (Yamamoto et al., 2007a). The plasma VTG concentration in exposed male medaka became similar to that in female blank medaka at 24,000, 630, 500, and 500 μg l^{-1} methyl, *n*-butyl, *i*-butyl, and benzylparaben, respectively. Again, the relationship between log D_{ow} values and $NOEC_{VTG}$ values are plotted in Fig. 2b and that of ethyl, *n*-propyl, and *i*-propylparaben was predicted and shown in Table 5 within the parenthesis.

The significantly up-regulated or down-regulated genes in the medaka DNA microarray analyses are summarized in Table 6. The lowest concentration series in the methylparaben test (10 μg l^{-1}) induced the significant up-regulation of 13 genes including estrogen-responsive genes such as vitellogenin 2, choriogenin L, choriogenin, and estrogen receptor α as with the positive control while 10 genes were down-regulated.

3.4. Screening level ecological risk assessment

Hazard quotients were determined for both Tokushima and Osaka sites for all individual parabens in Fig. 4a. These values were summed and also presented as "Σparabens" in Fig. 4a. As can be seen, the hazard quotient was the highest for *n*-propylparaben followed by *n*-butylparaben and methylparaben. The highest MEC/PNEC value for individual paraben was 0.010 for *n*-propylparaben while that for "Σparabens" was at most 0.017.

n-Butylparaben equivalence was determined for the seven parabens from the experimental or predicted toxicity values in Table S2. Based on these factors, the measured concentrations of seven parabens were converted into *n*-butylparaben equivalence and MEC/PNEC value was determined for acute/chronic tests for three aquatic organisms as shown in Fig. 4b. As shown in Fig. 4b, the maximum value was 0.018 for daphnia acute tests with assessment factor of 100.

Table 6
List of up-regulated and down-regulated genes in medaka DNA microarray tests.

Descriptions of significantly affected genes	Accession number	10 μg l^{-1} mean ratio (methylparaben/CTL)	0.1 μg l^{-1} mean ratio (E2/CTL)
Up-regulated			
Adult beta-type globlin	AB080120	5.3	2.3
Apolipoprotein E [*Oplegnathus fasciatus*]	AJ457362	3.5	1.9
Choriogenin H	D89609	2.7	17
Choriogenin H minor	AB025967	37	320
Choriogenin L (cds)	AF500194	18	120
Embryonic alpha-type globin	AB026052	7.1	2.0
Embryonic beta-type globin	AB080118	8.1	1.7
Estrogen receptor alpha	AB033491	8.3	21
Glyceraldehyde-3-phosphate dehydrogenase [*Rattus norvegicus*]	AU179061	2.4	1.7
HMG CoA-reductase (HMG-CoA), partial cds	EF537031	3.4	1.6
Neural precursor cell expressed, developmentally down-regulated 8 (NEDD8) [*Homo sapiens*]	BJ001162	5.5	7.4
Seryl-tRNA synthetase (SARS) [*Homo sapiens*]	BJ489333	4.4	7.8
Vitellogenin 2	AB074891	3.3	77
Down-regulated			
Annexin max 2	Y11253	0.23	0.86
Beta-alanine synthase [Rattus norvegicus]	AU179036	0.38	0.50
cdc42 homolog precursor [Salmo salar]	BJ492759	0.35	0.09
Cytidylate kinase (pig)	AV669628	0.37	0.22
KFH-R	AB001604	0.37	0.37
MC6AST3, astacin like metallo-protease	AB256947	0.35	ND
Reelin	AB072425	0.44	0.12
SH2 domain containing 4B [Gallus gallus]	AV668553	0.29	0.31
ZPC domain containing protein 4	AF128812	0.38	0.18
ZPC domain containing protein 5	AF128813	0.42	0.17

4. Discussion

The detected concentration of parabens in the present study in Osaka sites (Table 3) was at most 199 ng l^{-1} and was comparable to the reported concentrations in surface waters (Kasprzyk-Hordern et al., 2008; Jonkers et al., 2009; Ramaswamy et al., 2011). Some of the sampling sites in Osaka were affected by the effluent of WWTPs such as Sts. 7, 8, and 10 (Fig. 1), where relatively higher concentration was found. For the other sites, residual of parabens are sufficiently diluted or eliminated. Parabens are found to be highly degradable in WWTPs (Lee et al., 2005; Yamamoto et al., 2007a), moderately persistent in the river water (Yamamoto et al., 2007b), and only benzylparaben is easily photodegraded (Yamamoto et al., 2007b), which resulted in relatively low concentrations detected in Osaka sites, where more than 90% is with sewer system.

In contrast, the concentrations found in Tokushima sites (i.e., maximum of 676 ng l^{-1}) are much higher than the reported values in surface water (Kasprzyk-Hordern et al., 2008; Jonkers et al., 2009; Ramaswamy et al., 2011). The detected concentrations in Tokushima sites are rather similar to those reported in effluent or even influent of WWTPs (Paxeus, 1996; Lee et al., 2005; Canosa et al., 2006b). The selected urban creeks, especially Sts 1 to 4, are highly contaminated by treated or untreated domestic sewage with no sewerage system

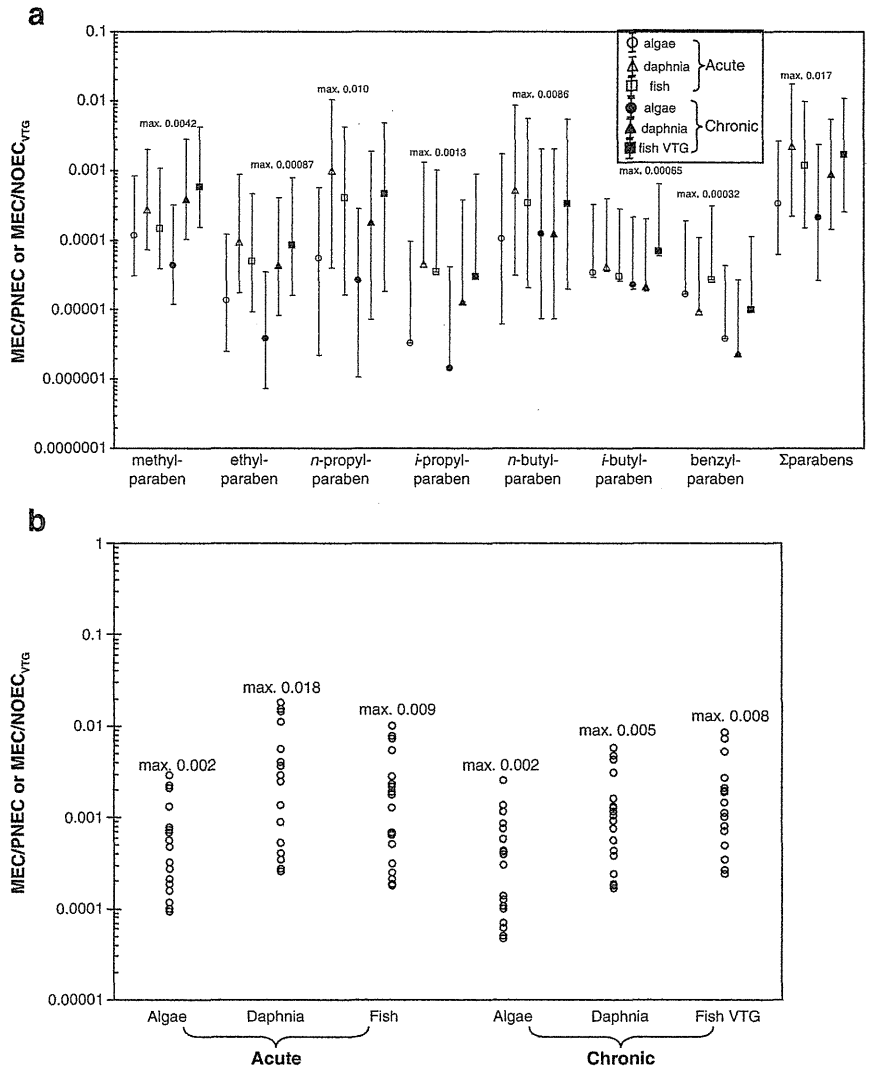


Fig. 4. Results of the preliminary ecological risk assessment for (a) seven individual parabens and their summation, and (b) *n*-butylparaben equivalence-based approach for three aquatic organisms (green algae, daphnia, and medaka) using acute and chronic toxicity values and the measured environmental concentrations in Tokushima and Osaka, Japan.

in the watershed area but approximately 40% of residents are using septic tanks. The reported concentrations may represent the hot spot contamination of parabens in streams in residential area with no sewer system both in developed and developing countries. In our previous study (Yamamoto et al., 2007b), butylparaben and benzylparaben were found to be moderately accumulative in river sediment (i.e., K_d is less than 100 L kg^{-1}), and the significant accumulation of the less hydrophobic methyl, ethyl, and propylparaben is unlikely.

Acute toxicity of parabens for three aquatic organisms was also examined in this study for four parabens in addition to our previous studies on three parabens (Yamamoto et al., 2007a). As can be seen from Table 4, the acute toxicity of methyl, ethyl, and propylparabens was comparable to the previous studies such as Madsen et al. (2001) and Dobbins et al. (2009). The linear relationship between log D_{ow} and logarithm of EC_{50} or LC_{50} was also clear for all the three aquatic organisms (Fig. 2a). Some antimicrobial agents such as triclosan and triclocarban are specifically toxic for algae (e.g., Tatarazako et al., 2003) but the similar phenomena were not observed for a class of preservatives, parabens. As for the mixture toxicity, our preliminary acute experiments suggest the applicability of the concentration (or dose) addition assumption as discussed by Kortenkamp (2007) for endocrine disruptors.

Chronic effects of methylparaben were investigated in present study in addition to the three parabens as reported in our previous paper (Yamamoto et al., 2007a). Some of these data are in screening level because the nominal concentrations are used instead of analytically measured ones to determine toxicity values. Parabens are not so persistent in the static system and the applicability of those values based on nominal exposure concentrations is limited. These tests were not conducted for ethylparaben and propylparabens because of the limitation of cost and time but the relatively high linear correlation between log D_{ow} and logarithm NOEC (Fig. 2b) can provide acceptably reliable estimate of the toxicity values of these paraben species. Dobbins et al. (2009) presented poor correlation between logarithm of the daphnia reproduction NOEC and log P but their duration of the exposure was 10-d instead of 21-d recommended in OECD No. 211 for *D. magna* (OECD, 2008). *D. magna* starts to deliver neonates after 7 to 8 days old and the 10-d reproduction is the counting of only first (and possibly second) brood, which may be unstable and therefore make it difficult to observe any significant effect. Our results also had deficiency in the volume of chamber, which may slightly affect the reproduction of daphnia although the DO concentrations of the testing solution were carefully checked at every replacement of testing waters.

As far as medaka VTG induction is concerned, the literature LOEC values for *n*-butylparaben (Alslev et al., 2005; Bjerregaard et al., 2008) were much higher than our NOEC values (Fig. 3, Table 5) but our dilution factor was 5 and the LOEC values were similar each other. Inui et al. (2003) did not investigate even lower concentration of propylparaben and the results are not comparable. The NOEC values for medaka VTG was one to two orders of magnitude lower than those for daphnia reproduction and algal growth, although the VTG induction is a biomarker and not directly means the "adverse" effect. The lowest methylparaben concentration of 10 $\mu g\,l^{-1}$ even provided with significant up-regulation of some estrogen-responsive genes such as VTG 2 and CHG L in the cDNA microarray analysis as similar to what we found for benzylparaben in our previous study (Yamamoto et al., 2007a). The induction of these estrogen-responsive genes could potentially affect the reproduction of medaka fish in full-life cycle or multigenerational test although the bioconcentration of methylparaben is limited compared to benzylparaben, which is considered as more hydrophobic. Several other genes are up-regulated or down-regulated as presented in Table 6 but further dose–response relationships need to be examined to confirm the relevance between the exposure to parabens and the targeted responses. The measured environmental concentrations of methylparaben is still two to three orders of magnitude lower than 10 $\mu g\,l^{-1}$ while the maximum concentration of benzylparaben reported (1 $\mu g\,l^{-1}$ by Paxeus, 1996) was comparable to the previously reported LOEC for the microarray analysis of 4.0 $\mu g\,l^{-1}$ (nominal value).

The individual ecological risk assessment for seven parabens suggests the sufficiently low risk of all the paraben species (Fig. 4). The highest risk was observed for *n*-propylparaben and the maximum and median MEC/PNEC was 0.010 and 0.0010, respectively, followed by *n*-butylparaben and methylparaben. Our results are slightly different from the results of probabilistic assessment conducted by Dobbins et al. (2009) using the 95 percentile of the limited sets of the detected concentrations of the parabens (e.g., Benijts et al., 2004; Lee et al., 2005; Kasprzyk-Hordern et al., 2008). Dobbins et al. (2009) presented the highest hazard quotient of 0.00023 for benzylparaben followed by *i*-butylparaben (0.00011) and methylparaben (0.000090). The summed hazard quotient of Dobbins et al. (2009) is slightly lower than our MEC/PNEC (maximum of 0.017) mainly because of the use of $NOEC_{VTG}$ and assessment factor of 100 or 10 for acute or chronic toxicity, respectively.

The *n*-butylparaben equivalence approach slightly changed the final MEC/PNEC value and became at most 0.018, 0.0099, and 0.0084 for daphnia acute, fish acute, and fish VTG induction test. The slight difference was probably attributed to the slight discrepancy of toxicity values for each paraben from the linear regression line shown in Fig. 2 and the slight difference in the dose–response curve in each paraben. This concentration addition approach is probably useful for the reasonable ecological risk assessment for structurally similar, presumably physiologically similar, group of PPCPs.

The use of detected concentrations in highly effluent-dominated urban streams in the present study may also provide more reliable and comprehensive ecological risk of seven parabens. Even though the sampling sites are highly affected by treated and untreated domestic sewage, the risk level was far below the trigger level for the further collection of the relevant information (i.e., 0.1) suggested by Japan Ministry of Environment (2011). However, this work lacks regular fish chronic tests such as early-life stage tests and the chronic effects of ethylparaben and propylparaben on daphnia were predicted. Furthermore, the concentration of parabens in surface water could be higher in extremely effluent-dominated streams in semi-arid or arid area or dry season and become the order of 1 $\mu g\,l^{-1}$ because the concentration of parabens in influent or effluent of WWTPs becomes micrograms per liter level (Paxeus, 1996; Lee et al., 2005; Canosa et al., 2006a, b).

Further detailed study may be necessary such as large-scale monitoring in the highly effluent-dominated streams all over the world in full season and chronic toxicity tests including partial or full-life cycle tests for fish because this class of compounds are weakly estrogenic and could affect the reproduction of vertebrates such as fishes. Further investigation is also necessary for synergistic effects for multiple paraben species and the other antibacterial agents such as triclosan and triclocarban, which are found to exert higher ecological risk than parabens (e.g., Ramaswamy et al., 2011; Brausch and Rand, 2011).

Supplementary materials related to this article can be found online at doi:10.1016/j.scitotenv.2011.09.040.

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