



## RESEARCH ARTICLE

# Summary of 17 chemicals evaluated by OECD TG229 using Japanese Medaka, *Oryzias latipes* in EXTEND 2016

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## Abstract

In June 2016, the Ministry of the Environment of Japan announced a program “EXTEND2016” on the implementation of testing and assessment for endocrine active chemicals, consisting of a two-tiered strategy. The aim of the Tier 1 screening and the Tier 2 testing is to identify the impacts on the endocrine system and to characterize the adverse effects to aquatic animals by endocrine disrupting chemicals detected in the aquatic environment in Japan. For the consistent assessment of the effects on reproduction associated with estrogenic, anti-estrogenic, androgenic, and/or anti-androgenic activities of chemicals throughout Tier 1 screening to Tier 2 testing, a unified test species, Japanese medaka (*Oryzias latipes*), has been used. For Tier 1 screening, the in vivo Fish Short-Term Reproduction Assay (OECD test guideline No. 229) was conducted for 17 chemicals that were nominated based on the results of environmental monitoring, existing knowledge obtained from a literature survey, and positive results in reporter gene assays using the estrogen receptor of Japanese medaka. In the 17 assays using Japanese medaka, adverse effects on reproduction (i.e., reduction in fecundity and/or fertility) were suggested for 10 chemicals, and a significant increase of hepatic vitellogenin in males, indicating estrogenic (estrogen receptor agonistic) potency, was found for eight chemicals at the concentrations in which no overt toxicity was observed. Based on these results, and the frequency and the concentrations detected in the Japanese environment, estrone, 4-nonylphenol (branched isomers), 4-tert-octylphenol, triphenyl phosphate, and bisphenol A were considered as high priority candidate substances for the Tier 2 testing.

## KEYWORDS

endocrine disrupting effect, Japanese medaka, OECD TG229, *Oryzias latipes*, reproduction

Yukio Kawashima and Yuta Onishi equally contributed on this study.

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## 1 | INTRODUCTION

The Ministry of the Environment of Japan (MOE) administered its fourth program on endocrine disrupting effects of chemical substances (EDC) “EXTEND2016” in June 2016, a continuation of EXTEND2010 (MOE, 2010, 2016). EXTEND2016 has the following five structures: (1) actions and effects assessment and test method development, (2) survey of environmental concentrations and exposure assessment, (3) risk assessment of environment and risk management, (4) accumulation of knowledge on endocrine disruptors, and (5) promotion of international cooperation and information. Under the EXTEND2016, the following three effects relevant to endocrine disruptive activity of chemicals has been assessed using aquatic organisms: (1) effects on reproduction of fish relevant to estrogenic, anti-estrogenic, androgenic, and anti-androgenic activities using Japanese medaka, *Oryzias latipes*, (2) effects on development of amphibians including metamorphosis relevant to thyroid hormone-like and/or anti-thyroid hormone activity using *Xenopus laevis*, and (3) effects on growth of invertebrates relevant to juvenile and/or molting (ecdysone) hormone-like activity using *Daphnia magna* (MOE, 2016; Iguchi et al., 2020).

In the EXTEND2016 program, in order to assess the environmental risks of chemicals with endocrine disruptive activity, not only the presence or absence of the actions on the endocrine system but also the degree of adverse effects need to be investigated. Thus, the framework for testing and assessment has been implementing a two-tiered strategy that is constructed involving ecotoxicological tests necessary to identify the activities of chemicals on endocrine systems and to characterize the adverse effects caused by endocrine disrupting chemicals. Also, the chemicals to be tested and assessed have been selected, considering both their hazardous properties and the potential for exposure in the aquatic environment, from the view of future environmental risk assessment, just like in the EXTEND2010 program (Iguchi et al., 2020). Principally, the chemicals detected in the environmental monitoring surveys conducted by the MOE have been pooled as a preliminary group of chemicals for evaluation. The chemicals in Class 1 Designated Chemicals of the Law of Pollutant Release and Transfer Register (PRTR) system, the substances listed in the Endocrine Disruptor Screening Program (EDSP) of the US Environmental Protection Agency (US EPA, 2009), and the substances of very high concern (SVHC) listed by the European Chemical Agency (Dinu, 2019) were added to the preliminary group in the EXTEND2016. Regarding the chemicals in the preliminary group, a literature search and reliability evaluation of the collected literature were performed, and then a comprehensive judgement, based on the existing knowledge obtained from the literature, was done on whether each chemical could be considered as “Chemicals that can be subjected to test for endocrine disrupting effects”, taking into account the definition of endocrine disruptors by Damstra et al. (2002) (MOE, 2016).

As described above, in the EXTEND2016 program, a two-tier framework, that is, Tier 1 and Tier 2, has been adopted for testing and

assessment of endocrine disrupting chemicals detected in the aquatic environment. For Tier 1 in vitro screenings, reporter gene assay systems (RGAs) using fish estrogen receptor (Esr, known as ER in mammals) (Katsu et al., 2010; Lange et al., 2012; Miyagawa et al., 2014), fish androgen receptor (Ar, known as AR in mammals) (Lange et al., 2015), thyroid hormone receptor (TR) of amphibians (Oka et al., 2013), juvenile hormone receptor (JhR) (Miyakawa et al., 2013; Miyakawa & Iguchi, 2017; Tanaka et al., 2019), or ecdysone receptor (EcdR) (Kato et al., 2007) of *Daphnia* species were established. Also, the applicability of OECD TG229 (Fish Short-Term Reproduction Assay, FSTRA) using Japanese medaka (OECD, 2012) in Tier 1 in vivo screening was demonstrated for identifying the effects of chemicals with Er agonistic and antagonistic activity, and Ar agonistic activity, potency on the reproduction of fish, as well as steroidogenesis inhibitory activity. In addition, the reference chemical studies demonstrated that the effects of the progestin that masculinized and reduced reproductive activity in females by activating Ar (Onishi et al., 2021) were similar to the reports on other small test fish (Ellestad et al., 2014; Frankel et al., 2016; Svensson et al., 2013). Regarding the endpoints adopted for the in vivo fish assay, vitellogenin (VTG) and secondary sex characteristics (SSC) in Japanese medaka were sensitive to Esr agonistic and antagonistic, and Ar agonistic, activities of chemicals (Onishi et al., 2021). On the other hand, the TG229 assay using Japanese medaka was less sensitive in the detection of anti-androgenic (Ar antagonistic) activity of chemicals (OECD, 2012; Onishi et al., 2021). Japan has currently been developing a new method for in vivo screening, that is, the Juvenile Medaka Anti-androgen Screening Assay (JMASA), which can detect anti-androgenic effects, as indicated in the OECD guidance document (GD) No. 150 (OECD, 2018).

Tier 1 assessment is performed based on existing knowledge obtained from the literature, and the results of the Tier 1 screening assays and “Chemicals with actions relevant to endocrine disruption” are judged to be candidates for Tier 2 testing. In the two-tier framework for testing and assessment of EXTEND2016, the same biological species (i.e., Japanese medaka, *Xenopus* species, and *Daphnia* species) are offered to both the Tier 1 screening and the Tier 2 testing for consistent assessment of each endocrine disrupting effect. For Tier 2 testing to characterize hazardous effects on reproduction, the Medaka Extended One Generation Reproduction Test (MEOGRT), which is published as OECD TG240 (OECD, 2015), is adopted.

Under the EXTEND2010 and 2016 programs, based on the strategy as explained above, literature surveys including reliability evaluation were performed for the chemicals selected based on the results of the national environmental monitoring. And the TG229 assay using Japanese medaka was conducted for twenty chemicals which had estrogenic, anti-estrogenic, androgenic, and/or anti-androgenic activities as suggested by RGAs using medaka Esr1 (mEsr1, known as ER $\alpha$  in mammals) or mAr $\beta$  (known as AR $\beta$  in mammals, Ogino et al., 2016). This manuscript describes the results of the Tier 1 screening assays for 17 chemicals, except three chemicals previously reported (Onishi et al., 2021).

## 2 | MATERIALS AND METHODS

### 2.1 | Procedure of test chemical selection

Environmental concentrations of chemicals have been investigated, utilizing the results of the environmental monitoring surveys, that is, Environmental Survey and Monitoring of Chemicals, Water Quality Survey of Public Water Areas, and Research on the Existence of Chemicals or Environmental Studies on the Pesticides, conducted by the MOE, and literature surveys were performed for the 209 substances of the preliminary group of chemicals for evaluation, in which the chemicals detected in the aquatic environment in the monitoring survey because FY1996 and the chemicals in Class 1 Designated Chemicals of Law of PRTR system were included. In the survey, literature on in vitro, in vivo, and epidemiological studies regarding the subject chemicals was searched with a designated set of keywords, using literature search engines (PubMed and JDream III), and reliability evaluation of the literature collected was performed for the chemicals identified in ten or more literatures found. Reliability of the studies described in the literature was evaluated by whether “Materials and Methods” were sufficiently described so that “Results” could be substantiated, as well as whether any plausible relationship to endocrine disrupting effects could be identified or not. Based on the existing knowledge obtained from reliable literature, a comprehensive judgment on whether the chemical nominated could be considered a chemical that could be subjected to testing for endocrine disrupting effects was carried out by experts (MOE, 2016).

Up until October 2019, the process of the evaluation has been completed for 198 chemicals, and 129 substances were identified as “chemicals that can be subjected to tests for endocrine disrupting effects.” Of these 129 substances, 90 were assayed by in vitro RGAs using mEsr1, mAr $\beta$ , TR $\beta$  of *X. tropicalis*, and/or EcdR of *D. magna* and positive responses in any of the targeted endocrine disruptive activities were detected in 37 of them (data unpublished). Within these 37 chemicals, an in vivo TG229 assay using Japanese medaka was conducted for 20 chemicals, which included 19 that RGAs using mEsr1 suggested as having either estrogenic or antiestrogenic activity and flutamide for which anti-androgenic potency was indicated in an mAr $\beta$  RGA. However, only the results of 17 assays are presented in this report because the results of three studies for 17 $\beta$ -estradiol (E2), 17 $\alpha$ -ethynylestradiol (EE2), and flutamide were previously reported (Onishi et al., 2021).

As for the 17 chemicals reported here, their concentrations detected in ambient surface water in Japan are summarized in Table 1. Sixteen of them, other than pendimethalin (PMD), were detected in the environmental surveys. Bisphenol A, estrone (E1), 4-nonylphenol (4NP), 4-*tert*-octylphenol (4tOP), and triclosan (TCS) were frequently detected and their maximum environmental concentration (MEC) was 280, 4.1, 320, 31, and 93 ng/L, respectively. 4-*tert*-Pentylphenol was not detected in ambient water at all sites but was detected in sediment samples at 6 out of 26 sites. PMD was included in the test chemicals due to being a PRTR substance, although no detection was confirmed in the environmental surveys.

### 2.2 | Existing knowledge of test chemicals

The existing knowledge for the basis on which the substances could be considered as “Chemicals that can be subjected to test for endocrine disrupting effects” is briefly summarized below for the 17 chemicals. It is noted that the literature survey was conducted between FY2008 and FY2017 (i.e., the implementation year of the survey varies depending on the substance) and any negative suggestions regarding endocrine disrupting effects in the literatures are omitted.

#### 2.2.1 | Benzophenone-2

Benzophenone-2 (BP2) is used largely as an ultraviolet (UV) filter in personal care products such as cosmetics and in numerous other materials for UV protection. Estrogenic, anti-estrogenic, and anti-androgenic activity was suggested by in vivo and in vitro studies. Estrogenic activity was demonstrated in RGAs using Chinese hamster ovarian (CHO) cells transfected with human ER (hER) linked with thermostable alkaline phosphatase gene (Matsumoto et al., 2005), MCF-7 human breast carcinoma cell line (Matsumoto et al., 2005; Suzuki et al., 2005), and HeLa cells stably transfected with rainbow trout (*Oncorhynchus mykiss*) Esr1 (Molina-Molina et al., 2008). Anti-androgenic activity of BP2 was demonstrated in RGAs using NIH3T3 cells transfected with hAR (Suzuki et al., 2005) and human prostate cancer PALM cells transfected with hAR (Molina-Molina et al., 2008). BP2 induced uterine weight gain in ovariectomized rats, administered by gavage (Schlecht et al., 2004, 2006), and in mice given subcutaneous injections (Koda et al., 2005; Ohta et al., 2012). BP2 increased uterine weight in immature female rats (Yamasaki, Takeyoshi, Yakabe, et al., 2003; Yamasaki, Takeyoshi, Sawaki, et al., 2003). Anti-estrogenic activity of BP2 was demonstrated using ovariectomized rats co-treated with E2 or with EE2; BP2 inhibited E2- and EE2-induced uterine weight gain (Yamasaki, Takeyoshi, Yakabe, et al., 2003; Yamasaki, Takeyoshi, Sawaki, et al., 2003). In reproductively mature fathead minnow (*Pimephales promelas*) exposed to BP2, VTG induction, reduction of number of nuptial tubercles, and inhibition of spermatocyte development in testes in males, and, in females, inhibition of oocyte and increased atretic follicles in ovaries, were found (Weisbrod et al., 2007). Kunz and Fent (2009) showed VTG production in juvenile fathead minnows. Haselman et al. (2016) showed BP2 exposure in *X. laevis* from Nieuwkoop and Faber (NF) Stage 8 to NF Stage 62 induced VTG production and hyperplasia in thyroid follicle epithelial cells.

#### 2.2.2 | Bisphenol A

Bisphenol A (BPA) has been used for the raw material of polycarbonate plastics and epoxy resins. Anti-androgenic activity of BPA was demonstrated in stickleback kidney cells in the presence of 5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT) (Jolly et al., 2009), in RGAs using

**TABLE 1** Concentration detected in ambient surface water in Japan

Chemical	Survey <sup>a</sup>	Year	Frequency <sup>b</sup>	Range <sup>c</sup> (ng/L)	LOD (ng/L)
Benzophenone-2 (BP2)	(A)	FY2014	1/21	nd-13	12
Bisphenol A (BPA)	(A)	FY2014	18/20	nd-280	1.7
Cyanazine (CZ)	(A)	FY2006	6/7	nd-2.5	0.4
Diazinon (DZ)	(A)	FY2006	7/10	nd-19	1
Diisobutyl phthalate (DIBP)	(A)	FY1996	0/11	nd	200
	(B)	FY2002	2/25	nd-30	20
Estrone (E1)	(A)	FY2016	10/15	nd-4.1	0.046
Fenvalerate (FV)	(A)	FY2007	0/12	nd	2.6
	(B)	FY2006	1/50	nd-41	10–50 <sup>g</sup>
4-Hydroxybenzoate methyl (MPB)	(A)	FY2008	1/3	nd-3	2
4-Hydroxybenzoate propyl (PPB)	(A)	FY2012	1/16	nd-16	14
1-Naphtol (1NT)	(A)	FY2017	3/20	nd-2.7	2.6
4-Nonylphenol (branched) (4NP) <sup>d</sup>	(A)	FY2014	16/30	nd-320	18
4- <i>tert</i> -Octylphenol (4tOP)	(A)	FY2012	19/24	nd-31	0.36
Pendimethalin (PDM) <sup>e</sup>	(A)	FY2007	0/84	nd	1.4
4- <i>tert</i> -Pentylphenol (4tPP) <sup>f</sup>	(A)	FY2008	0/33	nd	1.1
Phenitoin (PHT)	(A)	FY2016	2/15	nd-28	2.1
Triclosan (TCS)	(A)	FY2014	16/16	0.76–93	0.13
Triphenyl phosphate (TPP)	(A)	FY2017	3/18	nd-24	1.1

Abbreviations: 1NT, 1-naphtol; 4NP, 4-nonylphenol (branched); 4tOP, 4-*tert*-octylphenol; 4tPP, 4-*tert*-pentylphenol; BP2, benzophenone-2; BPA, bisphenol A; CZ, cyanazine; DIBP, diisobutyl phthalate; DZ, diazinon; E1, estrone; FV, fenvalerate; LOD, limit of detection; MPB, 4-hydroxybenzoate methyl; nd, not detected; PDM, pendimethalin; PHT, phenitoin; PPB, 4-hydroxybenzoate propyl; TCS, triclosan; TPP, triphenyl phosphate.

<sup>a</sup>Survey of environmental concentrations of the subject chemicals were utilized Environmental Survey and Monitoring of Chemicals (A) and/or Survey on the items for water environment preservation (B).

<sup>b</sup>Data suggest the number of the sites at which the target chemical was detected in the concentration exceeding the LOD and the number of sites surveyed.

<sup>c</sup>Data show the ranges of minimum-maximum concentrations determined within the survey sites.

<sup>d</sup>Concentration of 4NP was the sum of measured concentrations of typical isomers monitored (equivalent of CAS number 25154-52-3);

<sup>e</sup>PMD was not detected in the Environmental Survey but it was listed in the PRTR, therefore, it was included in the list.

<sup>f</sup>In the FY2008 survey, 4tPP was not detected in ambient water in all sites but was detected in sediment samples at 6 out of 26 sites (maximum concentration at 0.44 ng/g-dry).

<sup>g</sup>Limit of detections were depend on the samples.

mouse Sertoli 15p-1 cells in the presence of 10-nM testosterone (T) and human liver cancer HepG2 cells transfected with androgen response element (ARE) and reporter gene (Lee et al., 2003), African monkey kidney CV-1 cell line transfected with hAR in the presence of 5 $\alpha$ -DHT (Teng et al., 2013; Xu et al., 2005), and yeast cells transfected with hAR (YAS) (Sohoni & Sumpter, 1998). In Japanese medaka, BPA induced *choriogenin* mRNA (Lee et al., 2002) and *vitellogenin II* (*vtgII*) mRNA in adult male liver (Yamaguchi et al., 2005) and increased serum VTG in adult males (Tabata et al., 2004). Additionally, it was reported that BPA induced delay of hatching (Yokota et al., 2000), occurrence of testis-ova in adult males (Kang et al., 2002), expression of *aromatase b* and *esr2a* mRNA (Schiller et al., 2014), and mRNA expressions of various steroidogenesis related genes and *vtgII* mRNA (Sun et al., 2014) in Japanese medaka. In goldfish (*Carassius auratus*), BPA induced *vtg* mRNA in liver, reduced sperm count, sperm volume, mobility of sperm, serum 11-ketotestosterone (11KT), and T in adult males (Hatef, Alavi, et al., 2012; Hatef, Zare, et al., 2012), and induced

serum VTG in juveniles (Li et al., 2012). In fathead minnow, BPA induced serum VTG and reduced cell-type frequencies in testis (Mihaich et al., 2012) and serum VTG in males (Staples et al., 2011). Elevation of serum VTG by BPA in juvenile rainbow trout (van den Belt et al., 2003) and *vtg* mRNA expression in whole body zebrafish (*Danio rerio*) for 96 h from just after fertilization (Chow et al., 2013) were reported. In *X. laevis*, BPA induced male-biased sex ratio and *vtg* mRNA expression in whole body at 120 days of age (Levy et al., 2004).

### 2.2.3 | Cyanazine

Cyanazine (CZ), a herbicide, showed anti-estrogenic activity in a yeast expressing hER (YES screen) in the presence of E2. Competitive binding assays demonstrated that CZ displaced radiolabeled E2 from recombinant hER (Tran et al., 1996).

### 2.2.4 | Diazinon

Estrogenic activity of diazinon (DZ), an insecticide, was demonstrated in an RGA using human ovarian tumor BG1 cells transfected with hER $\alpha$  (Kojima et al., 2005) and cell proliferative activity of rat pituitary tumor MtT/Se cells, which respond to estrogen (Manabe et al., 2006).

### 2.2.5 | Estrone

E1, a natural estrogen, frequently found in the effluent from sewage treatment plants, showed estrogenic activity in a YES screen,  $\beta$ -galactosidase induction and MVLN assay (transformed MCF-7 cell line) (van den Belt et al., 2004). E1 exposure induced VTG production in mature male fathead minnow and mature male rainbow trout (Panter et al., 1998; Routledge, Sheahan, et al., 1998).

### 2.2.6 | Fenvalerate

Fenvalerate (FV) is a synthetic pyrethroid insecticide. Estrogenic activity of FV was demonstrated in an E-Screen assay using MCF-7 cell line (Chen et al., 2002; Go et al., 1999) and in Ishikawa Var-1 human endometrial cancer cell lines (Garey & Wolff, 1998). Anti-estrogenic activity was demonstrated in an E-Screen assay in the presence of E2 (Kim et al., 2004). FV reduced the binding of E2 to ER in rat uterine cytosol (Chen et al., 2002). Anti-androgenic activity was demonstrated in an RGA using CV-1 cell line in the presence of 5 $\alpha$ -DHT (Xu et al., 2006). Nassr et al. (2010) showed that FV exposure by gavage in adult male rats induced reduction of weights of testis and epididymis and sperm counts in both organs, suggesting anti-androgenic activity.

### 2.2.7 | Diisobutyl phthalate

Diisobutyl phthalate (DIBP) is commonly used as a plasticizer in a variety of household products. Estrogenic activity of DIBP was demonstrated in ZR-75 and MCF-7 cell lines, and a YES assay (Harris et al., 1997). Saillenfait et al. (2008) showed DIBP given to pregnant rats from gestation day (GD) 12–21 for 10 days resulted in reduction of the anogenital distance and prostate weight. Hannas et al. (2011) showed that DIBP given by gavage for 5 days from GD 14–18 to mother rats reduced expression of *cyp11a* and *Star* mRNAs and T levels in the fetal testis.

### 2.2.8 | 4-Hydroxybenzoate methyl (also known as methylparaben, MPB)

MPB has been used as a preservative in cosmetics, lotions, shampoos, and bath products. Estrogenic activity of MPB was demonstrated in RGAs using a YES screen (Routledge, Parker, et al., 1998) and MCF-7 cells transfected with a reporter gene with ERE (Pugazhendhi

et al., 2005). Pugazhendhi et al. (2005) also demonstrated anti-estrogenic activity of MPB using a competitive hER binding assay. MPB induced uterine weight gain and uterine luminal epithelial height in ovariectomized mice (Lemini et al., 2004), indicating estrogenic activity in vivo.

### 2.2.9 | 4-Hydroxybenzoate propyl (also known as propylparaben, PPB)

PPB has been used as a preservative in cosmetics, lotions, shampoos, and bath products. Estrogenic activity of PPB was reported using a YES assay (Routledge, Sheahan et al., 1998), and MCF-12A and MDF-7 cell lines (Marchese & Silva, 2012; Wróbel & Gregoraszczyk, 2013). Anti-estrogenic activity of PPB, inhibition of fluorescence-labeled estrogen binding to ERs, was reported (Vo et al., 2010). Estrogenic activity of PPB in vivo was reported as induction of VTG and/or *vtg* mRNA in juvenile rainbow trout (Bjerregaard et al., 2003; Pedersen et al., 2000) and in Japanese medaka (Inui et al., 2003). Male Wistar rats exposed to PPB through food from postnatal days 19–20 for 4 weeks showed reduction of sperm count in the testis, reduction of sperm counts in cauda epididymis and reduction of serum T levels (Oishi, 2002), indicating that PPB has estrogenic activity and induces adverse effect on male reproductive system in juvenile Wistar rats.

### 2.2.10 | 1-Naphtol

1-Naphtol (1NT) is a major urinary metabolite of both naphthalene and the insecticide carbaryl. Meeker et al. (2006) measured 1NT in urine in male human subjects ( $n = 262$ ) who visited a hospital for infertility diagnosis and found negative association between 1N levels in urine and serum T levels, suggesting anti-androgenic activity of 1NT. Meeker et al. (2007) reported associations between urinary 1NT levels and reduction of sperm motility and serum T levels and increased sperm DNA damage. Meeker et al. (2007) also found that 1NT from carbaryl exposure is likely responsible for the association between 1NT and sperm motility, whereas 1NT from naphthalene exposure is likely accountable for the association between 1NT and sperm DNA damage. Han et al. (2010), using male human subjects ( $n = 562$ ) who visited a hospital for infertility diagnosis, demonstrated a positive correlation between urine 1NT levels and abnormally higher plasma luteinizing hormone levels, suggesting a 1NT effect through the hypothalamus–pituitary–gonad (HPG) axis.

### 2.2.11 | Branched isomers of 4-nonylphenol

4NP has been used in manufacturing antioxidants, lubricating oil additives, laundry and dish detergents, emulsifiers, and solubilizers. For anti-androgenic activity, 4NP inhibited the following androgen-induced activity; androgen-dependent spigin protein production in

the presence of 5 $\alpha$ -DHT in stickleback kidney cells (Jolly et al., 2009), 5 $\alpha$ -DHT-induced androgenic effect in an RGA using CV-1 cells transfected with a reporter gene and ARE (Xu et al., 2005), androgen effect in RGA using human hepatoma HepG2 cells transfected AR with a reporter gene, and T-induced reporter gene in mouse Sertoli 15p-1 cells transfected with ARE and in a YAS screen (Lee et al., 2003). Androgenic activity of 4NP was also reported in a YAS screen (Lee et al., 2003). Estrogenic effect of 4NP was reported in several fish species *in vivo*; 4NP induced liver VTG and intersex in males (Seki, Yokota, Maeda, et al., 2003), liver VTG in males (Nozaka et al., 2004), and hepatic *vtg* mRNA expression in Japanese medaka (Jin et al., 2011; Lee et al., 2002; Yamaguchi et al., 2005). In addition, NP-induced VTG and *vtg* mRNA were reported in zebrafish (Jin et al., 2009, 2010), juvenile goldfish (Li et al., 2012), and juvenile rainbow trout (van den Belt et al., 2003).

### 2.2.12 | 4-*tert*-Octylphenol

4tOP is an intermediate in the production of phenol/formaldehyde resins and octylphenol ethoxylates that are used in rubber, pesticides, and paints. In fish species, 4tOP exposure induced VTG production in male Japanese medaka (Gronen et al., 1999; Nozaka et al., 2004), male juvenile common carp (Huang & Wang, 2001), juvenile male goldfish (Li et al., 2012), and juvenile rainbow trout (van den Belt et al., 2003). 4tOP also induced intersex in Japanese medaka (Seki, Yokota, Maeda et al., 2003). In amphibian species, bull frog (*Rana catesbeiana*) exposure to 4tOP from Gosner Stage 32 for 24 h accelerated the onset of sexual differentiation and increased the male ratio (Mayer et al., 2003). In Western clawed frog (*X. tropicalis*), 4tOP exposure from NF Stage 46 tadpoles to just prior to completion of metamorphosis (NF 65) induced VTG in immature froglets and development of oviducts in male adult frogs (Porter et al., 2011). Xu et al. (2005) reported that 4tOP has anti-androgenic activity in an RGA using CV-1 cells transiently transfected with hAR in the presence of 5 $\alpha$ -DHT, indicating that 4tOP acted as a hAR antagonist.

### 2.2.13 | Pendimethalin

Undeger et al. (2010) demonstrated that PMD administered by oral gavage to immature female rats induced uterine weight gain, indicating estrogenic activity, and upregulation of ER $\beta$  mRNA and downregulation of *ar* mRNA.

### 2.2.14 | 4-*tert*-Pentylphenol

4-*tert*-Pentylphenol (4tPP) is used as a chemical intermediate, mainly for phenolic resins. 4tPP induced VTG production in carp hepatocytes *in vitro* (Smeets et al., 1999). Gimeno et al. (1997), Gimeno, Komen, Jobling, et al. (1998), and Gimeno, Komen, Gerritsen, and Bowmer (1998) demonstrated serum VTG induction and reduction of

sperm count by 4tPP in adult male carp, together with serum VTG induction and reduced number of spermatogonia, reduced development of seminiferous tubules, and reduced number of primordial germ cells in juvenile male carps. Panter et al. (2010) demonstrated that 4tPP reduced total number of eggs in females and induced serum VTG production in males in fathead minnow. Seki, Yokota, Matsubara, et al. (2003) conducted a Japanese medaka full life-cycle test using 4tPP over two generations from 12 h after fertilization to 101 days of age. The lowest observed effect concentrations (LOECs) on abnormal sexual differentiation, hepatic VTG induction, and reproductive impairment were 224,  $\leq 51.1$ , and 224  $\mu\text{g/L}$ , respectively, indicating that 4tPP has estrogenic effects that reduce the reproductive potential of Japanese medaka.

### 2.2.15 | Phentoin

Phentoin (PHT) is used as an anti-seizure medication. Several anti-epileptic drugs including PHT are associated with anti-cancer activity, reducing free fractions of sex-steroid hormones, and anti-estrogenic activity of PHT in the presence of E2 in E-Screen assay using MCF-7 cell line has been demonstrated (Olsen et al., 2004). Gehlhaus et al. (2007) showed increased expression of *ar* mRNA and *cyp3A11* mRNA in mouse hepatic 1c1c7 cells by PHT, and Meyer et al. (2009) showed increased expression of AR and *cyp3A2* in AR expressing PC-12 cells by PHT, suggesting anti-androgenic activity of PHT *in vitro*.

### 2.2.16 | Triclosan

TCS is used as an antimicrobial agent in personal care, pharmaceutical, industrial, and household products. Stoker et al. (2010) demonstrated that TCS in rats advanced the age of onset of vaginal opening and increased uterine weight, indicating an estrogenic effect, and decreased serum E2, and total and free thyroxine levels, suggesting that TCS acts on the hypothalamus–pituitary–thyroid axis, in addition to the estrogenic activity. Jung et al. (2012) showed that TCS delivered to rats by gavage for 3 days from postnatal days 19 induced uterine weight gain, and co-treatment with ICI 182,780 and progesterone receptor antagonist RU 486 with TCS reversed TCS-induced uterine weight. Gee et al. (2008) showed a suppressive effect of TCS in an RGA using MCF-7 cells transfected with ERE, indicating anti-estrogenic activity, and Chen et al. (2007) demonstrated TCS inhibited T-induced transcriptional activity in HEK293 cells transfected with AR, indicating anti-androgenic activity. Gee et al. (2008) also showed anti-androgenic activity of TCS in two RGAs using mouse mammary tumor S115 cells and human mammary tumor T47D cells transfected with hAR. Ishibashi et al. (2004) showed that TCS exposure to adult Japanese medaka induced VTG production in males and low GSI in females and reduction of fertility in the F1 generation. Raut and Angus (2010) showed *vtg* mRNA expression in male liver of adult mosquitofish (*Gambusia affinis*) by TCS, indicating estrogenic activity of TCS *in vivo*.

## 2.2.17 | Triphenyl phosphate

Triphenyl phosphate (TPP) is commonly used as an additive flame retardant and a plasticizer in a wide range of materials. Meeker and Stapleton (2010) analyzed TPP concentrations in house dust with hormone levels and semen quality parameters in 50 men recruited in a U.S. infertility clinic. TPP was detected in 98% of samples, and it was associated with a 10% increase in prolactin and a 19% decrease in sperm concentration, suggesting that TPP is causing effects through the HPG axis.

## 2.3 | Test chemicals

For the Tier 1 in vitro and in vivo assays, commercially available reagents with the highest purity were acquired for the test substances. The details of the reagents tested in the in vitro RGAs and the in vivo OECD TG229 assays, such as CAS number, purity, and supplier, are available in Table 2.

## 2.4 | Tier 1 in vitro RGAs

As described above, a possible influence on reproduction, or activity through the mEsr and/or mAr $\beta$ , was suggested for the 17 chemicals, from existing knowledge. Potency in each estrogenic, anti-estrogenic, androgenic, and anti-androgenic activity, including the activities which existing knowledge suggested, was confirmed by RGAs using mEsr1 or mAr $\beta$ . The Tier 1 in vitro RGAs for 17 chemicals were conducted in one laboratory from FY2010 to FY2020 under the EXTEND2010 and EXTEND2016 programs.

The mEsr1 RGA was performed based on the methods previously reported (Katsu et al., 2010; Lange et al., 2012; Miyagawa et al., 2014) to assess an agonistic or an antagonistic activity on mEsr1 using HEK293 cells. For the agonist and the antagonist assays, E2 and 4-hydroxytamoxifen (4HTAM) were used as a positive control, to confirm the assay adequately worked, respectively. The mAr $\beta$  RGAs using HepG2 cells were also carried out based on the methods previously reported (Lange et al., 2015), and 11KT and 2-hydroxyflutamide (2HFLT) were used as a positive control for the

Chemical <sup>a</sup>	CAS no.	Reagents for RGAs <sup>b</sup>	Reagents for TG229
BP2	131-55-5	SIGMA, 98.6% <sup>(A)(B)(C)(D)</sup>	TCl, 99.6%
BPA	80-05-7	TCl, 100% <sup>(A)(B)(C)(D)</sup>	TCl, 99.9%
CZ	21725-46-2	FUJI, 99.2% <sup>(A)(C)(D)</sup> ; WAKO, 99.4% <sup>(B)</sup>	WAKO, 99.4%
DZ	333-41-5	WAKO, 99.6% <sup>(A)(B)(C)(D)</sup>	WAKO, 99.8%
DIBP	84-69-5	TCl, 99.2% <sup>(A)(D)</sup> ; FUJI, 98.9% <sup>(B)(C)</sup>	TCl, 98.9%
E1	53-16-7	WAKO, 100% <sup>(A)</sup> ; TCl, 99.4% <sup>(B)(C)(D)</sup>	WAKO, 98.0%
FV	51630-58-1	WAKO, 99.5% <sup>(A)(B)</sup> ; 99.4% <sup>(D)</sup> ; FUJI, 99.3% <sup>(C)</sup>	WAKO, 99.5%
MPB	99-76-3	WAKO, 100% <sup>(A)(B)</sup> ; TCl, 100% <sup>(C)(D)</sup>	WAKO, 100%
PPB	94-13-3	TCl, 99.9% <sup>(A)(B)</sup> ; TCl, 100% <sup>(C)(D)</sup>	TCl, 99.9%
1NT	90-15-3	WAKO, 100% <sup>(A)(D)</sup> ; TCl, 99.9% <sup>(B)(C)</sup>	WAKO, 100%
4NP	84852-15-3	SIGMA, 97.8% <sup>(A)(B)(D)</sup> ; SIGMA, 92.9% <sup>(C)</sup>	KNTC, 99.7%
4tOP	140-66-9	TCl, 97.2% <sup>(A)(B)(C)(D)</sup>	TCl, 97.2%
PDM	40487-42-1	KNTC, 98.8% <sup>(A)(B)(C)(D)</sup>	FUJI, 99.3%
4tPP	80-46-6	WAKO, >97.0% <sup>(A)</sup> ; TCl, 99.9% <sup>(B)(C)(D)</sup>	TCl, 99.9%
PHT	57-41-0	TCl, 100% <sup>(A)(D)</sup> ; WAKO, 99.3% <sup>(B)(C)</sup>	TCl, >98%
TCS	3380-34-5	TCl, 99.8% <sup>(A)(B)(C)(D)</sup>	WAKO, 98.0%
TPP	115-86-6	TCl, 99.9% <sup>(A)(B)(D)</sup> ; TCl, 100% <sup>(C)</sup>	WAKO, >97%
E2 <sup>c</sup>	50-28-2	WAKO, 98.6% or 100%	na
11KT <sup>c</sup>	53187-98-7	SIGMA, 99% or >98%	na
4HTAM <sup>d</sup>	68047-06-3	SIGMA, 99.9% or 99.0%	na
2HFLT <sup>d</sup>	52806-53-8	SIGMA, >99%	na

**TABLE 2** Supplier and purity of chemical reagents used in the RGAs and TG229

Abbreviations: na, not applicable; WAKO, Wako Pure Chemical Industries, Ltd.; TCl, Tokyo Chemical Industry Co., Ltd.; SIGMA, Sigma-Aldrich Co., LLC; FUJI, FUJIFILM Wako Pure Chemical Corporation; KNTC, Kanto Chemical Co., Inc.

<sup>a</sup>Abbreviations of chemical names are the same in Table 1.

<sup>b</sup>Reagents were subjected to mEsr1 agonist (A), mEsr1 antagonist (B), mAr $\beta$  agonist (C) and/or mAr $\beta$  antagonist (D) RGAs.

<sup>c</sup>17 $\beta$ -Estradiol (E2) and 11-ketotestosterone (11KT) were used as positive controls in agonist assays, respectively, and as competitive natural ligands in antagonist assays of mEsr1 and mAr $\beta$  RGAs, respectively.

<sup>d</sup>4-Hydroxy tamoxifen (4HTAM) and 2-hydroxy flutamide (2HFLT) were used as positive controls in antagonist assay of mEsr1 and mAr $\beta$  RGAs, respectively.

agonist and the antagonist assay, respectively. The details of methods of both mEsR1 and mAr $\beta$  RGAs can be found in Onishi et al. (2021).

In the EXTEND programs, all RGAs were conducted at least in four concentrations: The highest concentration was the lowest of which no insoluble particle in the test medium, and no cytotoxicity of test chemical was found or  $1.0 \times 10^{-4}$  M. For the agonist assays, if possible, a half maximal effective concentration (EC<sub>50</sub>) was estimated by a non-linear curve fitting (three parameters logistic regression curve) using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Similarly, IC<sub>50</sub> (i.e., a half maximal inhibitory concentration) at which the transcriptional activity induced by competitively spiked natural ligand, that is, E2 or 11KT, decreased by 50% of the transcriptional activity compared with the positive control assayed in the same plate, was calculated for the antagonistic assays. Additionally, in the agonist assays, in the case that a significant increase of fold activation, compared with solvent control (SC), was found at the highest test concentration but an EC<sub>50</sub> estimated was higher than the highest test concentration, that value was not adopted and a PC<sub>10</sub>, which was defined as the concentration in which an agonistic activity equivalent to 10% of maximum activity in the assay for positive control substance (E2 or 11-KT) performed in the same day was observed, was derived by a simple linear regression using data of the two highest test concentrations. In the case of antagonistic assays in which the transcriptional activity by competitively spiked natural ligand (E2 or 11-KT) was inhibited more than 30% but less than 50% compared with the positive control in the same plate, IC<sub>30</sub> was calculated via a simple linear regression.

## 2.5 | Tier 1 in vivo FSTRA (OECD TG229 assay)

The OECD TG229 assays for 17 chemicals (Table 2) were conducted in four laboratories in Japan, that is, National Institute for Environmental Studies, Institute of Environmental Ecology (NIES), IDEA Consultants Inc. (IDEA), Chemicals Evaluation and Research Institute (CERI), and Sumitomo Chemical Techno Service (SCTS), in accordance with the OECD TG229 (OECD, 2012). The exposure conditions specified in the TG229 are summarized in Table 3, and the details of each study are shown in Table S1.

### 2.5.1 | Test fish

The test species was orange-red varieties of Japanese medaka (*O. latipes*) in all 17 studies. Of these studies, six studies (BPA, E1, 4NP, PHT, TCS, and TPP) used a NIES-R strain (Horie & Kobayashi, 2014), nine studies (BP2, CZ, FV, MPB, PPB, 1NT, 4tOP, PDM and 4tPP) used the laboratory strains, which originated from the same fish as the NIES-R strain and have been subcultured within each test facility (CERI and IDEA) for more than 12 years, and the other two DZ and DIBP studies used a S-rR strain (Hagino et al., 2001). In each study, healthy and mature male and female Japanese medaka at

**TABLE 3** Test conditions for TG229 (FSTRA)

Type of exposure	Flow-through
Exposure duration	21 days
Test chamber size	Minimum 2 L
Test solution volume	Minimum 1.5 L
Volume exchange of test solution	Minimum 5 times/day
Loading rate	< 5 g/L
Number of test fish	6 fish (3 males and 3 females) per tank
Number of vessels per treatment	Minimum 4
Number of treatments	3 (plus appropriate controls)
Temperature of test solution	25 ± 2 °C
Photoperiod	16-h light and 8-h dark
Aeration	None unless DO concentration falls below 60% air saturation
Feeding	Brine shrimp nauplii two or three times daily ad libitum

16 ± 2 weeks old, selected from a single stock population, which were bred within the test facilities, were employed (as provided in Table S1). The test fish were maintained in conditions similar to the assay for at least 7 days before the study, and spawning status was checked during the acclimation period. On the day of the assay (chemical exposure) initiation, to ensure balanced distribution of replicates between treatment groups, the fish tanks (replicate vessels), each containing three male and female Japanese medaka, were distributed to each treatment and control group, by a randomized block design, based on reproduction status within the acclimation period (i.e., the number of fertilized eggs in the last 5–7 days of the acclimation in each replicate tank).

### 2.5.2 | Test concentrations

Three or four concentrations were set for each test chemical, referring to the results of the other validation studies previously conducted and toxicity tests using Japanese medaka (Table 3). To prevent excessive lethal effects, the highest test concentration was determined based on acute toxicity on Japanese medaka (96 h LC<sub>50</sub>). Also, the water solubility of each test chemical was considered when deciding the highest test concentration. To set the lower concentrations, a spacing factor ranging between two and five was used. All assays included a dilution water control (DWC), and three assays included a SC. In the DIBP, FV, and PMD studies, *N,N*-dimethylformamide (DIBP and PMD) or acetone (FV) was used to prepare the concentrated stock solutions of the test chemicals because of their poor water solubility. The testing laboratories chose these carrier solvents referring physico-chemical properties of test chemicals and chronic toxicities of carrier solvents (Hutchinson et al., 2006; OECD, 2019). The solvent concentrations in test solutions were set at 100 µl/L consistently in control and treatment groups for these three studies.



Test solutions at the selected concentrations were prepared by diluting a stock solution, which was prepared by an appropriate method depending on the physicochemical properties of the test chemical by simply mixing the test chemical in dilution water by stirring and/or sonicating for an appropriate time or by using a solid–liquid saturator. In each participating laboratory, flow-through exposure systems, in which a series of test solutions at the target concentration can be continuously prepared and derived to test vessels at the controlled flow rates, were used for the exposure experiment (Hagino et al., 2001; Haselman et al., 2016; Seki, Yokota, Maeda, et al., 2003; Watanabe et al., 2017). Dechlorinated tap water in which the water quality had been checked at an appropriate frequency in the laboratories was used as dilution water to prepare the test solutions, including the DWC and SC groups. The details of preparation of test solutions and the water quality of dilution water in each study are provided in Tables S1 and S2, respectively.

### 2.5.3 | Chemical analysis

Concentrations of test chemicals in test solutions including DWC and SC controls were quantitatively measured once a week during the exposure period. Water samples, collected from test vessels, were immediately subjected to chemical analysis or stored at 4°C until use. The sample water was pretreated by solid–liquid extraction or liquid–liquid extraction, if necessary, to obtain the limit of quantitation (LOQ) required. Analytical method and a LOQ in each study are summarized in Table 4, and the operating conditions applied to the analysis of test concentrations are provided in Table S3.

### 2.5.4 | Chemical exposure and observation

In the TG229 assays, each of three mature adult male and female Japanese medaka in a test vessel was exposed together to the test chemicals in flow through conditions for three weeks. In all studies, each treatment including the DWC and SC groups contained four replicate vessels. During the exposure period, the fish were fed ad libitum live brine shrimp nauplii (newly hatched *Artemia* sp.) two to three times a day on weekdays and one to three times a day on weekends. Water temperature of the test solutions was recorded at least for one vessel in each treatment and control group every day and for all test vessels at least once a week. Dissolved oxygen and pH of test solutions were measured for all test vessels at least once a week. In daily observation, mortality and any abnormal response in appearance and behavior, including a remarkable reduction of feeding activity, in the fish exposed to the test chemicals were daily observed and recorded. All eggs females spawned on the day were collected every day. Any fish that died were removed from the vessel as soon as possible, and fecal material was appropriately removed from the vessels by siphoning. On the day of the assay completion, the surviving male and female fish were subjected to sample collection after the daily observations and measurements. All the 17 chemical assays had been

**TABLE 4** Methods for sample pretreatment, analysis, and LOQs in test solution analysis for the TG229

Chemical <sup>a</sup>	Sample pretreatment	Analysis	LOQ
BP2	None	HPLC	0.0199 mg/L
BPA	None	HPLC	0.025 mg/L
CZ	Solid–liquid extraction	GC–MS	0.0003 mg/L
DZ	None	HPLC	12.5 µg/L
DIBP	Liquid–liquid extraction	GC–MS	1 µg/L
E1	Solid–liquid extraction	LC–MS/MS	0.0022 µg/L
FV	Liquid–liquid extraction	GC–MS	13 µg/L
MPB	None	LC–MS/MS	0.0008 mg/L
PPB	None	LC–MS/MS	0.0008 mg/L
1NT	Solid–liquid extraction	LC–MS	0.6 µg/L
4NP	Solid–liquid extraction	GC–MS	0.06 µg/L
4tOP	None	LC–MS/MS	0.6 µg/L
PDM	None	LC–MS	0.20 µg/L
4tPP	Liquid–liquid extraction	GC–MS	0.5 µg/L
PHT	None	LC–MS	0.05 mg/L
TCS	None	LC–MS	10 µg/L
TPP	Solid–liquid extraction	GC–MS	0.03 µg/L

Abbreviations: GC–MS, gas chromatography–mass spectrometer; GC–MS/MS, gas chromatography–tandem mass spectrometer; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometer; LC–MS/MS, liquid chromatography–tandem mass spectrometer; LOQ, limit of quantitation.

<sup>a</sup>Abbreviations of chemical names are the same in Table 1.

conducting in the conditions OECD TG229 specified (Table 3) and satisfied the test acceptance criteria provided by the OECD TG229 (OECD, 2012), except a slight deviation in water temperature for TCS study (Table S4).

### 2.5.5 | Endpoints measurements

#### *Spawning status (fecundity and fertility)*

In each tank, eggs and egg clutches released on the bottom of the tank were collected by siphoning and egg clutches on female abdomen were carefully removed from the body of the female, captured by a small net. All the eggs collected were observed under the microscope and counted separately as fertilized or unfertilized eggs. The means of the number of total eggs and fertilized eggs (per female per day) were quantified, respectively. In addition, the fertility rate, which was defined as the ratio (percentage) of the number of fertilized eggs to the number of total eggs over the 21 days of exposure period, was calculated.

#### *Necropsy and sample collection*

At the completion of the exposure, all surviving fish were anesthetized in ice-cold water. The fish were dissected and the whole liver sampled, after measuring body length and weight. The liver samples were weighed and immediately stored under –20°C or less until VTG

quantification. In addition, the anal fin was imaged in a flat and spread-out condition, or the posterior region of the body including the anal fin was collected and stored in 10% neutral buffered formalin for SSC assessment.

#### Hepatic VTG

Hepatic VTG concentrations were quantitatively determined based on the methods described in the OECD TG229 (OECD, 2012). Briefly, the liver sample was homogenized with assay buffer included in the ELISA kit used, and the supernatant obtained by centrifuging was collected in a microtube. The concentration of VTG in the hepatic extract was quantitatively determined using either commercially available Enbio Medaka vitellogenin ELISA System (EnBio Tec Laboratories Co. Ltd., Tokyo, Japan or Fujikura Kasei Co. Ltd., Tokyo, Japan) or Medaka VTG ELISA assay kit (Trans Genic Inc., Fukuoka, Japan) (Tatarazako et al., 2004). The determination limit for VTG in hepatic extracts was 2.0 or 5.0 ng/ml, depending on the assays (Table S1).

#### Secondary sex characteristics

As an SSC, the number of joint plates in which papillary processes were visibly formed were counted on the images or fixed samples of anal fin under the microscope (OECD, 2012).

### 2.5.6 | Statistical analysis for TG229 data

For each endpoint, differences between the chemical treatment and the DWC or SC groups were statistically analyzed based on the replicate means of the data (OECD, 2004, 2006a). Briefly, first, the homogeneity of variances was assessed by Levene's or Bartlett's test. The data in which the assumption of the homogeneity of variances was met were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test. The data in which a homogeneity of variances was not initially found were appropriately transformed (by log transformation, square root transformation, or arcsine transformation) and reanalyzed. If no homogeneity of variances was found even in the transformed data, the data were analyzed by a nonparametric Kruskal–Wallis followed by Steel's test or Dunn's test. The level of significance was set at  $p < 0.05$  in the statistical analyses. Hepatosomatic and gonadosomatic indices at the completion of the chemical exposure, which were not included in the endpoints specified by the OECD TG229, were statistically analyzed in the same procedure.

## 3 | RESULTS

### 3.1 | In vitro reporter gene assay

In the agonist assays of mEsr1 RGA, the EC<sub>50</sub> of E2, used as a positive control to confirm the verification of the assay, was  $1.8 \times 10^{-10}$  M in geometric mean and ranged between  $6.6 \times 10^{-11}$  and  $3.8 \times 10^{-10}$  M (Table 5). Regarding the 17 chemicals, 15 chemicals (other than CZ

and PHT) exhibited an agonistic activity on mEsr1, and an EC<sub>50</sub> was obtained for 12 chemicals. As illustrated in Figure 1, among them, EC<sub>50</sub>s varied from  $5.4 \times 10^{-9}$  to  $7.8 \times 10^{-5}$  M, and the five chemicals with relatively strong mEsr1 agonistic activity were E1, 4NP, 4tOP, BPA, and 4tPP, showing an EC<sub>50</sub> of  $5.4 \times 10^{-9}$ ,  $2.2 \times 10^{-8}$ ,  $2.3 \times 10^{-8}$ ,  $8.6 \times 10^{-8}$ , and  $9.7 \times 10^{-7}$  M, respectively. Only CZ and PHT, on one hand, inhibited the transcriptional activity on mEsr1 by E2 (i.e., suggesting an anti-estrogenic potency on mEsr1) in the antagonist assays.

In the RGA using mAr $\beta$ , a positive agonistic activity was only found in two chemicals (BP2 and 1NT), only PC<sub>10</sub> and not EC<sub>50</sub> could be estimated. The results of the antagonistic assays suggested that five chemicals, that is, BP2, BPA, E1, PDM, and 4tPP, have an inhibitory potency to transcriptional activity on mAr $\beta$  by 11KT. Interestingly, four of these chemicals (except PDM) also showed relatively potent mEsr1 agonistic activity, less than  $1.0 \times 10^{-7}$  M of EC<sub>50</sub>. Regarding BP2, especially, the results suggested the possibility of three different MOAs other than anti-estrogen activity (Table 5).

### 3.2 | Fish short-term reproduction assay (TG229)

#### 3.2.1 | Measured concentrations of test chemicals

The mean measured concentrations of test chemicals in test solutions were ranged within  $\pm 20\%$  of the nominal concentration in all studies, except BPA (at the lowest test concentration), PMD, and TPP (Table 6). For PMD, it is presumed that the decreasing test concentrations was caused by adsorption onto the materials of exposure equipment such as test tanks, due to its poor water solubility around 0.3 mg/L or less (Kovdienko et al., 2010; Lewis et al., 2016). In the TPP study, stock solution of test chemical to prepare test solutions was generated using a saturator (Sibata Scientific Technology, Ltd., Saitama, Japan). In the preliminary examination, the saturator could prepare an aqueous solution in which TPP concentration ranged within the literatures suggested water solubility, 1–1.9 mg/L (Brooke et al., 2009; Saeger et al., 1979). Therefore, in the TG229 assay, the test solutions at 20.0, 64.0, 200 and 640  $\mu\text{g/L}$  of nominal were continuously prepared by diluting the stock solution assuming TPP concentration of 1.5 mg/L with dilution water by 2.3, 7.5, 23.4, or 75 times on a flow-through exposure system. However, when TPP concentration of the stock solution was checked after the initiation of the exposure, it was always around a tenth of the assuming value and thus the mean measured TPP concentrations in test solutions ranged between 7.0% and 11% of the nominal. Though the cause of the low concentration in stock solution is uncertain, the environmental conditions such as water quality of dilution water might have affected the solubility of TPP (OECD SIDS, 2002). Because the TPP concentrations measured during the exposure ( $n = 4$ ), on the one hand, did not much varied with less than 7.5% of coefficient of variation in all four treatment groups and an adverse effect on reproduction was caused in the exposure concentrations, it was concluded the results of this assay was acceptable.

**TABLE 5** Results of agonist and antagonist reporter gene assays with medaka Esr1 and medaka AR $\beta$ 

Chemical <sup>a</sup>	Medaka Esr1 reporter gene assay		Medaka AR $\beta$ reporter gene assay	
	Agonistic activity	Antagonistic activity <sup>b</sup>	Agonistic activity	Antagonistic activity <sup>c</sup>
BP2	EC <sub>50</sub> = 1.6 × 10 <sup>-6</sup> M	NE (MAC = 1.0 × 10 <sup>-4</sup> M) <sup>(B)</sup>	PC <sub>10</sub> = 1.2 × 10 <sup>-5</sup> M	IC <sub>50</sub> = 4.0 × 10 <sup>-5</sup> M <sup>(D)</sup>
BPA	EC <sub>50</sub> = 8.6 × 10 <sup>-8</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	IC <sub>50</sub> = 7.0 × 10 <sup>-6</sup> M <sup>(D)</sup>
CZ	NE (MAC = 1.0 × 10 <sup>-4</sup> M)	IC <sub>50</sub> = 6.1 × 10 <sup>-7</sup> M <sup>(A)</sup>	NE (MAC = 1.0 × 10 <sup>-4</sup> M)	NE (MAC = 1.0 × 10 <sup>-4</sup> M) <sup>(D)</sup>
DZ	PC <sub>10</sub> = 1.0 × 10 <sup>-5</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(A)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(C)</sup>
DIBP	PC <sub>10</sub> = 3.0 × 10 <sup>-6</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(D)</sup>
E1	EC <sub>50</sub> = 5.4 × 10 <sup>-9</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	IC <sub>50</sub> = 1.5 × 10 <sup>-6</sup> M <sup>(D)</sup>
FV	EC <sub>50</sub> = 2.4 × 10 <sup>-6</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(A)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(D)</sup>
MPB	EC <sub>50</sub> = 7.0 × 10 <sup>-5</sup> M	NE (MAC = 1.0 × 10 <sup>-4</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-4</sup> M)	NE (MAC = 1.0 × 10 <sup>-4</sup> M) <sup>(D)</sup>
PPB	EC <sub>50</sub> = 7.0 × 10 <sup>-6</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(A)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(D)</sup>
1NT	EC <sub>50</sub> = 7.8 × 10 <sup>-5</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	PC <sub>10</sub> = 3.3 × 10 <sup>-5</sup> M	NE (MAC = 1.0 × 10 <sup>-4</sup> M) <sup>(C)</sup>
4NP	EC <sub>50</sub> = 2.2 × 10 <sup>-8</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(D)</sup>
4tOP	EC <sub>50</sub> = 2.3 × 10 <sup>-8</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(D)</sup>
PDM	EC <sub>50</sub> = 3.3 × 10 <sup>-6</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	IC <sub>50</sub> = 3.1 × 10 <sup>-6</sup> M <sup>(D)</sup>
4tPP	EC <sub>50</sub> = 9.7 × 10 <sup>-7</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-4</sup> M)	IC <sub>30</sub> = 1.7 × 10 <sup>-5</sup> M <sup>(D)</sup>
PHT	NE (MAC = 1.0 × 10 <sup>-4</sup> M)	IC <sub>50</sub> = 2.1 × 10 <sup>-6</sup> M <sup>(A)</sup>	NE (MAC = 1.0 × 10 <sup>-4</sup> M)	NE (MAC = 3.2 × 10 <sup>-5</sup> M) <sup>(D)</sup>
TCS	PC <sub>10</sub> = 2.0 × 10 <sup>-6</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(D)</sup>
TPP	EC <sub>50</sub> = 9.7 × 10 <sup>-6</sup> M	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(B)</sup>	NE (MAC = 1.0 × 10 <sup>-5</sup> M)	NE (MAC = 1.0 × 10 <sup>-5</sup> M) <sup>(D)</sup>
PC <sup>d</sup>	[E2]	[4HTAM]	[11KT]	[2HFLT]
	EC <sub>50</sub> = 1.8 × 10 <sup>-10</sup> M (n = 10)	IC <sub>50</sub> = 4.4 × 10 <sup>-10</sup> M (n = 3) <sup>(A)</sup>	EC <sub>50</sub> = 5.8 × 10 <sup>-9</sup> M (n = 5)	IC <sub>50</sub> = 1.6 × 10 <sup>-7</sup> M (n = 2) <sup>(C)</sup>
	(6.6 × 10 <sup>-11</sup> to 3.8 × 10 <sup>-10</sup> M)	(2.9 × 10 <sup>-10</sup> to 8.7 × 10 <sup>-10</sup> M) <sup>(A)</sup>	(3.5 × 10 <sup>-9</sup> to 9.3 × 10 <sup>-9</sup> M)	(1.3 × 10 <sup>-7</sup> to 2.0 × 10 <sup>-7</sup> M) <sup>(C)</sup>
		IC <sub>50</sub> = 1.4 × 10 <sup>-9</sup> M (n = 5) <sup>(B)</sup>		IC <sub>50</sub> = 4.5 × 10 <sup>-7</sup> M (n = 4) <sup>(D)</sup>
		(4.9 × 10 <sup>-10</sup> to 7.0 × 10 <sup>-9</sup> M) <sup>(B)</sup>		(3.3 × 10 <sup>-7</sup> to 5.9 × 10 <sup>-7</sup> M) <sup>(D)</sup>

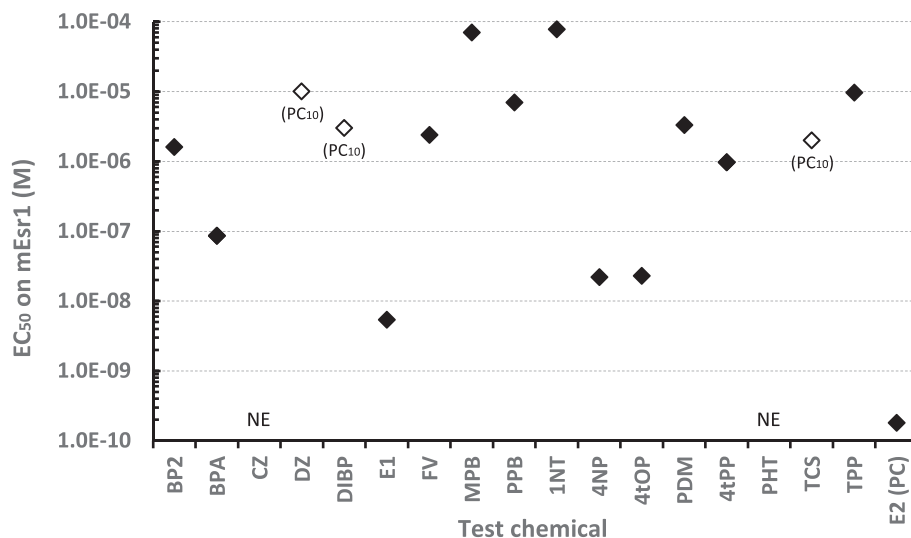
Abbreviations: MAC, maximum assayed concentration; NE, no effect was indicated even at the MAC.

<sup>a</sup>Abbreviations of chemical names are the same in Table 1.

<sup>b</sup>In antagonist assay of mEsr1 RGA, E2 was competitively spiked at 0.2 (A) or 1.0 nM (B).

<sup>c</sup>In antagonist assay of mAR $\beta$  RGA, 11-KT was competitively spiked at 10 (C) or 50 nM (D).

<sup>d</sup>Data of positive controls (PC) denote the geometric means and the ranges (minimum-maximum) of EC<sub>50</sub>s or IC<sub>50</sub>s.



**FIGURE 1** Results of agonist assays of mEsr1 RGA. Abbreviations of chemical names are the same in Table 1. Open squares (CZ, DZ and TCS) indicate PC<sub>10</sub> values and NE denotes that neither EC<sub>50</sub> nor PC<sub>10</sub> was obtained

**TABLE 6** Nominal and measured test concentrations for the TG229 and the fiscal year of implementation of each exposure study

Chemical <sup>a</sup>		Test concentrations <sup>b</sup>				Year of implementation
		conc. 1	conc. 2	conc. 3	conc. 4	
BP2	Nominal (mg/L)	0.100	1.00	10.0	na	FY2018
	Measured (mg/L)	0.0943 (94)	0.939 (94)	9.53 (95)		
BPA	Nominal (mg/L)	0.20	1.00	5.00	na	FY2015
	Measured (mg/L)	0.155 (78)	0.826 (83)	4.67 (93)		
CZ	Nominal (mg/L)	0.100	0.320	1.00	na	FY2011
	Measured (mg/L)	0.11 (110)	0.349 (109)	1.02 (102)		
DZ	Nominal (µg/L)	40	200	600	1,000	FY2015
	Measured (µg/L)	38 (95)	196 (98)	598 (100)	952 (95)	
DIBP	Nominal (µg/L)	40	200	1,000	na	FY2017
	Measured (µg/L)	35 (88)	184 (92)	836 (84)		
E1	Nominal (ng/L)	32	100	320	1,000	FY2011
	Measured (ng/L)	29.3 (91)	112 (112)	272 (85)	1,009 (101)	
FV	Nominal (µg/L)	0.120	0.60	3.00	na	FY2015
	Measured (µg/L)	0.0619 (52)	0.294 (49)	1.30 (43)		
MPB	Nominal (mg/L)	0.400	2.00	10.0	na	FY2014
	Measured (mg/L)	0.357 (89)	1.9 (95)	9.75 (98)		
PPB	Nominal (mg/L)	0.320	1.00	3.20	na	FY2017
	Measured (mg/L)	0.311 (97)	0.926 (93)	2.94 (92)		
1NT	Nominal (µg/L)	100	316	1,000	na	FY2012
	Measured (µg/L)	80 (80)	258 (82)	857 (86)		
4NP	Nominal (µg/L)	6.4	20	64	200	FY2014
	Measured (µg/L)	5.63 (88)	18.8 (94)	51.8 (81)	170 (85)	
4tOP	Nominal (µg/L)	25.0	79.1	250	na	FY2014
	Measured (µg/L)	25.3 (101)	82.3 (104)	250 (100)		
PDM	Nominal (µg/L)	8.00	40.0	200	na	FY2018
	Measured (µg/L)	5.69 (71)	28.8 (72)	100 (50)		
4tPP	Nominal (µg/L)	62.5	250	1,000	na	FY2012
	Measured (µg/L)	58.4 (93)	227 (91)	940 (94)		
PHT	Nominal (mg/L)	2.25	4.5	9.00	18.0	FY2011
	Measured (mg/L)	2.25 (100)	4.76 (106)	8.72 (97)	18.51 (103)	
TCS	Nominal (µg/L)	50.0	100	200	400	FY2016
	Measured (µg/L)	54.5 (109)	104 (104)	177 (89)	353 (88)	
TPP	Nominal (µg/L)	20.0	64.0	200	640	FY2012
	Measured (µg/L)	2.13 (11)	7.19 (11)	17.1 (8.5)	44.9 (7.0)	

Abbreviation: na, not applicable (because the assays were conducted in three concentrations).

<sup>a</sup>Abbreviations of chemical names are the same in Table 1.

<sup>b</sup>The ratios in percentage of the mean measured concentrations to the nominal show in parentheses.

### 3.2.2 | Mortality

The validity criterion for control mortality in the OECD TG229, less than 10%, was satisfied in all studies (Table 7). A significant increase of mortality compared with the control was found at 4.67 mg/L of BPA (71%), at 598 and 952 µg/L of DZ (42% and 58%, respectively), PHT at 18.5 mg/L (33%), and at 177 and 353 µg/L of TCS (29% and 50%, respectively). It is noted that, in the DZ assay, the testing

laboratory found the symptoms of presumed paralysis of motor function (clumsy swimming, twitching of pectoral fins) in all fish at the 196 µg/L level and above, and, in some individuals (12.5%), at 38 µg/L (data not shown). Due to the reduced food intake associated with the symptoms observed, the weight and the HSI of both male and female Japanese medaka exposed to DZ at more than 196 µg/L were significantly smaller than the controls at the completion of the exposure (Table 8). These results indicate that the toxicity of DZ, which was not

TABLE 7 Mortality, hepatic VTG concentration, SCC, numbers of total and fertilized eggs, and fertility of test fish at the completion of the exposure in TG229 assays

Chemical <sup>a</sup>	Measured concentration	Number of fish		Mortality	VTG (ng/mg-liver) <sup>b</sup>		SSC		Number of total eggs	Number of fertilized eggs	Fertility rate (%)
		Male	Female		Male	Female	Male	Female			
BP2	DWC	12	12	0 (0.0%)	0.6 ± 0.1	345 ± 152	80 ± 4.8	0 ± 0	14 ± 1.8	14 ± 1.6	95 ± 1.4
	0.0943 mg/L	12	12	0 (0.0%)	0.5 ± 0.0	354 ± 105	73 ± 7.0	0 ± 0	15 ± 2.3	15 ± 2.2	95 ± 2.3
	0.939 mg/L	12	12	1 (4.2%)	0.9 ± 0.5	398 ± 230	77 ± 20	0 ± 0	15 ± 2.7	14 ± 2.3	94 ± 2.5
	9.53 mg/L	12	12	0 (0.0%)	↑ 393 ± 306	449 ± 114	79 ± 8.4	0 ± 0	14 ± 1.7	13 ± 1.7	↓ 90 ± 3.5
BPA	DWC	12	12	0 (0.0%)	0.5 ± 0.0	438 ± 60	68 ± 11	0 ± 0	14 ± 2.1	Na	97 ± 0.4
	0.155 mg/L	12	12	4 (17%)	0.5 ± 0.0	435 ± 65	74 ± 3.6	0 ± 0	14 ± 1.8	Na	96 ± 2.1
	0.826 mg/L	12	12	2 (8.3%)	0.5 ± 0.0	768 ± 297	71 ± 4.2	0 ± 0	14 ± 1.6	Na	94 ± 1.9
	4.67 mg/L	12	12	↑ 17 (71%)	↑ 2,804 ± 811	3,456	76 ± 5.7	0 ± 0	## ± 1.2	Na	↓ 59 ± 2.5
CZ	DWC	12	12	0 (0.0%)	1.9 ± 2.8	2,360 ± 617	98 ± 9.9	0 ± 0	20 ± 4.9	18 ± 4.2	90 ± 3.3
	0.110 mg/L	12	12	0 (0.0%)	0.5 ± 0.0	2,570 ± 528	99 ± 9.1	0 ± 0	14 ± 3.3	12 ± 3.6	85 ± 6.6
	0.349 mg/L	12	12	1 (4.2%)	0.5 ± 0.0	2,550 ± 896	96 ± 5.9	0 ± 0	14 ± 5.1	12 ± 5.8	80 ± 1.9
	1.02 mg/L	12	12	2 (8.3%)	0.5 ± 0.0	2,860 ± 679	94 ± 2.3	0 ± 0	## ± 5.1	## ± 5.5	↓ 71 ± 2.0
DZ	DWC <sup>c</sup>	11	13	1 (4.2%)	0.94 ± 1.61	984 ± 374	81 ± 14	0 ± 0	20 ± 2.6	19 ± 4.0	98 ± 0.7
	38 µg/L	12	12	1 (4.2%)	0.13 ± 0.16	2,665 ± 1942	82 ± 16	0 ± 0	19 ± 0.6	18 ± 0.7	96 ± 0.8
	196 µg/L	12	12	1 (4.2%)	0.08 ± 0.14	1722 ± 1,394	84 ± 19	0 ± 0	## ± 0.1	## ± 0.1	↓ 12 ± 6.3
	598 µg/L	12	12	↑ 10 (42%)	0.06 ± 0.04	661 ± 552	89 ± 17	0 ± 0	## ± 0.1	## ± 0.1	↓ 55 ± 6.7
DIBP	952 µg/L	12	12	↑ 14 (58%)	0.89 ± 1.67	1,621 ± 1,314	89 ± 15	0 ± 0	## ± 0.2	## ± 0.1	↓ ## ± 1.4
	DWC	12	12	0 (0.0%)	0.4 ± 0.4	763 ± 109	## ± 14	0 ± 0	24 ± 8.6	23 ± 8.5	97 ± 4.1
	SC	12	12	1 (4.2%)	2.3 ± 2.6	604 ± 114	99 ± 15	0 ± 0	23 ± 7.0	22 ± 6.9	97 ± 3.2
	35 µg/L	12	12	0 (0.0%)	0.3 ± 0.4	596 ± 144	96 ± 11	0 ± 0	24 ± 7.0	23 ± 6.6	97 ± 4.8
E1	184 µg/L	12	12	0 (0.0%)	0.2 ± 0.1	404 ± 85	96 ± 10	0 ± 0	24 ± 7.2	23 ± 6.9	97 ± 3.9
	836 µg/L	12	12	0 (0.0%)	2.2 ± 3.2	421 ± 73	97 ± 18	0 ± 0	25 ± 5.8	24 ± 5.9	96 ± 5.4
	DWC	12	12	0 (0.0%)	3.7 ± 4.3	955 ± 189	## ± 22	0 ± 0	18 ± 2.7	16 ± 3.9	90 ± 9.1
	29 ng/L	12	12	0 (0.0%)	47.9 ± 54.0	652 ± 391	97 ± 14	0 ± 0	18 ± 3.2	17 ± 4.4	92 ± 9.8
FV	112 ng/L	12	12	0 (0.0%)	382 ± 351	977 ± 274	98 ± 25	0 ± 0	19 ± 2.6	19 ± 2.4	96 ± 1.3
	272 ng/L	12	12	0 (0.0%)	↑ 3,649 ± 665	1,017 ± 238	85 ± 14	0 ± 0	17 ± 2.5	16 ± 2.5	96 ± 2.8
	1,009 ng/L	12	12	0 (0.0%)	↑ 5,429 ± 687	1,994 ± 1,480	85 ± 18	0 ± 0	10 ± 2.0	## ± 1.8	86 ± 3.6
	DWC	12	12	0 (0.0%)	2.1 ± 3.2	296 ± 154	82 ± 13	0 ± 0	20 ± 5.1	18 ± 3.6	91 ± 5.2
SC	SC	12	12	0 (0.0%)	7.2 ± 13	298 ± 193	88 ± 3.9	0 ± 0	22 ± 4.0	20 ± 2.9	90 ± 5.7
	0.0619 µg/L	12	12	0 (0.0%)	0.5 ± 0.0	390 ± 68	80 ± 5.6	0 ± 0	19 ± 3.6	18 ± 2.7	92 ± 3.2
	0.294 µg/L	12	12	0 (0.0%)	0.5 ± 0.0	370 ± 212	88 ± 7.2	0 ± 0	21 ± 3.1	20 ± 3.5	93 ± 4.3
	1.30 µg/L	12	12	0 (0.0%)	0.9 ± 0.4	237 ± 152	83 ± 4.8	0 ± 0	24 ± 1.8	23 ± 1.7	94 ± 2.8

TABLE 7 (Continued)

Chemical <sup>a</sup>	Measured concentration	Number of fish		Mortality	VTG (ng/mg·liver) <sup>b</sup>		SSC		Number of total eggs	Number of fertilized eggs	Fertility rate (%)
		Male	Female		Male	Female	Male	Female			
MPB	DWC	12	12	1 (4.2%)	1.3 ± 0.2	3,260 ± 481	98 ± 4.6	0 ± 0	28 ± 4.3	26 ± 3.9	94 ± 1.3
	0.357 mg/L	12	12	0 (0.0%)	1.7 ± 1.2	3,450 ± 724	## ± 8.3	0 ± 0	29 ± 3.3	27 ± 3.5	93 ± 2.9
	1.90 mg/L	12	12	0 (0.0%)	↑ 153 ± 113	3,890 ± 992	99 ± 12	0 ± 0	28 ± 3.3	24 ± 3.0	88 ± 7.7
	9.75 mg/L	12	12	3 (13%)	↑ 2,640 ± 1720	3,930 ± 963	93 ± 15	0 ± 0	↓ 20 ± 2.2	↓ 16 ± 2.3	↓ 78 ± 4.8
PPB	DWC	12	12	1 (4.2%)	0.5 ± 0.0	1,170 ± 72	## ± 7.8	0 ± 0	26 ± 1.0	25 ± 0.7	96 ± 1.0
	0.311 mg/L	12	12	0 (0.0%)	↑ 23.7 ± 9.0	1,190 ± 238	## ± 3.8	0 ± 0	28 ± 1.6	26 ± 0.8	93 ± 3.1
	0.926 mg/L	12	12	0 (0.0%)	↑ 42.2 ± 36.8	1,590 ± 271	## ± 15	0 ± 0	23 ± 2.0	↓ 22 ± 1.6	94 ± 3.7
	2.94 mg/L	12	12	0 (0.0%)	↑ 4,170 ± 513	↑ 3,540 ± 1,080	## ± 8.0	0 ± 0	↓ 10 ± 4.7	↓ ## ± 4.1	↓ 41 ± 2.3
1NT	DWC	12	12	0 (0.0%)	1.4 ± 0.9	3,080 ± 204	## ± 7.8	0 ± 0	25 ± 6.3	24 ± 6.4	95 ± 2.6
	80.0 µg/L	12	12	0 (0.0%)	2.2 ± 1.3	3,220 ± 623	## ± 12	0 ± 0	27 ± 10	24 ± 10	89 ± 4.0
	258 µg/L	12	12	0 (0.0%)	81 ± 75	3,840 ± 579	## ± 16	0 ± 0	21 ± 7.9	19 ± 8.3	90 ± 5.2
	857 µg/L	12	12	0 (0.0%)	1.3 ± 0.8	↑ 5,170 ± 1,530	## ± 11	0 ± 0	17 ± 3.6	11 ± 4.4	↓ 65 ± 14
4NP	DWC	12	12	0 (0.0%)	2.1 ± 4.0	716 ± 411	87 ± 14	0 ± 0	21 ± 2.5	20 ± 2.8	95 ± 3.5
	5.63 µg/L	12	12	0 (0.0%)	↑ 32 ± 31	866 ± 277	92 ± 15	0 ± 0	21 ± 0.9	20 ± 1.2	97 ± 2.2
	18.8 µg/L	12	12	0 (0.0%)	↑ 375 ± 320	↑ 1,153 ± 300	79 ± 21	0 ± 0	18 ± 4.9	17 ± 5.4	94 ± 7.7
	51.8 µg/L	12	12	0 (0.0%)	↑ 2,431 ± 1,455	↑ 1,348 ± 258	97 ± 13	0 ± 0	↓ 13 ± 3.0	↓ ## ± 2.7	↓ 61 ± 10
4tOP	170 µg/L	12	12	1 (4.2%)	↑ 5,009 ± 1830	↑ 1,212 ± 506	87 ± 12	0 ± 0	↓ ## ± 1.7	↓ ## ± 0.8	↓ 33 ± 9.7
	DWC	12	12	0 (0.0%)	1.6 ± 0.8	3,740 ± 699	## ± 3.5	0 ± 0	25 ± 4.8	23 ± 5.2	94 ± 3.9
	25.3 µg/L	12	12	0 (0.0%)	3.2 ± 2.2	3,710 ± 617	99 ± 4.1	0 ± 0	26 ± 3.2	24 ± 3.5	91 ± 5.1
	82.3 µg/L	12	12	1 (4.2%)	↑ 5.6 ± 1.9	4,380 ± 1,350	## ± 7.7	0 ± 0	27 ± 1.3	24 ± 1.5	92 ± 2.1
PDM	250 µg/L	12	12	0 (0.0%)	↑ ##### ± 3,040	5,290 ± 2030	98 ± 8.5	0 ± 0	23 ± 2.6	21 ± 2.6	89 ± 3.3
	DWC	12	12	1 (4.2%)	0.5 ± 0.0	570 ± 248	87 ± 6.7	0 ± 0	21 ± 4.7	19 ± 4.1	91 ± 4.0
	SC	12	12	0 (0.0%)	1.4 ± 1.0	515 ± 328	80 ± 7.4	0 ± 0	22 ± 2.1	18 ± 2.1	85 ± 6.7
	5.69 µg/L	12	12	0 (0.0%)	0.5 ± 0.0	649 ± 200	85 ± 11	0 ± 0	21 ± 3.4	19 ± 3.1	93 ± 0.9
4tPP	28.8 µg/L	12	12	1 (4.2%)	5.7 ± 6.6	933 ± 692	80 ± 8.2	0 ± 0	20 ± 3.9	18 ± 3.6	88 ± 4.9
	100 µg/L	12	12	2 (8.3%)	↑ 774 ± 290	813 ± 157	80 ± 13	0 ± 0	19 ± 2.1	↓ 12 ± 2.3	↓ 62 ± 11
	DWC	12	12	1 (4.2%)	1.8 ± 1.7	2,310 ± 267	## ± 12	0 ± 0	24 ± 2.2	21 ± 3.0	90 ± 6.0
	58.4 µg/L	12	12	1 (4.2%)	↑ 970 ± 858	2,930 ± 737	## ± 6.1	0 ± 0	21 ± 3.1	20 ± 3.2	94 ± 1.7
940 µg/L	227 µg/L	12	12	1 (4.2%)	↑ 3,700 ± 2,300	3,070 ± 686	## ± 6.3	0 ± 0	20 ± 2.7	19 ± 2.9	94 ± 2.5
	940 µg/L	12	12	3 (13%)	↑ ##### ± 6,440	↑ 8,100 ± 3,340	## ± 11	0 ± 0	↓ 16 ± 4.7	↓ 13 ± 3.5	81 ± 8.9

(Continues)

TABLE 7 (Continued)

Chemical <sup>a</sup>	Measured concentration	Number of fish		Mortality		VTG (ng/mg·liver) <sup>b</sup>		SSC		Number of total eggs	Number of fertilized eggs	Fertility rate (%)	
		Male	Female	Male	Female	Male	Female	Male	Female				
PHT	DWC	12	12	0 (0.0%)		3.0 ± 1.5		780 ± 72	## ± 22	0 ± 0	24 ± 5.6	22 ± 6.7	91 ± 8.6
	2.25 mg/L	12	12	0 (0.0%)		1.4 ± 0.5		685 ± 110	95 ± 14	0 ± 0	20 ± 5.0	18 ± 4.3	92 ± 2.8
	4.76 mg/L	12	12	0 (0.0%)		4.2 ± 6.7		1,013 ± 183	## ± 20	0 ± 0	20 ± 2.0	17 ± 3.2	86 ± 9.3
	8.72 mg/L	12	12	1 (4.2%)		1.0 ± 0.4		661 ± 199	99 ± 21	0 ± 0	## ± 1.8	## ± 1.1	↓ 49 ± 12
	18.5 mg/L	12	12	↑ 8 (33%)		1.4 ± 0.9		693 ± 243	85 ± 15	0 ± 0	## ± 1.1	## ± 0.4	↓ 18 ± 4.1
TCS	DWC	12	12	0 (0.0%)		5.4 ± 5.4		581 ± 142	## ± 6.0	0 ± 0	25 ± 5.1	25 ± 5.2	97 ± 1.3
	54.4 µg/L	12	12	1 (4.2%)		3.3 ± 4.7		616 ± 220	## ± 6.0	0 ± 0	26 ± 3.8	25 ± 4.0	96 ± 3.3
	104 µg/L	12	12	3 (13%)		4.5 ± 2.7		698 ± 32	## ± 14	0 ± 0	24 ± 1.1	24 ± 1.0	98 ± 1.4
	177 µg/L	12	12	↑ 7 (29%)		0.9 ± 0.1	↑	955 ± 73	## ± 4.0	0 ± 0	19 ± 7.2	18 ± 7.0	97 ± 1.5
	353 µg/L	12	12	↑ 12 (50%)		1.0 ± 0.3	↑	1,111 ± 265	## ± 19	0 ± 0	10 ± 2.0	10 ± 1.9	98 ± 0.8
TPP	DWC	12	12	0 (0.0%)		9.1 ± 5.4		764 ± 220	88 ± 7.5	0 ± 0	23 ± 3.7	22 ± 4.0	93 ± 5.3
	2.13 µg/L	12	12	0 (0.0%)		6.9 ± 4.0		542 ± 115	83 ± 7.4	0 ± 0	20 ± 2.7	18 ± 3.1	89 ± 4.9
	7.19 µg/L	12	12	0 (0.0%)		8.4 ± 9.0	↓	360 ± 60	84 ± 6.9	0 ± 0	19 ± 3.1	17 ± 3.4	91 ± 3.8
	17.1 µg/L	12	12	0 (0.0%)		11 ± 9.4	↓	420 ± 77	83 ± 7.2	0 ± 0	20 ± 2.0	19 ± 1.8	93 ± 2.2
	44.9 µg/L	12	12	1 (4.2%)		8.8 ± 8.1	↓	417 ± 70	90 ± 8.0	0 ± 0	16 ± 3.2	15 ± 3.5	91 ± 3.9

Note. Data denote in mean ± standard deviation in the replicate mean basis and arrows indicate that a significant increase (↑) or decrease (↓) from the control was found ( $p < 0.05$ ).

Abbreviations: DWC, dilution water control; SC, solvent control; SSC, secondary sex characteristics (number of joint plates with papillary processes on anal fin); VTG, hepatic vitellogenin concentration.

<sup>a</sup>Abbreviations of chemical names are the same in Table 1.

<sup>b</sup>A half value of lower limit of quantification (LLQ) was used in replicate mean calculation for the males of which VTG lower than LLQ was detected.

<sup>c</sup>In DZ study, one test tank of DWC group accidentally included two males and four females.

TABLE 8 Length, weight, HSI, and GSI of the test fish at the completion of the exposure in TG229

Chemical <sup>a</sup>	Measured Concentration	Length (mm) <sup>b</sup>		Weight (mg) <sup>b</sup>		HSI (%)		GSI (%)	
		Male	Female	Male	Female	Male	Female	Male	Female
BP2	DWC	30.9 ± 0.7	30.1 ± 1.0	288 ± 38	317 ± 39	1.9 ± 0.28	3.4 ± 0.42	0.86 ± 0.11	7.5 ± 1.4
	0.0943 mg/L	30.8 ± 1.2	30.2 ± 0.4	291 ± 27	296 ± 16	1.8 ± 0.37	2.8 ± 0.87	1.07 ± 0.14	6.9 ± 0.3
	0.939 mg/L	30.6 ± 1.1	30.3 ± 1.0	290 ± 32	304 ± 48	1.9 ± 0.24	3.1 ± 0.68	0.69 ± 0.15	7.6 ± 1.2
	9.53 mg/L	31.9 ± 1.1	30.4 ± 0.5	327 ± 34	295 ± 8.0	2.0 ± 0.55	3.5 ± 0.58	0.79 ± 0.23	8.6 ± 0.4
BPA	DWC	33.0 ± 0.3	32.0 ± 0.9	325 ± 17	318 ± 25	2.0 ± 0.46	3.5 ± 0.33	0.93 ± 0.33	6.5 ± 1.5
	0.155 mg/L	33.2 ± 0.9	31.7 ± 0.9	290 ± 23	313 ± 23	2.1 ± 0.40	3.4 ± 0.46	0.91 ± 0.13	6.8 ± 0.5
	0.826 mg/L	32.5 ± 0.9	32.0 ± 0.9	310 ± 10	342 ± 20	2.3 ± 0.45	3.3 ± 0.75	0.80 ± 0.23	6.0 ± 1.0
	4.67 mg/L	33.6 ± 1.9	30.6	370 ± 72	281	3.4 ± 1.15	4.8	0.77 ± 0.23	2.3
CZ	DWC	35.4 ± 0.6	36.4 ± 0.5	476 ± 8.9	592 ± 18	2.3 ± 0.34	4.8 ± 0.72	0.71 ± 0.09	10.0 ± 1.7
	0.110 mg/L	34.6 ± 0.4	35.8 ± 0.3	437 ± 31	546 ± 15	2.2 ± 0.34	4.2 ± 0.30	0.67 ± 0.04	10.0 ± 1.6
	0.349 mg/L	35.3 ± 1.1	35.8 ± 1.0	483 ± 50	515 ± 28	1.8 ± 0.18	3.4 ± 0.69	0.67 ± 0.11	8.3 ± 0.9
	1.02 mg/L	34.4 ± 0.8	35.0 ± 1.0	419 ± 40	470 ± 49	1.7 ± 0.14	3.5 ± 0.33	0.55 ± 0.08	7.6 ± 0.9
DZ	DWC	30.8 ± 0.4	31.7 ± 0.9	305 ± 35	376 ± 24	3.6 ± 1.20	5.8 ± 1.50	1.2 ± 0.2	12.9 ± 2.1
	38 µg/L	30.1 ± 0.6	30.7 ± 1.0	294 ± 21	338 ± 36	2.8 ± 1.70	5.3 ± 1.20	0.9 ± 0.4	12.3 ± 1.3
	196 µg/L	28.5 ± 0.6	28.5 ± 1.1	200 ± 14	217 ± 19	1.6 ± 0.60	2.0 ± 0.70	0.6 ± 0.2	6.8 ± 3.5
	598 µg/L	29.6 ± 0.2	28.7 ± 0.0	190 ± 13	203 ± 47	1.2 ± 0.40	1.9 ± 0.30	0.4 ± 0.1	3.3 ± 0.7
DIBP	952 µg/L	28.3 ± 1.2	27.5 ± 0.8	183 ± 20	203 ± 26	1.5 ± 0.60	1.6 ± 0.50	0.5 ± 0.1	6.9 ± 2.4
	DWC	32.4 ± 1.5	32.6 ± 1.1	345 ± 58	395 ± 30	3.5 ± 3.00	5.8 ± 1.50	1.2 ± 0.4	12.7 ± 1.7
	SC	32.6 ± 1.2	32.5 ± 1.0	367 ± 46	395 ± 42	2.7 ± 0.70	5.8 ± 1.10	1.1 ± 0.3	13.1 ± 1.8
	35 µg/L	31.9 ± 1.2	33.6 ± 1.5	335 ± 39	446 ± 48	2.7 ± 0.40	6.3 ± 1.60	1.2 ± 0.2	13.3 ± 2.3
E1	184 µg/L	31.8 ± 1.4	32.6 ± 1.5	329 ± 55	405 ± 52	3.1 ± 0.40	6.3 ± 0.90	1.0 ± 0.3	13.6 ± 1.9
	836 µg/L	32.4 ± 1.7	32.6 ± 1.4	348 ± 62	413 ± 61	3.4 ± 0.40	5.9 ± 1.30	1.2 ± 0.4	12.0 ± 2.0
	DWC	36.1 ± 1.6	34.4 ± 1.1	469 ± 55	453 ± 35	2.1 ± 0.49	6.0 ± 0.93	1.00 ± 0.32	10.8 ± 1.9
	29 ng/L	35.8 ± 1.7	33.8 ± 1.0	468 ± 85	454 ± 56	2.0 ± 0.28	5.4 ± 0.61	1.15 ± 0.31	10.7 ± 1.4
FV	112 ng/L	36.5 ± 1.2	34.3 ± 1.6	481 ± 44	460 ± 86	2.1 ± 0.54	5.5 ± 0.77	1.17 ± 0.27	11.2 ± 1.8
	272 ng/L	36.5 ± 1.3	34.0 ± 1.0	464 ± 62	417 ± 41	2.7 ± 0.38	6.0 ± 0.76	0.84 ± 0.32	11.3 ± 1.0
	1,009 ng/L	35.9 ± 1.4	34.2 ± 2.1	468 ± 54	443 ± 97	3.5 ± 0.50	5.1 ± 1.24	0.48 ± 0.23	11.4 ± 7.1
	DWC	34.5 ± 0.7	34.1 ± 0.4	435 ± 20	471 ± 40	2.4 ± 0.30	3.9 ± 1.20	0.72 ± 0.16	10.4 ± 0.5
SC	DWC	34.7 ± 0.9	33.7 ± 0.2	449 ± 28	442 ± 29	2.7 ± 0.40	5.0 ± 0.30	0.72 ± 0.07	10.0 ± 0.9
	0.0619 µg/L	33.8 ± 1.4	33.5 ± 1.1	418 ± 51	454 ± 36	2.7 ± 0.30	4.3 ± 0.70	0.75 ± 0.07	9.9 ± 0.7
	0.294 µg/L	34.0 ± 0.2	34.4 ± 0.9	422 ± 22	484 ± 33	2.3 ± 0.50	4.7 ± 0.40	0.67 ± 0.11	9.7 ± 0.5
	1.30 µg/L	33.9 ± 1.9	34.1 ± 0.5	416 ± 56	491 ± 33	2.5 ± 0.20	4.4 ± 0.80	0.72 ± 0.10	10.0 ± 0.1

(Continues)



TABLE 8 (Continued)

Chemical <sup>a</sup>	Measured Concentration	Length (mm) <sup>b</sup>		Weight (mg) <sup>b</sup>		HSI (%)		GSI (%)	
		Male	Female	Male	Female	Male	Female	Male	Female
MPB	DWC	36.1 ± 1.0	37.1 ± 0.5	527 ± 16	597 ± 46	2.0 ± 0.38	4.5 ± 0.72	0.69 ± 0.08	10.0 ± 1.4
	0.357 mg/L	36.0 ± 0.5	37.0 ± 1.3	514 ± 28	589 ± 39	2.1 ± 0.16	4.4 ± 0.44	0.74 ± 0.18	11.0 ± 1.2
	1.90 mg/L	36.4 ± 1.3	36.2 ± 1.2	530 ± 47	574 ± 27	2.4 ± 0.21	4.7 ± 0.59	0.81 ± 0.06	11.0 ± 0.9
	9.75 mg/L	36.2 ± 1.1	36.6 ± 1.4	504 ± 62	603 ± 47	2.3 ± 0.60	5.4 ± 0.38	0.69 ± 0.15	9.8 ± 0.9
PPB	DWC	36.8 ± 0.6	36.1 ± 0.7	536 ± 33	520 ± 19	2.1 ± 0.26	5.0 ± 0.43	0.79 ± 0.08	9.7 ± 1.2
	0.311 mg/L	36.4 ± 1.3	36.1 ± 0.2	525 ± 13	525 ± 27	2.7 ± 0.98	5.4 ± 0.75	0.74 ± 0.14	10.0 ± 0.2
	0.926 mg/L	37.1 ± 1.7	36.8 ± 0.8	515 ± 49	539 ± 21	2.7 ± 0.23	5.1 ± 0.20	0.78 ± 0.05	9.9 ± 1.1
	2.94 mg/L	36.5 ± 0.9	36.5 ± 1.5	490 ± 26	549 ± 83	2.3 ± 0.14	4.3 ± 0.77	0.81 ± 0.21	10.0 ± 1.1
1NT	DWC	39.1 ± 0.9	38.6 ± 0.5	669 ± 52	653 ± 39	2.0 ± 0.21	4.0 ± 0.14	0.80 ± 0.05	11.4 ± 0.7
	80.0 µg/L	39.2 ± 1.1	39.3 ± 0.3	645 ± 53	702 ± 48	1.9 ± 0.15	4.2 ± 0.32	0.67 ± 0.15	11.1 ± 0.4
	258 µg/L	38.2 ± 1.4	39.0 ± 0.3	598 ± 30	697 ± 43	2.3 ± 0.48	4.1 ± 0.48	0.81 ± 0.18	11.0 ± 1.7
	857 µg/L	38.9 ± 1.3	39.2 ± 1.0	638 ± 81	807 ± 126	2.3 ± 0.26	4.1 ± 0.75	0.61 ± 0.03	17.1 ± 6.1
4NP	DWC	37.0 ± 0.8	35.6 ± 0.6	507 ± 33	492 ± 18	1.4 ± 0.10	3.4 ± 0.20	0.89 ± 0.06	7.2 ± 0.5
	5.63 µg/L	36.8 ± 1.3	36.7 ± 1.3	515 ± 45	535 ± 82	1.7 ± 0.10	3.4 ± 0.55	1.02 ± 0.07	7.8 ± 1.6
	18.8 µg/L	37.5 ± 0.6	35.5 ± 1.2	552 ± 34	500 ± 46	1.7 ± 0.28	4.1 ± 0.59	1.08 ± 0.06	8.9 ± 0.6
	51.8 µg/L	37.6 ± 1.2	36.4 ± 0.8	552 ± 69	537 ± 45	2.0 ± 0.22	4.2 ± 0.66	1.15 ± 0.20	7.7 ± 0.6
4tOP	170 µg/L	38.6 ± 1.5	36.0 ± 1.6	602 ± 64	540 ± 64	2.4 ± 0.10	3.9 ± 0.58	1.05 ± 0.15	7.9 ± 1.5
	DWC	38.1 ± 1.2	36.5 ± 0.5	664 ± 73	578 ± 29	2.6 ± 0.50	4.2 ± 0.63	0.92 ± 0.33	10.3 ± 1.5
	25.3 µg/L	37.0 ± 0.9	37.8 ± 0.5	608 ± 31	625 ± 55	2.1 ± 0.16	4.6 ± 0.82	0.83 ± 0.11	11.4 ± 1.6
	82.3 µg/L	37.9 ± 0.6	37.0 ± 0.2	630 ± 44	599 ± 16	2.6 ± 0.10	4.1 ± 0.58	0.81 ± 0.08	10.7 ± 0.4
PDM	250 µg/L	37.4 ± 0.4	36.7 ± 0.5	626 ± 21	605 ± 10	2.9 ± 0.28	4.5 ± 0.29	0.81 ± 0.08	11.1 ± 2.0
	DWC	31.8 ± 1.1	31.8 ± 1.2	367 ± 21	393 ± 43	2.5 ± 0.39	3.8 ± 0.75	0.74 ± 0.23	10.6 ± 1.2
	SC	31.0 ± 0.9	31.2 ± 1.3	342 ± 31	369 ± 52	2.8 ± 0.45	3.9 ± 0.34	0.65 ± 0.10	10.6 ± 1.0
	5.69 µg/L	31.9 ± 0.7	32.4 ± 0.7	366 ± 25	427 ± 25	2.7 ± 0.16	4.5 ± 0.70	0.64 ± 0.11	10.7 ± 0.8
4tPP	28.8 µg/L	32.2 ± 0.2	33.1 ± 0.9	393 ± 20	455 ± 46	2.7 ± 0.28	7.7 ± 0.58	0.55 ± 0.11	11.3 ± 0.8
	100 µg/L	31.9 ± 0.7	31.4 ± 0.8	389 ± 28	423 ± 30	2.9 ± 0.45	4.4 ± 1.04	0.60 ± 0.20	9.7 ± 0.3
	DWC	36.4 ± 0.6	36.2 ± 0.4	509 ± 21	552 ± 32	1.8 ± 0.14	3.9 ± 0.59	0.76 ± 0.09	11.0 ± 1.8
	58.4 µg/L	37.8 ± 1.2	36.0 ± 0.5	590 ± 39	558 ± 48	2.3 ± 0.18	4.3 ± 0.50	0.89 ± 0.11	10.0 ± 0.6
940 µg/L	227 µg/L	36.2 ± 0.6	35.5 ± 0.7	546 ± 23	523 ± 48	2.2 ± 0.33	4.2 ± 0.44	0.73 ± 0.16	9.9 ± 1.0
	940 µg/L	35.6 ± 0.8	36.1 ± 1.6	509 ± 47	588 ± 130	3.6 ± 0.50	3.9 ± 0.73	0.73 ± 0.13	8.9 ± 1.2

TABLE 8 (Continued)

Chemical <sup>a</sup>	Measured Concentration	Length (mm) <sup>b</sup>		Weight (mg) <sup>b</sup>		HSI (%)		GSI (%)	
		Male	Female	Male	Female	Male	Female	Male	Female
PHT	DWC	36.6 ± 0.8	35.1 ± 0.6	450 ± 3	467 ± 26	1.3 ± 0.55	3.6 ± 1.19	0.78 ± 0.14	8.5 ± 1.0
	2.25 mg/L	35.9 ± 0.6	35.9 ± 0.4	435 ± 26	466 ± 26	1.4 ± 0.36	2.8 ± 1.03	1.32 ± 0.77	8.3 ± 0.8
	4.76 mg/L	36.5 ± 1.5	35.5 ± 1.0	435 ± 63	463 ± 10	1.6 ± 0.35	2.9 ± 1.18	1.00 ± 0.26	9.0 ± 1.4
	8.72 mg/L	36.5 ± 0.1	36.0 ± 0.5	415 ± 31	576 ± 28	↑ 2.2 ± 0.30	3.0 ± 1.31	1.04 ± 0.31	↑ 19.3 ± 10
	18.5 mg/L	38.4 ± 1.4	37.5 ± 1.4	507 ± 83	609 ± 67	↑ 3.4 ± 0.47	3.4 ± 0.88	0.44 ± 0.26	↑ 17.0 ± 9.7
TCS	DWC	34.7 ± 1.0	34.1 ± 0.8	419 ± 30	461 ± 25	1.4 ± 0.18	4.4 ± 0.48	1.10 ± 0.17	8.5 ± 0.6
	54.4 µg/L	33.6 ± 0.7	33.5 ± 0.7	379 ± 28	449 ± 11	1.6 ± 0.23	3.8 ± 0.40	1.00 ± 0.13	7.3 ± 0.4
	104 µg/L	34.8 ± 0.7	32.8 ± 1.1	417 ± 13	409 ± 20	1.5 ± 0.09	3.7 ± 0.79	1.00 ± 0.23	7.5 ± 0.6
	177 µg/L	33.3 ± 0.9	32.4 ± 1.3	380 ± 66	425 ± 45	1.4 ± 0.41	3.8 ± 0.30	0.90 ± 0.17	7.9 ± 1.4
	353 µg/L	33.3 ± 1.5	33.2 ± 0.9	361 ± 57	437 ± 30	2.4 ± 1.08	4.5 ± 0.23	1.20 ± 0.73	8.7 ± 1.1
TPP	DWC	38.0 ± 0.5	36.5 ± 0.5	543 ± 45	512 ± 37	1.2 ± 0.19	2.7 ± 0.52	0.86 ± 0.08	8.0 ± 0.7
	2.13 µg/L	37.9 ± 1.1	35.3 ± 0.7	532 ± 44	449 ± 19	1.4 ± 0.12	3.5 ± 0.39	0.87 ± 0.17	7.9 ± 0.5
	7.19 µg/L	38.0 ± 1.1	35.5 ± 0.3	544 ± 49	464 ± 27	↑ 1.7 ± 0.29	3.1 ± 0.21	0.95 ± 0.09	8.1 ± 0.6
	17.1 µg/L	37.7 ± 1.0	35.2 ± 1.0	539 ± 31	468 ± 34	↑ 1.7 ± 0.24	3.6 ± 0.46	0.94 ± 0.10	7.7 ± 0.7
	44.9 µg/L	38.3 ± 1.1	34.4 ± 0.4	583 ± 68	432 ± 32	↑ 1.7 ± 0.13	3.6 ± 0.60	0.80 ± 0.07	9.4 ± 1.3

Note. Data denote in mean ± standard deviation in the replicate mean basis and arrows indicate that a significant increase (↑) or decrease (↓) from the control was found ( $p < 0.05$ ).

Abbreviations: DWC, dilution water control; GSI, gonadosomatic index; HSI, hepatosomatic index; SC, solvent control.

<sup>a</sup>Abbreviations of chemical names are the same in Table 1.

<sup>b</sup>No statistical analysis was conducted for length and weight data.

related to endocrine disruption, showed adverse effects on the test fish, even at 196 µg/L at which there was no statistically significant increase in mortality. In addition, at 4.76 mg/L and above in the PHT treatment, fish frequently showed abnormal behavior such as loss of balance, although feeding activity did not reduce.

### 3.2.3 | Hepatic VTG

VTG induction is generally at a low level in male fish and thus has been widely used as a biomarker to screen chemicals with estrogenic activity over the years (Hansen et al., 1998; Kime et al., 1999; OECD, 2009b; Onishi et al., 2021; Sumpter & Jobling, 1995; Tyler et al., 1996). In the present study, the mean hepatic VTG concentrations of male Japanese medaka in control groups (i.e., 17 DWC and three SC groups) were all less than 10 ng/mg-liver. On the other hand, hepatic VTG concentrations in females varied from 296 to 3,740 ng/mg-liver among the control groups (Table 7).

In males, nine chemicals (BP2, BPA, E1, MPB, PPB, 4NP, 4tOP, PDM, and 4tPP) significantly elevated hepatic VTG levels. The LOECs of them were 9.53 mg/L, 4.67 mg/L, 272 ng/L, 1.90 mg/L, 0.311 mg/L, 5.63 µg/L, 82.3 µg/L, 100 µg/L, and 58.4 µg/L, respectively and a positive correlation was evident in the relationship with the EC<sub>50</sub>s obtained from mEsr1 RGAs (Figure 2). In females, DIBP and TPP significantly reduced VTG levels, whereas a significant increase was found in the PPB, 1NT, 4NP, 4tPP, and TCS assays. In the TCS assay, two exposure concentrations that significantly reduced VTG in both males and females resulted in a significant increase in mortality.

### 3.2.4 | Secondary sex characteristics

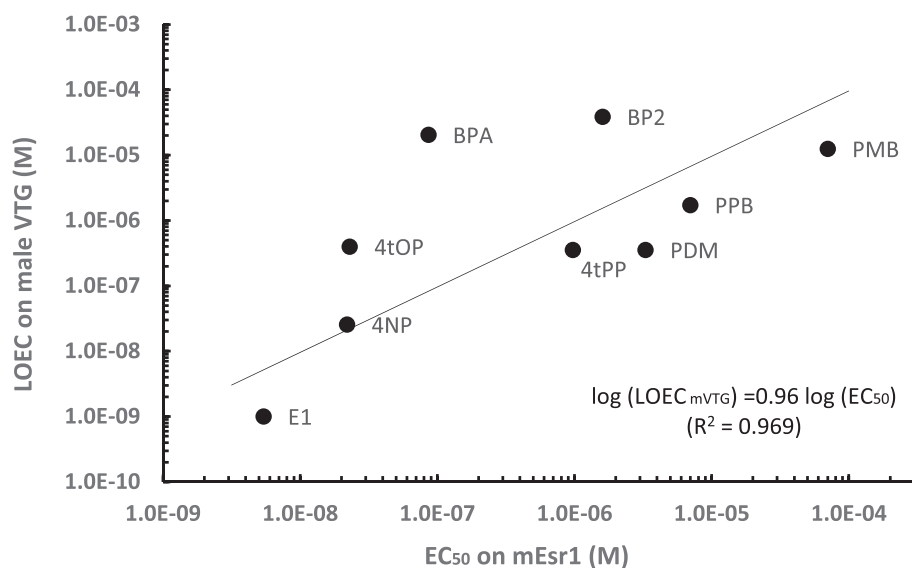
The papillary processes on the anal fin rays are masculine SSC in Japanese medaka and can be induced in females by exogenous

androgen exposure has been reported (Ogino et al., 2014; Onishi et al., 2021). In the present study, the mean values of the number of joint plates with papillary processes on anal fin rays ranged from 68 to 126 in 17 control male groups, and none were observed in females. A significant change in SSC was found in neither males nor females by the exposure of the 17 chemicals (Table 7).

### 3.2.5 | Reproduction (spawning status)

The number of total eggs ranged from 14 to 28 eggs in the control groups among the 17 assays. In addition, there were differences between the DWC and SC groups in the DIBP, FV, and PMD assays as shown in Table 7. A statistically significant decrease in fecundity was observed in the BPA, CZ, DZ, E1, MPB, PPB, 4NP, 4tPP, PHT, TCS, and TPP studies, whereas on the other hand, a significant increase in mortality or abnormal symptoms, suggesting an overt toxicity by the chemicals, was also found at the same concentrations of BPA, DZ, PHT, and TCS. The observed effects on number of fertile eggs were almost the same as those on the number of total eggs, but a significant effect could statistically be detected at lower concentrations in the PPB and PDM assays.

The mean fertility rates at 21 days were more than 90% in almost all the DWC and SC controls among the 17 assays. Only the SC group of the PDM assay showed a less than 90% (85%) fertility rate but was not statistically different from the DWC control (91%). The chemicals in which a significant difference compared with the control could be detected in fertility rate were slightly different from the chemicals that exhibited an effect on daily fecundity. In the BPA, 1NT, and PMD treatments, a significant decrease in fertility rate was detected at the concentrations that did not affect fecundity, that is, number of total eggs. On the contrary, no significant effect on fertility rate was suggested for CZ, E1, 4tPP, TCS, and TPP in which a significant decrease of number of total eggs was found.



**FIGURE 2** Relationship between EC<sub>50</sub> on mEsr1 and LOEC on VTG induction in male medaka. Abbreviations of chemical names are the same in Table 1

### 3.2.6 | LOEC/MEC ratio

To reference in the considering high-priority candidates for the Tier 2 study, the results of *in vitro* RGAs and *in vivo* TG229 assays, including ratios of LOECs on male VTG induction (LOEC<sub>mVTG</sub>) or reproduction (LOEC<sub>REPRO</sub>) to MECs, are summarized for the 17 test chemicals. As shown in Table 9, the LOEC<sub>mVTG</sub>/MEC ratio was relatively small for 4NP, E1, and 4tOP (18, 66, and 2,660, respectively) and the ratio of LOEC<sub>REPRO</sub>/MEC was relatively small for E1, 4NP, and TPP (246, 162, and 1870, respectively).

## 4 | DISCUSSION

Based on the EXTEND 2016 strategy, OECD TG229 assays, (FSTRA using Japanese medaka) were conducted for 17 chemicals in which RGAs using mEsr1 suggested either an estrogenic (Esr agonistic) or an anti-estrogenic (Esr antagonistic) potency. In the short-term reproduction assay using fish, fecundity and fertility can be the most useful indicators of the general reproductive condition of mature fish because disturbances in the HPG axis that directly or indirectly impair gamete maturation and/or interfere with reproductive behavior will reduce spawning frequency and fecundity, although these endpoints are not intended to unequivocally identify specific cellular mechanisms of action (OECD, 2012; US EPA, 2007). In the Tier 1 *in vivo* screening for 17 chemicals, an adverse effect on reproduction

(i.e., statistically significant reduction in either fecundity or fertility) was found at concentrations at which no overt toxicity, such as increasing mortality and/or abnormal symptoms during the exposure, was observed for BP2, CZ, E1, MPB, PPB, 1NT, 4NP, PDM, 4tPP, and TPP, and as shown in Table 8, the LOEC<sub>REPRO</sub> of 9,530, 1,020, 1,009, 9,750, 926, 857, 51.7, 100, 940, and 44.9 µg/L were obtained, respectively. Within these chemicals, the LOEC<sub>REPRO</sub>/MEC ratio was relatively small for E1, 4NP, and TPP (246, 162, and 1,870, respectively), indicating a concern of environmental risk related to endocrine disrupting activities.

In fish assay for endocrine disrupting chemicals, estrogenic activities can be detected via measurement of VTG induction in males (OECD, 2012), and its applicability has been confirmed by several validation studies (OECD, 2006b, 2006c, 2009a, 2011; US EPA, 2007). In the present study, the VTG levels in control males were quite low, less than 10 ng/mg-liver, in all the 17 assays. As a result, a significant increase of VTG was detected in males exposed to nine of the test chemicals (Table 7). In the mEsr1 RGAs, on the other hand, a positive agonistic activity was detected, and thus, an effective concentration was obtained for 15 chemicals, excepting CZ and PHT (Table 5). For the six chemicals that did not increase male VTG in the TG229 studies, PC<sub>10</sub>, but not EC<sub>50</sub>, could be estimated for the following three, DZ, DIBP, and TCS in the mEsr1 RGAs, indicating estrogenic potency was weaker than for the other test substances. In a two-tiered testing strategy such as EXTEND2016 program, a reasonable estimation of strength of endocrine disruptive activity on test

**TABLE 9** Summary of RGAs and TG229 assays and LOEC/MEC ratio

Chemical <sup>a</sup>	MEC (µg/L)	In vitro positive <sup>b</sup>	LOEC <sub>TOX</sub> (µg/L)	LOEC <sub>mVTG</sub> (µg/L)	LOEC <sub>REPRO</sub> (µg/L)	LOEC <sub>mVTG</sub> /MEC	LOEC <sub>REPRO</sub> /MEC
BP2	0.013	Esr, Ar, aAr	ND (>9,530)	9,530	9,530	733,000	733,000
BPA	0.28	Esr, aAr	4,670	4,670	4,670	16,700	16,700
CZ	0.0025	aEsr	ND (>1,020)	ND (>1,020)	1,020	ND (>408,000)	408,000
DZ	0.019	Esr	196	ND (>952)	196	ND (>50,100)	10,300
DIBP	0.03	Esr	ND (>836)	ND (>836)	ND (>836)	ND (>27,900)	ND (>27,900)
E1	0.0041	Esr, aAr	ND (>1,009)	0.272	1,009	66	246
FV	0.041	Esr	ND (>1.3)	ND (>1.30)	ND (>1.30)	ND (>32)	ND (>32)
MPB	0.003	Esr	ND (>9,750)	1900	9,750	633,000	3,250,000
PPB	0.016	Esr	ND (>2,940)	311	926	19,400	57,900
1NT	0.0027	Esr, Ar	ND (>857)	ND (>857)	857	ND (>317,000)	317,000
4NP	0.32	Esr	ND (>170)	5.63	51.8	18	162
4tOP	0.031	Esr	ND (>250)	82.3	ND (>250)	2,660	ND (>8,060)
PDM	nd (<0.0014)	Esr, aAr	ND (>100)	100	100	ND (>71,400)	ND (>71,400)
4tPP	nd (<0.0011)	Esr, aAr	ND (>940)	58.4	940	ND (>53,100)	ND (>671,000)
PHT	0.028	aEsr	4,760	ND (>18,500)	8,720	ND (>661,000)	311,000
TCS	0.093	Esr	177	ND (>353)	353	ND (>353)	3,800
TPP	0.024	Esr	ND (>44.9)	ND (>44.9)	44.9	ND (>1870)	1870

Abbreviations: LOEC, LOEC<sub>TOX</sub>, lowest observed effect concentration (LOEC) on mortality; LOEC<sub>mVTG</sub>, LOEC on VTG induction in males; LOEC<sub>REPRO</sub>, LOEC on reproduction (fecundity and/or fertility); MEC, maximum environmental concentration; ND, could not be determined (because neither MEC nor LOEC could be determined); nd, not detected.

<sup>a</sup>Abbreviations of chemical names are the same in Table 1.

<sup>b</sup>The mode of action in which effective or inhibitory concentration (EC<sub>50</sub>, PC<sub>10</sub>, IC<sub>50</sub> or IC<sub>30</sub>) obtained in RGA was listed (Esr, mEsr1 agonistic; aEsr, mEsr1 antagonistic; Ar, mArβ agonistic; aAr, mArβ antagonistic activity).

organisms *in vivo*, based on the results from preliminary *in vitro* assay, is helpful for prioritization of test chemicals and prediction of effective concentrations in preparation for Tier 1 and 2 *in vivo* assays. Teleost fish including Japanese medaka have at least three Esr subtypes and several *in vivo* and *in vitro* studies suggested that Esr1 might be the key receptor mediating hepatic VTG gene induction (Lee Pow et al., 2016; Mushiobira et al., 2020; Nelson & Habibi, 2010; Tohyama et al., 2017). Therefore, the estrogenic activity determined by the mEsr1 RGA should correlate with the effective concentrations on VTG induction in male medaka *in vivo* assay. For the nine chemicals that significantly induced male VTG, as illustrated in Figure 2, a positive correlation between the EC<sub>50</sub> obtained from the mEsr1 RGAs and the LOECs on male VTG induction from the TG229 assays was evident. These results demonstrated that estrogenic potency *in vivo* is predictable from *in vitro* data such as EC<sub>50</sub> from mEsr1 RGA as well as the reference chemical studies of FSTRA previously reported by Onishi et al. (2021). Our previous study also suggested that the exposure concentrations that might have significant effects on reproduction in long-term exposure studies, such as Medaka Multi-Generation Test (MMT) and Full-life Cycle Test (FLCT), were closer to the LOECs on VTG induction in males than to the LOECs on reproduction in the FSTRA (TG229 study), for instance the ER agonists (E2 and EE2) (Flynn et al., 2017; MOE, 2006; Onishi et al., 2021). In the 17 chemicals of the present study, as shown in Table 9, the LOEC<sub>mVTG</sub>/MEC ratios of 4NP, E1, and 4tOP were relatively smaller than other chemicals, indicating that there is a concern of adverse effects on reproduction in long-term exposure through the full life cycle history of fish.

In addition to the chemicals mentioned above, confirming the adverse effects by a comprehensive long-term reproduction test (i.e., MEOGRT and TG240) might be needed for BPA. This chemical was the most frequently detected in surface water samples within the national survey for the 17 chemicals by the MOE (Table 1), and a combined effect on fecundity and VTG induction, according to the LOEC/MEC ratios, was not so large (Table 9). On the other hand, it should understandably be borne in mind that effects on fecundity might be caused by systemic toxicity rather than endocrine disruption (OECD, 2018), because a significant increase in mortality was found at the same concentration (as shown in Table 9). In the present study, DZ at exposure concentrations more than 196 µg/L caused a statistically significant decrease in fecundities and fertilities. At the same concentrations, on the other hand, a significant increase of mortalities and/or fish with abnormal symptoms, presumed to be caused by inhibition of acetylcholine esterase enzyme at neuromuscular junctions and synapses of nervous system (Čolović et al., 2013), was also found (Table S4). As for DZ, Flynn et al. (2018) conducted a MEOGRT (at the exposure concentrations of 2.9, 5.2, 10.3, 19.8, and 40.2 µg/L) and suggested that the LOECs on fecundity and growth (2.9 and 5.2 µg/L, respectively) were more than 10 times lower than the TG229 assay. With chemicals for which adverse effects on reproduction and non-endocrine toxic effects are found at similar concentrations *in vivo* short-term assay, it might be necessary to evaluate carefully how these toxicities interact with each other and to

evaluate the LOEC on fish reproduction (including secondary effects of growth toxicity) in long-term exposure like the MEOGRT. The MEOGRT data obtained from a BPA study could provide useful information for application of this definitive testing in risk assessment, because the number of the chemicals tested and the laboratories involved in the validation of MEOGRT have so far been limited (OECD, 2015).

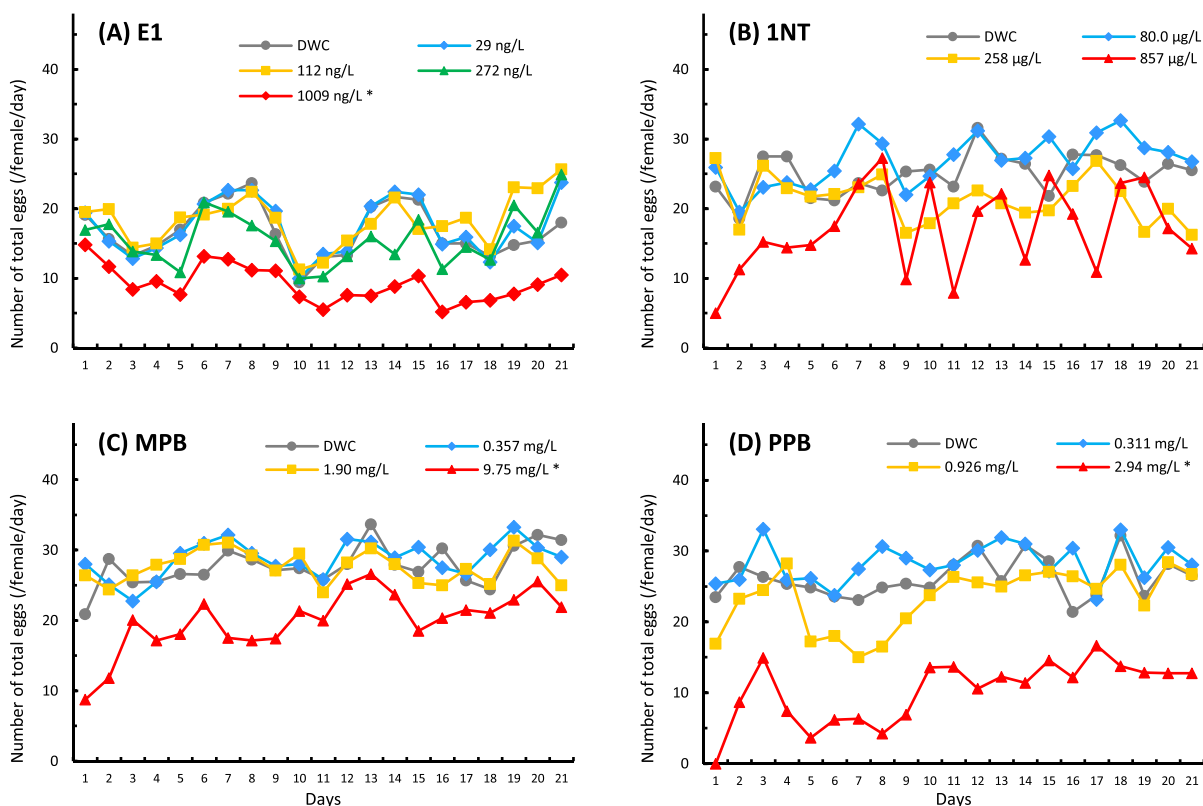
Among the 17 chemicals reported in the present study, antagonistic activity on mEsr1 was detected in CZ and PHT, but both these chemicals did not reduce hepatic VTG in females, although a reduction in fecundity was found in the TG229 assays. On the one hand, DIBP or TPP in which a statistically significant VTG reduction was found in the females *in vivo* did not inhibit the transcriptional activity induced by competitively spiked E2 *in vitro* RGAs. DIBP also significantly reduced the number of spawned eggs during the exposure period of the TG229 assay. The RGAs using mEsr1 are a useful tool in the screening of endocrine disrupting chemicals, but uncertainties remain in extrapolating from *in vitro* to *in vivo* toxicity, due to differences in the physicochemical properties or the external and internal concentrations of the chemicals tested (Groothuis et al., 2015; Onishi et al., 2021; Stadnicka-Michalak et al., 2014). Similarly, in the RGA using mArβ, a weak agonistic activity was suggested for BP2 and 1NT. As stated previously, 1NT was selected as a test chemical based on the results of environmental monitoring as well as the results from the literature survey conducted in FY2011 which epidemiological studies suggested a potential effect on the HPG axis. A later literature (Tange et al., 2016) reported that 1NT showed an estrogenic activity *in vitro* MCF7 cells transfected with ERE-luciferase reporter, supporting the results of present study. Tange et al. (2016) also suggested an anti-androgenic activity of 1NT using CHO cells transfected with ARE-luciferase reporter and hAR. In the TG229 assay, the number of total eggs was quite reduced compared with the control at the highest concentration (857 µg/L) during the first week of chemical exposure although no statistical significance was found, and additionally a large fluctuation in daily egg production was observed during the exposure period (Figure 3B). In the reference chemical studies of TG229 reported by Onishi et al. (2021), a similar response was observed in the medaka exposed to methyltestosterone and levonorgestrel that have an agonistic activity on mArβ, and it was assumed that treatment by an AR agonist might not only interfere with oogenesis but also disrupt maturational and ovulatory mechanisms resulting in inhibition of the normal release of mature oocytes during spawning (Hemmer et al., 2008). The significant increase in GSI in the females treated with 1NT at 857 µg/L (Table 8), probably due to residual mature oocytes, as well as the results from methyltestosterone and levonorgestrel studies in Onishi et al. (2021), would support this explanation. On the other hand, 1NT could not induce masculine SSC, that is, formation of papillary processes on anal fin, on female Japanese medaka *in vivo* TG229 assay, as well as BP2. BP2, for which both agonistic and antagonistic activities on mArβ were detected in the RGAs, did not cause any remarkable alteration in the daily fecundity during the exposure (data not shown). These results suggest that the *in vivo* effects of chemicals that may have

various MOAs and the interaction of each MOA need to be further investigated.

In the TG229 assay (FSTRA using Japanese medaka), because Japanese medaka is a daily spawner (Ankley & Johnson, 2004; Leaf et al., 2011; Padilla et al., 2009), the daily fecundity in controls (which was the average of the number of total eggs spawned by 12 females) was fairly stable over the exposure period in suitable feeding and environmental conditions; therefore, it is assumed that the variation in daily fecundity caused by the chemical treatments reflected the MOA of the chemicals tested and reproductive response of the fish exposed. Specifically, E2 and EE2, natural and synthetic estrogens in vertebrates, gradually decreased the daily fecundities approximately 1 week after from the initiation to the completion of the chemical treatment but did not reduce them immediately and significantly just after the exposure start (Onishi et al., 2021). In the present study, 15 chemicals suggested an agonistic potency in the mEsr1 RGAs and 8 of them significantly induced hepatic VTG in males and reduced the number of eggs spawned in females in the TG229 studies. The tendency in daily fecundity observed with E1, a kind of natural estrogen, was similar to E2 and EE2 (Figure 3A). Of these chemicals, on the one hand, MPB steeply reduced the fecundity on the first day to less than 10 eggs/female, but the daily number of total eggs was gradually increasing until the 21st day (Figure 3C). It is noteworthy that a similar transition in daily fecundity was also evident

in the exposure by PPB at 2.94 mg/L (Figure 3D). It is assumed that these results indicate that the parabens (MPB and PPB) have not only Esr-mediated estrogenic effects as clearly suggested by the VTG induction in males, but also other effects through the HPG axis (inhibition of steroid synthesis) on the reproduction of Japanese medaka. In addition, these results demonstrated that the change in daily fecundity over the exposure duration could support identifying an MOA for the test chemical although it is necessary to consider the possibility of non-endocrine toxic effects (Onishi et al., 2021).

In conclusion, under the EXTEND2016, continued from the EXTEND2010, program, the TG229 assay with Japanese medaka as Tier 1 in vivo screening was conducted on 17 chemicals selected based on the results of environmental monitoring, a literature survey and in vitro RGAs. In the 17 assays, an adverse effect on reproduction in Japanese medaka was suggested for 14 chemicals and a significant increase of hepatic VTG in males was found for nine chemicals indicating estrogenic (Esr agonistic) potency (Table 9). Based on these results, and the frequency and the concentration detected in the aquatic environment in Japan, E1, 4NP (branched isomers), 4tOP, TPP, and BPA were considered as candidate substances, which have a high priority, for Tier 2 testing. Currently, the MEOGRT (TG240) studies for E1, 4tOP, and BPA are being conducted in the laboratories contracted with the MOE



**FIGURE 3** Daily change in the mean number of total eggs during the 21-day exposure period. Data denote the mean of the daily fecundity ( $n = 4$ ). The exposure concentrations in which a significant reduction from the control was statistically detected in the number of total eggs throughout the exposure period were marked with an asterisk ( $p < 0.05$ ). (A) Estrone, (B) 1-naphtol, (C) 4-hydroxybenzoate methyl, and (D) 4-hydroxybenzoate propyl

([http://www.env.go.jp/chemi/post\\_142.html](http://www.env.go.jp/chemi/post_142.html), in Japanese), following the 4NP study (Watanabe et al., 2017).

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## CONFLICT OF INTEREST

The Authors did not report any conflict of interest.

## DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs.

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