Transgenic (*cyp19a1b*-GFP) zebrafish embryos as a tool for assessing combined effects of oestrogenic chemicals

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Abstract

Endocrine disrupting chemicals and especially oestrogen receptor (ER) agonists have been extensively studied over the years due to their potential effects on sexual development and reproduction in vertebrates, notably fish. As ER agonists can exist as complex mixtures in the aquatic environment, evaluating the impact of combined exposure on oestrogenic effects has become increasingly important. Use of predictive models such as concentration addition (CA) and independent action (IA), has allowed assessment of combined estrogenic effects of complex multi-compound mixtures of ER agonists in various fish *in vitro* and *in vivo* experimental models. The present work makes use of a transgenic zebrafish strain, tg(*cyp19a1b*-GFP), which expresses the green fluorescent protein (GFP) under the control of the *cyp19a1b* (brain aromatase or aromatase B) gene, to determine the oestrogenic potency of ER agonists alone or in mixtures. In these studies, tg(*cyp19a1b*-GFP) zebrafish embryos were exposed for four days (from one to five days post fertilization) to five different oestrogenic chemicals; 17α-ethinylestradiol (EE2), 17β-estradiol (E2), estrone (E1), bisphenol A (BPA) and 4-*tert*-octylphenol (OP), and three mixtures of up to four of these compounds. The mixture of BPA, OP and E2 was also tested with primary cultures of rainbow trout hepatocytes by analysing the ER-mediated induction of the oestrogenic biomarker vitellogenin in order to compare the performance of the two methods for assessing oestrogenic effects of complex mixtures. The three tested mixtures were predominantly acting in an additive manner on the expression of GFP. Additivity was indicated by the overlap of the 95% confidence interval of the concentration response curves for the observed data with the CA and IA prediction models, and model deviation ratios within a factor of two for a majority of the mixture concentrations. However, minor deviations determined as more than additive effects for the mixture of EE2, E1 and E2 and less than additive effects for the mixture of BPA, OP, EE2 and E1 were observed at the higher mixture concentrations tested. The successful prediction of additivity by CA and IA in tg(*cyp19a1b*-GFP) zebrafish embryos and deviations at high mixture concentrations seemed to correspond well to results obtained in the rainbow trout hepatocyte assay. The present results clearly show the usefulness of combining predictive modelling and use of *in vitro* bioassays for rapid screening of oestrogenic effects of complex mixtures and environmental samples.

Key words: concentration addition; independent action; tg(*cyp19a1b*-GFP) zebrafish; *Danio rerio*; rainbow trout hepatocytes; *Oncorhynchus mykiss*; oestrogen; mixture

1. Introduction

Endocrine disrupting chemicals (EDCs) and especially oestrogen receptor (ER) agonists have been extensively studied over the years due to the potential effects on reproduction and development of aquatic organisms such as fish. Fish may be simultaneously exposed to a number of different ER agonists (Jeffries et al. 2010; Yan et al., 2012), and mixtures of oestrogenic chemicals have been shown to induce oestrogenic effects *in vivo* (production of Vtg in fish) and *in vitro* (yeast estrogen screen), even when individual compounds were administered at concentrations below their NOEC (reviewed in Kortenkamp, 2008). Hence, assessing combined effects of multiple compounds in mixtures has been a growing field of interest. Two prediction models for combined effects, concentration addition (CA) and independent action (IA) (Altenburger and Greco, 2009; Backhaus et al. 2004), have been used to evaluate the effect of different mixtures of oestrogen agonists, both *in vivo* with exposure of fish (Brian et al., 2005; Thorpe et al., 2001) and *in vitro* by exposure of primary fish cells (Petersen and Tollefsen, 2011). The principle assumption is that these prediction models work as a reference for additive effects of similar acting (CA) and independently acting (IA) chemicals, whereas deviations from these predictions indicate either antagonism or synergy.

While screening for endocrine disruption is often conducted with *in vitro* systems, *in vivo* studies on endocrine disruption in fish are conditionally required by various regulations worldwide, such as the US Toxic Substance Control Act, the US Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the US Federal Food, Drug, and Cosmetic Act (FFDCA) and European Regulations on industrial chemicals, plant protection products, biocides and pharmaceuticals. Various testing schemes are proposed for the assessment of endocrine disruption (with focus on reproductive hormones) ranging from abbreviated short term tests (e.g. OECD 230 fish screening assay) to full life cycle tests (OECD, 2012a). *In vivo* fish studies are considered the most environmentally relevant test strategy for assessment of endocrine disruption (OECD, 2010). However, combined toxicity studies often require testing of a large number of concentrations of the single compounds and mixtures, leading to a substantial resource demand in terms of experimental animal usage, workload and cost. Alternative test methods using embryos and *in vitro* test approaches offer more ethical and cost-efficient alternatives for toxicity assessment, and comply with the aim of implementing the 3Rs (reduction, replacement and refinement) in ecotoxicological and regulatory testing (Castano et al., 2003). Alternative approaches such as primary cells, permanent cell lines and

fish embryo tests (FET) are thus becoming increasingly important tools for performing screening for EDCs and ER agonists in particular.

The fish embryo test has emerged as a promising alternative to (adult) fish *in vivo* testing (Embry et al., 2010) and a draft OECD test guideline (OECD, 2012b) for acute toxicity testing is currently under development for inclusion into regulatory testing. For acute toxicity, the zebrafish FET has demonstrated a high predictive accuracy with an almost 1:1 correlation between acute toxicity in the zebrafish FET and acute toxicity to fish *in vivo* (Belanger et al., 2013; Knöbel et al., 2012; Lammer et al., 2009). Inclusion of additional sub-lethal toxicity endpoints like protein and mRNA measurements could however, also enable to use the FET for investigation of chronic effects and/or endocrine disruption (Brion et al., 2012; Volz et al., 2011). For assessment of endocrine disruption, a transgenic zebrafish strain tg(*cyp19a1b*-GFP) (Tong et al., 2009) has been developed and recently proven suitable for screening of ER agonists and binary mixtures of these (Brion et al., 2012). The tg(*cyp19a1b*-GFP) zebrafish strain is stably transfected with a green fluorescent protein (GFP) gene that is regulated by the zebrafish *cyp19a1b* (brain aromatase) promoter (Tong et al., 2009). The brain aromatase is a key enzyme in the endogenous oestrogen synthesis and catalyses the aromatization of androgens into oestrogens (Diotel et al., 2010). Constitutive expression of *cyp19a1b* in the zebrafish embryo is detectable from between 24 and 48 hpf. In the same time window a significant increase is also observed for the expression of zebrafish ERs (Mouriec et al., 2009b), indicating that basal expression of *cyp19a1b* most likely relies upon expression of ERs. It has been found that the upregulation of *cyp19a1b* is dependent on both the oestrogen-responsive element (ERE) and a region named GxRE of the *cyp19a1b* promoter (Le Page et al., 2008; Menuet et al., 2005). The GxRE recruits glial specific transcription factors that act in synergy with ERs, indicating the presence of an oestrogen responsive unit in the *cyp19a1b* promoter in the radial glial cells (Le Page et al., 2008). The constitutive brain aromatase expression in embryos is low, but can be induced as early as 24 hours post fertilization (hpf) if exposed to 17β-estradiol (E2) (Mouriec et al., 2009b). The expression of GFP matches the expression of cyp19a1b (Tong et al., 2009), and is easily detected by *in vivo* fluorescent microscopy as early as 25 hpf (Mouriec et al., 2009b). Certain androgens can also up-regulate the *cyp19a1b* gene expression in zebrafish and induce the expression of GFP in the tg(*cyp19a1b*-GFP) zebrafish embryos in an ER-dependent manner (Brion et al., 2012; Mouriec et al. 2009a). The up-regulation by androgens may result from catalytic conversion to oestrogens or oestrogenic metabolites by basal levels of cyp19a1b and/or other

136 metabolizing enzymes (Mouriec et al., 2009a), thus allowing the bioassay to also detect the
137 (weak) oestrogenic effects of androgens.
138
139 Also primary liver cell cultures and fish cell lines are discussed as alternatives to *in vivo*
140 testing. For studying ER agonists, the liver is often used as donor for primary cell cultures,
141 since the liver is the site for ER-mediated synthesis of the oestrogenic biomarker vitellogenin
142 (Vtg) (Yaron, 1995). Cell lines are often believed to be more efficient and to produce more
143 reproducible results than primary cell cultures and have the advantage that they can be
144 cultivated from the order of months or up to decades (Bols et al., 2005). Only a few fish cell
145 lines originating from liver are used in environmental toxicology including cell lines such as
146 PLHC-1, R1, RTL-W1 and ZF-L (Bols et al., 2005). Although quite efficient for detecting
147 cytotoxic, genotoxic and CYP1A-inducing compounds, most piscine cell lines are not very
148 responsive to oestrogens (Bols et al., 2005) and exhibit lower metabolic activity/capacity than
149 primary hepatocytes (Thibaut et al., 2009). However, reporter gene assays based on stable
150 expression of subtypes of trout or zebrafish ER coupled to oestrogen response element driven
151 luciferase in PLHC-1 (Cosnefroy et al., 2009) and ZF-L (Cosnefroy et al., 2012) cell lines
152 have successfully been used to screen various xeno-oestrogens. Use of primary hepatocytes
153 are suitable for screening of oestrogenic chemicals as they maintain most of their native
154 properties related to cellular integrity, conservation of biochemical mechanisms and
155 pathways, liver specific gene expression and responsiveness to various hormones (Braunbeck
156 and Storch, 1992; Pesonen and Andersson, 1997; Segner, 1998). They also have the ability to
157 detect environmental oestrogens that require metabolic activation (Bickley et al., 2009).
158 Primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes have been used extensivly for
159 screening of ER agonists (Navas and Segner, 2006; Petersen and Tollefsen, 2011; Tollefsen et
160 al., 2008b).
161
162 Combined effects of ER agonists have previously only been studied to a limited extent in the
163 tg(*cyp19a1b*-GFP) zebrafish embryo and primary rainbow trout hepatocytes (Brion et al.
164 2012; Petersen and Tollefsen, 2011).The present study aimed to assess the applicability of the
165 zebrafish tg(*cyp19a1b*-GFP) strain for determination of combined effects of multi-compound
166 mixtures of the common ER agonists 17β-estradiol, 17α-ethinylestradiol, estrone, bisphenol A
167 and 4-*tert*-octyphenol. This was achieved by i) characterisation of the oestrogenic response of
168 the mixtures by use of the CA and IA prediction models, and ii) by comparing results
169 obtained for the tg(*cyp19a1b*-GFP) zebrafish embryos to results from primary cultures of

rainbow trout (*Onchorhynchus mykiss*) hepatocytes and available *in vivo* data to determine if these *in vitro* methods represent an alternative to *in vivo* testing for mixtures of ER agonists.

2. Materials and method

2.1 Compounds

17*α*-ethinylestradiol (EE2, ≥98%, CAS RN: 57-63-6), 17*β*-estradiol (E2, ≥98%, CAS RN: 50-28-2), estrone (E1, ≥99%, CAS RN: 53-16-7), 4-*tert*-octylphenol (OP, 97%, CAS RN: 140-66-9), tricaine-S (98%, CAS RN: 886-86-2) and methylcellulose (2% viscosity at 25 °C, CAS RN: 9004-67-5) were obtained from Sigma-Aldrich (St. Louis, MI, US). Bisphenol A (BPA, ≥97%, CAS RN: 80-05-7) was obtained from Merck (Darmstadt, Germany). The test chemicals were selected based on their environmental relevance and well known mode of oestrogenic action. They were all dissolved in dimethylsulfoxide (DMSO- ROTIDRY® ≥99.5 %; ≤200 ppm H_2O) and stored in the dark at room temperature until use.

2.2 Zebrafish maintenance and breeding

The tg(*cyp19a1b*-GFP) transgenic zebrafish (Tong et al., 2009), generation F6, were cultured at the Helmholtz Centre for Environmental Research (UFZ, Leipzig, Germany) with a light:dark cycle 14:10 and temperature 26±1˚C. The fish were fed twice daily with hatched *Artemia* nauplii (Sanders Great Salt Lake Artemia Cysts). Eggs were obtained from tanks with 7-8 females and 5-6 males of heterozygous transgenic zebrafish. Glass trays covered with a 3 mm mesh to protect the eggs from predation by their parents were placed in the aquaria in the evening before spawning was scheduled. Spawning and fertilisation was triggered by the onset of light (Nagel 2002; Lammer et al., 2009), and glass trays containing fertilised eggs were collected 1 hour after the onset of light the next morning. Only fertilized eggs showing normal development were selected for the tests. Fertilisation was indicated by regular cell divisions and only eggs that had reached at least the 8 cell stage within 2 hpf were used in the experiments.

2.3 Exposure and *in vivo* imaging of zebrafish embryos

At one day post fertilization (dpf), approximately 20-30 embryos were placed in separate glass Petri dishes with 20-25ml of embryonic media (294.0mg/L $CaCl_2*2H_2O$, 123.3mg/L $MgSO_4*7H_2O$, 64.7mg/L $NaHCO_3^-$ and 5.7mg/L KCl) according to the ISO 15088:2007 guideline (ISO 2006). Embryonic medium was supplemented with ≤1% DMSO (solvent

control), 100nM E2 (positive control) or different concentrations of the test chemicals. The used solvent concentration (maximum 1%) was below reported maximum-tolerated concentrations of DMSO in zebrafish embryo and larvae (Maes et al., 2012), and did not affect the GFP fluorescence in the tg(*cyp19a1b*-GFP) embryos (results not shown). The embryos were kept in an incubator at 26±1˚C with a light:dark cycle of 12:12h throughout the exposure experiments. Renewal of exposure media and removal of dead embryos, if any, were performed daily. The total number of dead, non-hatched or deformed embryos was noted at the end of exposure (see supplementary data, table S2 and S3 for these effect data after exposure to the mixture of BPA, OP, and E2 and the mixture of BPA, OP, EE2 and E1 respectively). *In vivo* imaging was performed according to Brion et al. (2012) with some modifications regarding embedding chemical, camera settings and background grey-value as specified below. Embryos were anesthetized with tricaine-S (600mg/L), embedded in 5% (m/v) methylcellulose, oriented in dorsal view under a fluorescent microscope (Leica 4000 DMI Microscope) equipped with an external light source (Leica EL6000) and a 10X objective (Leica PH1, 506507) and photographed for documentation and quantification of the GFP fluorescence. Due to the variability in GFP fluorescence among the individuals exposed to the same concentration, ten randomly selected and appropriately positioned transgenic embryos from each concentration replicate were used for imaging and subsequent quantification. Embryos were imaged always in dorsal position in order to avoid that a difference in the position such as slight tilting could affect the quantification of fluorescence. Each test was conducted independently three to five times. Each embryo was photographed under the same exposure conditions (200 ms of fluorescent light exposure, 30% of the maximal light intensity, a gain of 10, gamma value of 0.99 and grey scale from 0-255). The stability of the external fluorescent light source was assessed before and after each measurement using fluorescent calibration slides (AF, Analysentechnik, CHROMA). A black and white camera (Leica digital camera DFC 350FX) was used with the software LASV3.7 (Leica Microsystems, Heerbrugg, Switzerland). After the microscopic observation and image recording, the embryos were quickly sacrificed by mechanical pressure. Quantification of fluorescence was performed with Image J 1.44p software (available at: http://rsb.info.nih.gov/ij/) with background set to 45 (from maximum grey level 255).

In preceding tests, the transgenic embryos exposed to 50pM EE2 and 7.5µM BPA from 1-5dpf showed higher mean GFP expression than embryos exposed from 2hpf-5dpf or with

exposure started later than 1dpf (see supplementary data, figure S1 and S2). Thus an exposure window from 1-5dpf (4 days) was chosen for all chemicals and mixtures.

2.4 Rainbow trout hepatocytes: isolation, exposure and detection of vitellogenin

Juvenile rainbow trout (200-500g) from Valdres Ørretoppdrett (Valdres, Norway) were kept in tanks at the Department of Biology, University of Oslo (Norway) at a water temperature of 6±2ºC, a 12h:12h light:dark cycle, 100% oxygen saturation and a pH of 6.6. The fish were fed twice daily with pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of total body mass.

Primary cultures of hepatocytes were obtained by a two-step liver perfusion as described in Tollefsen et al. (2003). Only cell isolations with viability ≥90% determined by the trypan blue exclusion test were used. Cells were diluted to 500 000 cells/ml in serum free L-15 medium containing L-Glutamine (0.29mg/ml), $NaHCO_3$ (4.5nM), penicillin (100Units/ml), streptomycin (100µg/l) and amphotericin (0.25µg/ml) and plated in 96-well plates. After 24h, cells were exposed to solvent control (DMSO) or different concentrations of the test compounds for 96h. After 48h of exposure the cell culture medium containing test compounds or solvent control was renewed. At the end of exposure the culture media were transferred to Maxisorp Nunc-immunoplates (Nunc, Roskilde, Denmark), sealed with sealing tape (Nunc, Roskilde, Denmark) and stored at -80ºC for subsequent Vtg analysis.

Cytotoxicity was measured at the end of the experiments as described by Tollefsen et al. (2008a). Cells were incubated with Tris buffer (50mM, pH 7.5) containing 5% Alamar blue and 4µM 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) on an orbital shaker (30 min, 100 rpm). Fluorometric readings were performed with a Victor V^3 multilabel counter (Perkin Elmer, Waltham, MA, USA) using excitation and emission wavelength pairs of 530-590 (Alamar blue) and 485-530 (CFDA-AM), respectively. Cell viability was normalized between a positive ($CuSO_4$*$5H_2O$, 5mg/ml) and a negative control (DMSO), and expressed as percentage of the solvent control.

The relative amount of Vtg proteins were detected in the growth media from the primary rainbow trout hepatocytes by a capture ELISA as described by Tollefsen et al. (2003). In brief, 96-well microtiter plates were thawed and incubated overnight in a refrigerator to allow Vtg to bind to the surface of the wells of the plates. The relative amount of Vtg was measured

using the monoclonal mouse-anti salmon Vtg (BN-5, Biosense Laboratories AS, Bergen, Norway) and goat-anti mouse linked to Horseradish peroxidase, HRP (Bio-Rad, Herculaes, CA, USA) as primary and secondary antibody, respectively, both diluted 1:6000. A solution of TMB was used as a substrate for the HRP and the reaction was stopped with H_2SO_4 (1M). The absorbance was measured at 450nm with a Thermomax microplate reader (Molecular Devices, USA). Three independent replicates (exposure of cells from three different cell isolations) were used for deriving effect data.

2.5 Data analysis and mixture design

The induction of GFP, measured as integrated density, in the tg(*cyp19a1b*-GFP) zebrafish embryos was normalized between the DMSO control (0%) and 100nM of E2 (100%). The induction of Vtg in the fish hepatocytes was normalized between the DMSO control (0%) and maximum induction obtained by 30nM of E2 (100%). The concentration-response curves (CRCs) for the mixtures and single compound exposures were fitted with non-linear regression using GraphPad Prism v4.03 software (GraphPad Software Inc., La Jolla, CA, USA). Single compounds were fitted with a general equation for dose-response curves, the sigmoidal dose-response curve with variable slope (equation 2) which is a four parametric logistic equation. This model was not optimal for fitting the mixture effects data as one of the datasets could not be fitted. The extra sum-of squares F-test was used to compare the fit of the four parametric logistic equation (equation 2) with the simpler sigmoidal dose-response curve (equation 1) for all data sets for the mixture effects. The resulting P values (all above 0.05) indicated that the sigmoidal dose-response curve (equation 1) provided the best fit for the mixture effects data and was thus applied to these data sets. Concentrations causing delayed hatching or lethality in zebrafish embryo or cytotoxicity in primary hepatocytes were omitted from the non-linear regression analysis.

$$Y = \min + ((\max-\min) / (1 + 10^{(\text{LogEC50}-\text{LogX})})) \quad (1)$$

$$Y = \min + ((\max-\min) / (1+10^{(\text{LogEC50}-\text{LogX})*\text{slope}})) \quad (2)$$

Constraints for top and bottom were set at 100 and 0 respectively for the single compound exposures. X refers to the concentration of the compound or mixture.

A fixed ratio ray design was used to design the mixtures. The parameters are presented in table 1. Mixture composition was calculated by the concentration addition prediction model (equation 3) which is based on the concept of dose additivity introduced by Loewe and Muischnek (1926) and Loewe (1927).

$$EC_{x(Mix)} = \left(\sum_{i=1}^{n}(p_i/EC_{x_i})\right)^{-1} \quad (3)$$

EC_{x_i} is the concentration of substance i provoking a certain effect x when applied alone, $EC_{x(mix)}$ is the predicted total concentration of the mixture that provokes x % effect and p_i is the relative fraction of component i in the mixture.

For the exposure studies with rainbow trout hepatocytes, the mixture of BPA, OP and E2 was designed based on the EC_{50} and slope values reported in Petersen and Tollefsen (2011). An overview of effect levels for the mixture design and the ratios between the compounds for each mixture can be found in supplementary data (table S1).

The IA predicted effects were calculated by equation 4 and was first applied to biological data by Bliss (1939).

$$E_{Mix} = 1 - \prod_{i=1}^{n}(1-E_i) \quad (4)$$

E_{Mix} is the effect of the mixture of n compounds and E_i is the fractional effect of substance i when applied singly.

The model deviation ratio (MDR) was calculated as the ratio between the predicted concentration and the actual concentration for the different effect levels. The mixture was said to act by additivity if the 95% confidence interval of the sigmoidal dose-response curve overlapped with the prediction models and/or if the MDRs were within a factor of two ($0.5 \leq MDR \leq 2.0$). The MDRs were only calculated for effect levels covered by both the observed and predicted CRCs.

3. Results

3.1 Effects of single compounds on GFP expression in tg(*cyp19a1b*-GFP) zebrafish embryos

All tested chemicals induced the expression of GFP in a concentration-dependent manner (figure 1). The average GFP expression per independent exposure replicate (based on 10 embryos per concentration) was consistent between experiments despite a relatively high individual variation (coefficient of variation above 100% in some cases, data not shown). As expected, the most potent chemical was EE2 with an EC_{50} of 33.3pmol/L. The order of potency based on the EC_{50} values was EE2> E2>E1>OP>BPA (table 1), with the EC_{50} for E2, E1 and OP in the nanomolar range (3.48, 4.31 and 622 nM, respectively) and the EC_{50} for BPA in the micromolar range (7.36µM). The EC_{50} for the most potent of the tested chemicals (EE2) was more than 5 orders of magnitude lower than the least potent of the tested chemicals (BPA). The GFP expression induced by OP and BPA decreased at the highest concentrations, being consistent with an increase in mortality (figure 1). 4-*tert*-octylphenol was more acute toxic than BPA with LC_{50} for OP being approximately 20 times lower than for BPA (table 2). Delayed hatching was also observed at the higher tested concentrations (at 60 µM for BPA and at 3 µM for OP) of these two compounds (results not shown). No effects on lethality or time to hatch were observed after exposure to the compounds EE2, E2 or E1 (results not shown).

3.2 Combined effects of estrogenic compounds in tg(*cyp19a1b*-GFP) zebrafish embryos

All tested mixtures induced a concentration dependent increase in the expression of GFP (figure 2). The effect of the mixture of EE2, E2 and E1 was generally well characterised by the prediction models for CA and IA. The 95% confidence interval of the CRC fitted to the experimental data ($R^2 = 0.72$) overlapped with the prediction models for most of the concentrations tested. The resulting MDRs, the ratio between observed and predicted effect, were within a factor of two for all but the 3 highest tested concentrations (table 3), for which observed effects appeared to be higher than predicted. No effect on the viability of the embryos was observed at any of the tested concentrations (results not shown).

The oestrogenic effect of the mixture of BPA, OP and E2 was well predicted by the prediction models for CA and IA. The 95% CI of the CRC fitted to the experimental data ($R^2 = 0.90$) overlapped with the predictions for a majority of the tested concentrations and all but one of

the MDRs were within a factor of 2. Delayed hatching was observed at the highest tested concentration which was omitted from the curve fitting (see supplementary data, table S2).

The oestrogenic effect of the mixture of BPA, OP, EE2 and E1 could be predicted for most of the concentrations as the 95% CI of the CRC fitted to the experimental data ($R^2 = 0.82$) overlapped with the prediction models except at the 3 highest tested concentrations. The resulting MDRs were within a factor of two for all but the two highest concentrations. The MDR values of 0.19-0.35 at the higher tested mixture concentrations suggested that the mixture had lower oestrogenic effects than predicted by the CA and IA models. No effects on survival of the embryos were observed at the higher concentrations of these mixtures. Although not consistent, malformations (embryos with curved spinal cord) were observed at frequencies between 10 and 30% at the highest concentrations of this mixture in two of the four replicate tests (see supplementary data, table S3).

3.3 Combined effects of estrogenic compounds on the vitellogenin induction in rainbow trout hepatocytes

The mixture of BPA, OP and E2 was designed based on effect values for the individual compounds reported in Petersen and Tollefsen (2011). The mixture induced the production of Vtg in a concentration-responsive manner until a decrease in Vtg production was observed at the three highest tested concentrations (figure 3). The observed decrease in Vtg production co-occurred with a decrease in viability of the hepatocytes (figure 3). Onset of cytotoxicity in primary hepatocytes and delayed hatching of zebrafish embryos were observed at similar total mixture concentrations (25µM and 20µM, respectively). As observed for tg(*cyp19a1b*-GFP) zebrafish embryos, the combined effect of the mixture of BPA, OP and E2 was well characterised by the prediction models when using data from Vtg production in the primary rainbow trout hepatocytes as a marker for oestrogenicity (figure 3). The 95% CI of the CRC fitted to the experimental data ($R^2 = 0.77$) overlapped with CA and IA for a large part of the concentration range, and the MDRs (table 4) were within a factor of two for all but one of the lowest exposure concentrations.

4. Discussion

A comparative analysis of mixture effects of weak and strong oestrogens was performed in two assays regarded as potential alternatives to (adult) *in vivo* fish tests for screening of oestrogenic effects.

Effects of single compounds on GFP expression in tg(*cyp19a1b*-GFP) zebrafish embryos

In this study, oestrogens and environmental contaminants induced the expression of GFP in the tg(*cyp19a1b*-GFP) zebrafish embryos, and high-quality CRCs (R^2-values > 0.8) were obtained for the single compound exposures (table 1). Many studies have observed upregulation of cyp19a1b gene and protein after exposure to oestrogenic chemicals (Cheshenko et al., 2007; Menuet et al., 2005; Vosges et al., 2012, 2010), and the zebrafish *cyp19a1b* gene has been proposed to be a suitable biomarker for exposure to xeno-oestrogens (Brion et al., 2012; Le Page et al., 2006). In this study we monitored cyp19a1b expression using a transgenic zebrafish reporter (GFP) strain. Average GFP expression between replicates was highly consistent but the individual expression of GFP in single embryos within the same exposure replicate was quite variable. The observed variance could reflect individual differences in aromatase expression, differences in ratio of heterozygous and homozygous embryos, and/or subtle differences in the orientation of the embryos during imaging. High inter-individual variability of the native brain aromatase expression in whole embryos and larvae, as well as brains of adult stages, have been observed in previous studies and could not be linked to the gender of the fish (Goto-Kazeto et al., 2004; Trant et al., 2001). Hence, the variability in GFP expression observed in this study is likely reflecting the variability of the native brain aromatase.

All compounds tested in this study elicit the oestrogenic effect through ER binding and transcriptional activation. Upregulation of brain aromatase gene expression in zebrafish embryos have previously been observed for BPA (Brion et al., 2012; Chung et al., 2011), E2, EE2 and E1 (Brion et al., 2012). Both BPA and OP were lethal to the embryos at high concentrations, but a high-quality CRC for oestrogenicity was obtained at the non-lethal concentrations. Hence, masking of the *in vitro* oestrogenic response by acute toxicity (Tollefsen, 2008b; Petersen and Tollefsen, 2011) is unlikely to have affected the total response of the mixtures in the tg(*cyp19a1b*-GFP) zebrafish embryo assay.

The EC_{50} values for the GFP induction were similar to previously reported results for tg(*cyp19a1b*-GFP) zebrafish embryos exposed to EE2, E1, E2, OP and BPA (Brion et al., 2012) resulting in an identical ranking according to their oestrogenic potency. This confirms that the tg(*cyp19a1b*-GFP) embryo assay based on fluorescence microscopy can produce reproducible results between different laboratories. However, some differences were observed as the EC_{50} for E2 was about 7 times higher in our study than previously reported for the GFP

protein, but was similar to the EC_{50} for cyp19a1b and GFP mRNA induction (Brion et al., 2012). This difference might be partly linked to the different exposure windows used between the studies, i.e. 1-5dpf in this study and 2hpf-5dpf in the study by Brion et al. (2012). During the first 4hpf, embryos contain a high level of maternally inherited estrogen receptor 2a (Mouriec et al., 2009b) thus potentially making embryos exposed from 2hpf more sensitive towards estrogenic exposure than later in development. In addition, waves of expression of different sets of CYP genes over the course of development have been observed, with higher expression level of certain CYPs (CYP11A1, CYP26A1, CYP2P6, CYP2AA9 and CYP2AA12) during the first 24hpf (Goldstone et al., 2010). Although not investigated, expression of biotransformation enzymes might influence the degradation of estrogenic compounds differently, possibly rendering embryos exposed from 2hpf more sensitive for oestrogenic compounds than embryos exposed later in development. Furthermore, it must be noted that EC_{50} values from both studies are based on nominal concentrations and that deviations from intended concentrations cannot be excluded and may have contributed to the observed differences.

Effects of combined exposure on cyp19a1b expression

All tested mixtures appeared to act by additivity based on the comparison of the modelled CRCs compared to the CA and IA predictions and the calculated MDRs. The CA and IA predictions for the tested mixtures were very similar and are consistent with findings for estrogenic compounds elsewhere (Petersen and Tollefsen, 2011). The results are in agreement with previous studies showing additive effects of oestrogenic mixtures on the expression of GFP in the tg(*cyp19a1b*-GFP) assay (Brion et al., 2012). However some deviations were observed at the higher concentrations tested. The highest concentrations of the mixture of E2, EE2 and E1 induced a higher GFP expression than predicted by the CA and IA models and no MDRs could be calculated for these effect levels. As the variation between the replicate tests for these concentrations of the mixture was quite large, the 95% CI of the CRC fitted to the observed data overlapped with the CA and IA prediction models making it difficult to draw any definite conclusion as to whether the difference between observed and predicted effects was due to assay artefacts or interactions of the compounds in the mixture leading to synergistic effects. As binary mixtures with these compounds previously have been well predicted by the CA model when measuring GFP induction in tg(*cyp19a1b*-GFP) zebrafish (Brion et al., 2012) and Vtg production in male crucian carp (Zhang et al., 2009), the

deviations from predictions may warrant further studies to elucidate the rationale for the observed effect.

Lower than predicted oestrogenic effects were observed at high mixture concentrations of BPA, OP, EE2 and E1. The underlying mechanism for this deviation has not been investigated, but several potential explanations may be provided. It has previously been observed that oestrogenic mixtures containing OP deviate from additive predictions showing a weak antagonistic effect on the cell proliferation in the human MCF-7 breast cancer cell line (Rajapakse et al., 2004). However, contradictory results have been observed in male fathead minnow, where the oestrogenic effect of a mixture of OP, BPA, nonylphenol, E2 and EE2 on the Vtg production was well predicted by the CA model (Brian et al., 2005). Interestingly, deviation from CA was only detected in one of the two tested mixtures containing OP in our study, and then only at the higher total mixture concentrations, indicating that the lower than predicted oestrogenic effects was not solely caused by OP. Although not pursued in this study, it can be speculated that the higher total mixture concentrations could activate alternative toxicity pathways or biotransformation processes possibly interfering with the activation of the aromatase enzyme activity or expression of GFP. It is well documented that zebrafish exposed to increasing concentration of oestrogens such as EE2 show a concentration-dependent increase in the magnitude and number of differentially expressed genes, including up-regulation of sulfotransferases that are involved in biotransformation of steroids (Fisher, 2004; Hoffmann et al., 2006). Finally, the deviations may also result from toxic interference with the transcriptional activation of cyp19a1b. Some embryos in the highest concentration of two out of the four replicates showed a deformed spinal cord (in a frequency of 10-30%). This could be considered as an indicator for the onset of systemic toxicity albeit no mortality or other effects such as hatching delay were observed. Additional studies to elucidate the biological rationale for the observed deviations may be warranted as concentration-dependent deviations from predictions of complex mixtures of estrogenic compounds have also been observed in studies with primary cultures of rainbow trout hepatocytes (Petersen and Tollefsen, 2011).

Interestingly, delayed hatching was observed at the highest tested concentration of the mixture of BPA, OP and E2. Both BPA and OP led to apparent delayed hatching of the zebrafish embryos at high concentrations of these compounds when tested alone, thus suggesting that these two compounds were contributing to the observed effects. This seems to be consistent

with observations that both OP and BPA have affected hatching in fish (Fei et al., 2010; Kelly and Di Giulio, 2000).

Effects of combined exposure on *in vitro* Vtg production and assay comparison

To exemplarily compare the tg(*cyp19a1b*-GFP) transgenic embryo test with other screening *assays*, a mixture of BPA, OP and E2 was tested on a primary culture of rainbow trout hepatocytes. The primary culture of rainbow trout hepatocytes was less sensitive to the oestrogenic mixture of BPA, OP and E2 than the tg(*cyp19a1b*-GFP) zebrafish embryo as higher total mixture concentrations were needed to induce oestrogenic responses in the hepatocytes than in the zebrafish embryos. This could be explained by the difference in sensitivity of the two assays for exposure to OP. Data reported by Petersen and Tollefsen (2011) showed that the primary cultures of rainbow trout hepatocytes were approximately 20 times less sensitive to OP than the tg(*cyp19a1b*-GFP) zebrafish embryo, whereas the sensitivity of the other two compounds differed by less than a factor of six between the two assays. Thus a higher concentration of OP was needed in order to prepare an equi-oestrogenic mixture for the primary hepatocytes than for the zebrafish embryos, leading to a higher total mixture concentration necessary to induce the same oestrogenic effects in primary hepatocytes as in the zebrafish embryo. The relatively pronounced differences with respect to the sensitivity to OP in the assays might be attributed metabolic conversion as primary rainbow trout hepatocytes are known to metabolize OP rapidly (Pedersen and Hill, 2000). Interestingly, the effect of the mixture on the GFP-expression in the zebrafish embryos and the Vtg production in the rainbow trout hepatocytes were both well characterised by the prediction models at the concentrations not causing delayed hatching, lethality or cytotoxicity, indicating the usefulness of both of these assays for screening of oestrogenic compounds and mixtures of these. The two models offer different advantages compared to other alternative assays. The zebrafish embryo shares the screening capacity of *in vitro* tests but exhibits parts of the toxicokinetic and -dynamic characteristics of *in vivo* models (Strähle et al. 2012). Primary hepatocytes partially share the metabolic capacity of an intact organ or organism (Pesonen and Anderson, 1997). Furthermore, the two assays offer a unique opportunity to evaluate the combined oestrogenic effect in different cellular contexts, i.e. analysis of aromatase expression in radial glial cells using tg(*cyp19a1b*-GFP) zebrafish embryos and analysis of Vtg in a genuine *in vitro* assay, i.e. primary cultures of rainbow trout hepatocytes. Although extrapolation from these two assays to environmental scenarios are challenging, studies using these assays will increase the knowledge of the combined effects of ER agonists

(and other compounds) and possibly identify scenarios that will need to be further investigated with *in vivo* or field studies.

Tg(*cyp19a1b*-GFP) zebrafish embryos as an alternative to *in vivo* (adult) fish tests for oestrogenicity screening.

Zebrafish embryos have detectable expression and activity of phase I and II detoxification enzymes already at 2 hpf (Goldstone et al., 2010; Wiegand et al., 2000a). However, full capacity of enzymes protecting against chemical damage are not obtained until after hatching, and the expression activity of some biotransformation enzymes can be differentially expressed during embryonic development (e.g. CYPs) or between embryos, larvae and adult fish (e.g. glutathione S-transferase and glutathione peroxidase) (Goldstone et al., 2010; Wiegand et al., 2000b,). This might lead to differential sensitivity to chemicals in different life stages.

The fish embryo test (FET) has been proposed as an alternative to the acute toxicity fish test (OECD 203) as data obtained by FET correlates well to acute fish toxicity data obtained according to the OECD 203 test despite possible differences in sensitivity between different life stages (Belanger et al., 2013; Knöbel et al., 2012; Lammer et al., 2009). The FET could also be useful for prediction of endocrine disruption, particularly if transgenic strains such as the tg(*cyp19a1b*-GFP) is used. Although only a limited number of studies have been performed with the tg(*cyp19a1b*-GFP) zebrafish model to date, the obtained results are comparable with those previously reported in this model (Brion et al., 2012) and mostly within one order of magnitude of Vtg production data reported with *in vivo* fish studies with zebrafish exposed to the same oestrogenic chemicals (Scholz and Mayer 2008; Zhang et al., 2010). The present findings indicate the usefulness of the tg(*cyp19a1b*-GFP) assay for screening of oestrogenic chemicals and mixtures. The usefulness of the tg(*cyp19a1b*-GFP) as replacement method of animal experiments could be further evaluated by comparing results with upcoming data from *in vivo* screening programs, such as the US-EPA endocrine disrupter screening program (http://epa.gov/endo/, Fenner-Crisp et al., 2000).

5. Conclusion

The tg(*cyp19a1b*-GFP) zebrafish embryo appeared to be a suitable model for screening of oestrogenic chemicals and mixtures. The model provided reproducible results and had similar sensitivity for most of the oestrogenic compounds as other *in vitro* and *in vivo* assays. Both potent and weak oestrogens could be detected by the tg(*cyp19a1b*-GFP) zebrafish embryo as the oestrogenic effect of both endogenous and synthetic steroids as well as non-steroidal

environmental oestrogens was identified. The combined effects of the oestrogenic mixtures were mainly additive and the CA and IA produced good estimates for the observed effects. The tg(*cyp19a1b*-GFP) zebrafish embryo seems to offer a sensitive and reliable alternative to (adult) *in vivo* fish tests for screening of oestrogenic effect of single compounds and synthetic mixtures. A comparative analysis using transgenic embryos and hepatocytes could be particularly useful to address potential species differences and the role of metabolic conversion of test compounds for the identification and/or prediction of oestrogenic effects to fish.

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Ethics

All zebrafish husbandry and experimental procedures performed in this study are in accordance with the German animal protection standards and were approved by the Government of Saxony, Landesdirektion Leipzig, Germany (Aktenzeichen 75-9185.64). Based on the *Directive on the protection of animals* used for scientific purpose by the European Union (EU 2010), zebrafish embryos up to the stage of independent feeding (approximately 5 days after fertilization) are considered as non-protected stages (Strähle et al., 2012). They are considered as alternative to the testing of (adult) animals and no license was required for conducting the experiments. Use of transgenic fish was in compliance with guidelines of the German Ministry of Food, Agriculture and Consumer Protection (former Ministry of Food, Agriculture and Forestry) established originally for transgenic mice and rat (BML 1996). All transgenic fish used for embryo production were of generations F6.

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Highlights

- We used transgenic (*cyp19a1b*-GFP) zebrafish embryos to assess oestrogenic effects
- Mixture effects were assessed by CA and IA prediction models and were mostly additive
- Deviations from predictions occurred at higher total mixture concentrations
- One mixture was also tested on primary cultures of rainbow trout hepatocytes
- The effect of the oestrogenic mixture was similar and well predicted in both assays

Supplementary Data

Transgenic (*cyp19a1b*-GFP) zebrafish embryos as a tool for assessing combined effects of oestrogenic chemicals

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Comparison of different time points for the start of the exposure

The sensitivity of the tg(*cyp19a1b*-GFP) zebrafish embryos was compared using different time points for the start of the exposure to 17*α*-ethinylestradiol (EE2, 50pM) or bisphenol A (BPA 7.5µM) ranging from 2-98 hours post fertilization (hpf). All exposures were ceased at 5 days post fertilization. No significant differences in the fold induction of green fluorescent protein (GFP) were seen between embryos exposed from 2hpf and embryos exposed from later time-points. Although not significant, embryos exposed from 26hpf had higher mean fold induction of GFP than embryos exposed from 2hpf (figure S1 and S2). Therefore, this time point has been used for start of the exposure in all subsequent experiments.

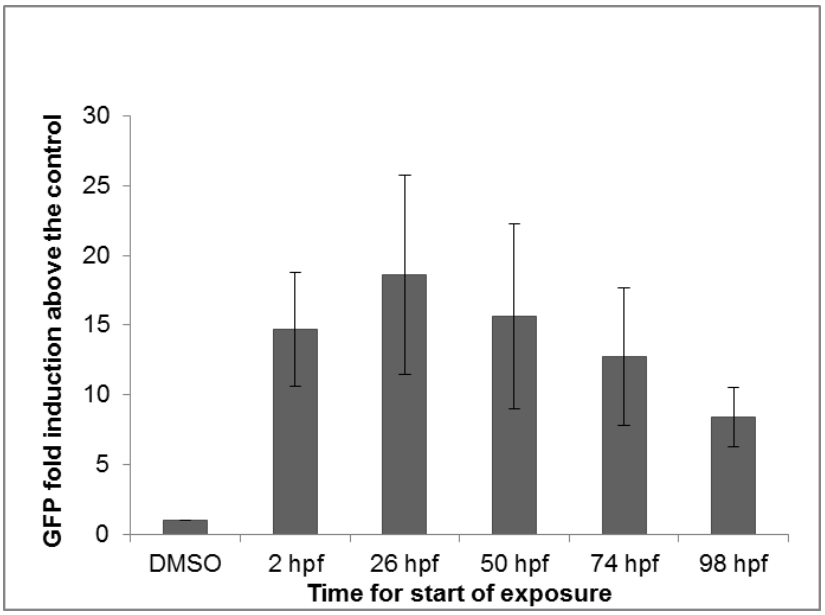


Figure S1. Comparison of different time points for the start of the exposure of tg(cyp19a1b-GFP) zebrafish embryos to 50pM EE2. Each column represents the mean GFP fluorescence of three independent experiments ± standard deviation. Exposure was performed from the indicated stage until 5 days post fertilization. No significant difference in the GFP fold induction was found between embryos exposed from 2hpf and embryos exposed from a later time-point (non-parametric ANOVA with Dunn's multiple comparison test).

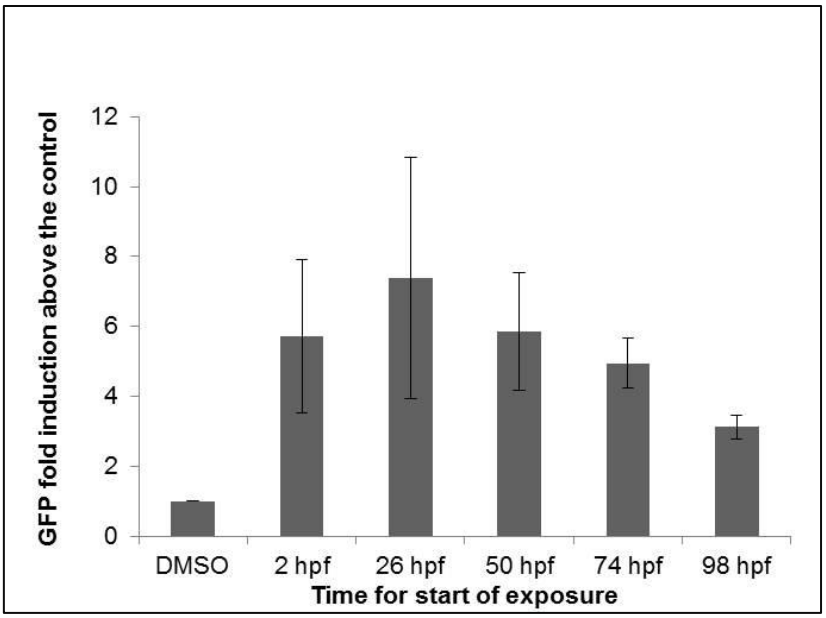


Figure S2. Comparison of different time points for the start of the exposure of tg(cyp19a1b-GFP) zebrafish embryos to 7.5µM bisphenol A. Each column represents the mean GFP fluorescence of three independent experiments ± standard deviation. Exposure was performed form the indicated stage until 5 days post fertilization. No significant difference in the GFP fold induction was found between embryos exposed from 2hpf and embryos exposed from a later time-point (non-parametric ANOVA with Dunn's multiple comparison test2-tailed t-test, p=0.05)

Mixture compositions

A fixed ratio ray design was used for the mixture experiments. The effect concentration (EC_x) and corresponding concentration ratios that resulted in the most equal contribution from all compounds to the anticipated mixture effect were chosen (table S1). The tg(*cyp19a1b*-GFP) zebrafish embryos were exposed to mixtures A, B and C, whereas primary cultures of rainbow trout hepatocytes were exposed to mixture D.

Table S1. Mixture compositions and concentration ratios of the mixture constituents in the four tested mixtures.

		Mixture concentration ratios			
Compound	Abbreviation	A[a] (EC_{50})	B[b] (EC_{20})	C[c] (EC_{50})	D[b] (EC_{20})
17*α*-ethynylestradiol	EE2	0.00426		0.0000113	
17*β*-estradiol	E2	0.432	0.000239		0.0000350
Estrone	E1	0.564		0.000642	
4-*tert*-octylphenol	OP		0.0500	0.0486	0.440
Bisphenol A	BPA		0.950	0.951	0.560
Sum		1.00	1.00	1.00	1.00

[a] 17*α*-ethinylestradiol, 17*β*-estradiol and estrone

[b] 17*β*-estradiol, bisphenol A and 4-*tert*-octylphenol

[c] 17*α*-ethinylestradiol, estrone, bisphenol A and 4-*tert*-octylphenol

Survival, hatching rate and malformations in zebrafish embryos exposed to mixtures

Effects on survival, hatching rate and malformations were not observed in embryos exposed to mixtures of steroidogenic estrogens.

Table S2: Rate (%) of survival, malformations (body curvature) and hatching in zebrafish embryos at 5 days post fertilization after exposure to a mixture of bisphenol A, 4-*tert*-octylphenol and 17*β*-estradiol. The table shows the results from three different replicates (R1-R3). Twenty-five (R1) or 20 embryos (R2, R3) were used per replicate (n.a. = not analysed).

	Survival rate			**Rate of malformations**			**Hatching rate**		
Replicate	R1	R2	R3	R1	R2	R3	R1	R2	R3
Concentration (mol/L)									
0 (Solvent control)	n/a	100	100	n.a.	0	0	n.a.	95	70
$1.56*10^{-7}$	100	100	100	0	0	0	100	55	95
$3.13*10^{-7}$	100	100	100	8	0	0	100	100	95
$6.25*10^{-7}$	100	100	100	0	0	0	100	100	95
$1.25*10^{-6}$	100	100	100	0	0	0	100	80	75
$2.50*10^{-6}$	100	100	100	0	0	5	100	80	95
$5.00*10^{-6}$	100	100	100	8	0	0	100	80	85
$1.00*10^{-5}$	100	95	100	28	25	0	92	85	90
$2.00*10^{-5}$	100	100	100	16	0	5	37.5	0	5

Table S4: Rate (%) of survival, malformations (body curvature) and hatching in zebrafish embryos at 5 days post fertilization after exposure to a mixture of bisphenol A, 4-*tert*-octylphenol, ethinylestradiol and estrone. Table shows the results from four different replicates (R1-R4). Twenty embryos were used per replicate (n.a. = not analysed).

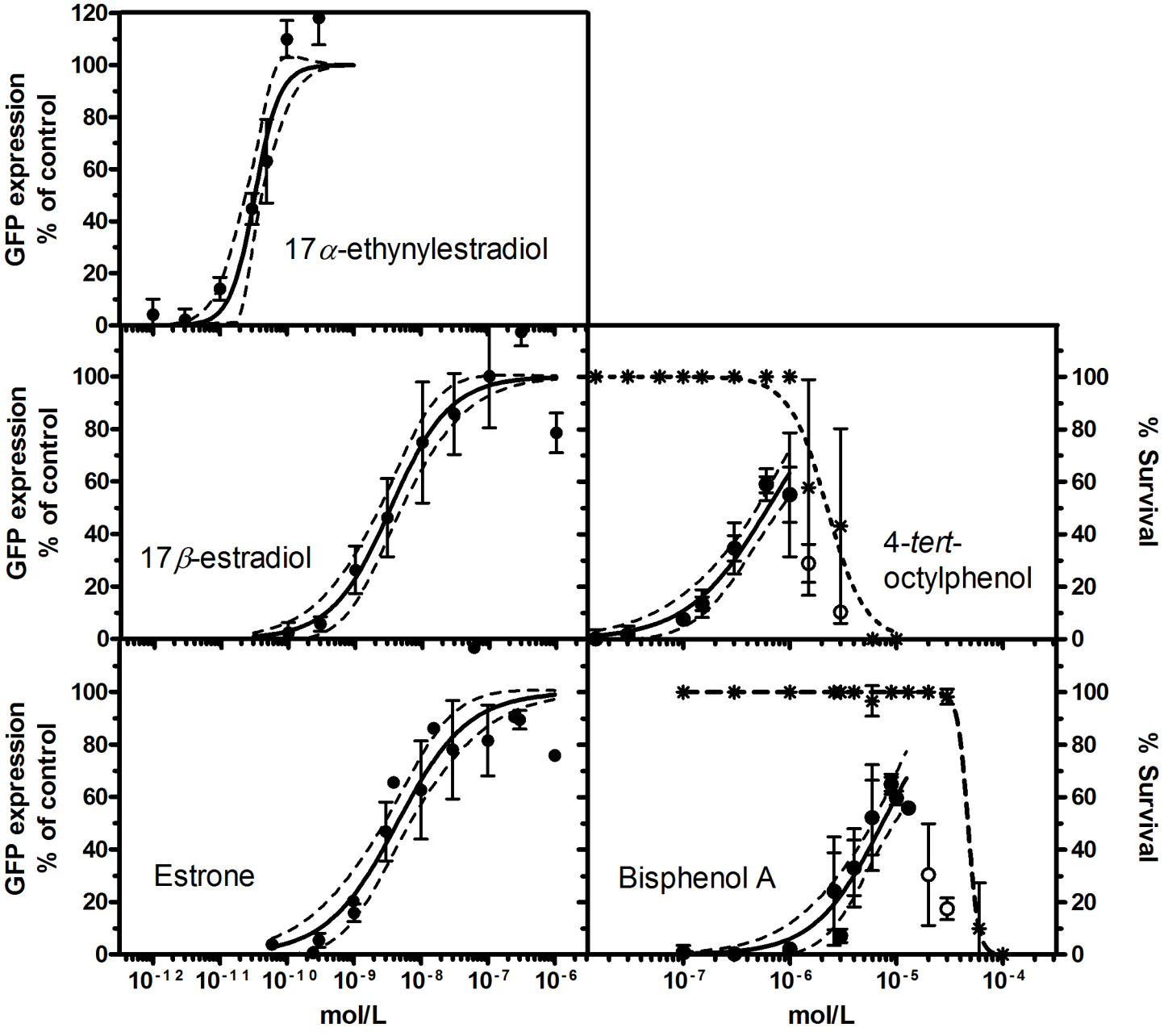
	Survival rate				**Rate of malformations**				**Hatching rate**			
Replicate	R1	R2	R3	R4	R1	R2	R3	R4	R1	R2	R3	R4
Concentration (mol/L)												
0 (DMSO-control)	n.a.	100	100	100	n.a.	0	0	0	n.a.	85	90	70
$7.72*10^{-8}$	100	n.a.	95	100	0	n.a.	0	0	100	n.a.	58	85
$1.54*10^{-7}$	100	100	100	95	0	0	0	0	100	80	75	100
$3.09*10^{-7}$	100	95	100	100	0	0	0	0	100	74	85	95
$6.18*10^{-7}$	100	100	100	100	0	0	0	0	95	85	60	95
$1.24*10^{-6}$	100	100	100	100	0	5	0	0	100	80	75	100
$2.47*10^{-6}$	100	100	100	100	0	0	0	0	95	65	80	70
$4.94*10^{-6}$	100	100	100	100	0	10	0	0	100	80	70	100
$9.89*10^{-6}$	100	95	100	100	0	30	10	0	100	58	55	85

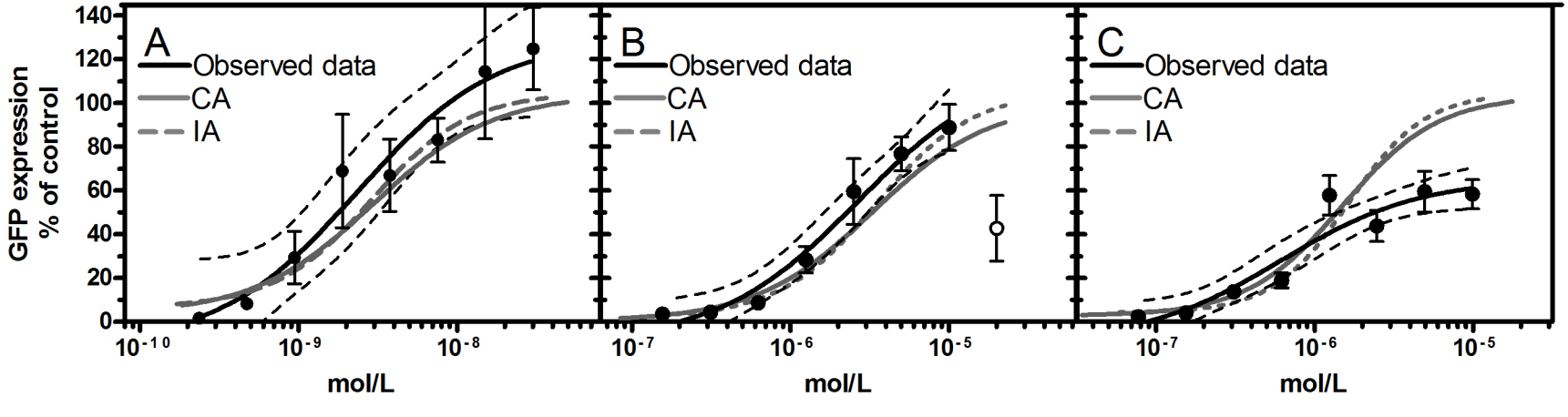
Legends to figures

Figure 1. Induction of green fluorescent protein (GFP) expression in tg(*cyp19a1b*-GFP) zebrafish embryos after exposure to oestrogens and xenoestrogens from one to five days post fertilization. The results are shown as mean values (●) ± standard deviation after exposure to 17*α*-ethinylestradiol (n=3), 17*β*-estradiol (n=4), estrone (n=4), 4-*tert*-octylphenol (n=5) and bisphenol A (n=4). The concentration-response curves with 95% confidence interval were modeled by non-linear regression using a sigmoidal concentration-response curve with variable slope. Data not included in the curve fitting of bisphenol A and 4-*tert*-octylphenol due to possible onset of systemic toxicity are shown as open circles (○). Survival data (*) are only included for compounds affecting viability.

Figure 2 Induction of green fluorescent protein (GFP) expression in tg(*cyp19a1b*-GFP) zebrafish embryos after exposure to oestrogenic mixtures from one to five days post fertilization. The results are presented as mean values (●) ± standard deviation after exposure to a mixture (A) of 17*α*-ethynylestradiol (EE2), 17*β*-estradiol (E2) and estrone (E1) (n=3), a mixture (B) of bisphenol A (BPA), 4-*tert*-octylphenol (OP) and E2 (n=3), and a mixture (C) of BPA, OP, E1 and EE2 (n=4). The concentration response curve with 95% confidence interval was modeled by non-linear regression using a sigmoidal concentration-response curve. Data not included in the curve fitting due to possible onset of systemic toxicity are shown as open circles (○). The concentration addition (CA) and independent action (IA) prediction models are presented as grey solid line and grey dotted line respectively.

Figure 3. Induction of vitellogenin (Vtg) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes presented as mean values (●) ± standard deviation after 96 hours exposure to a mixture of bisphenol A, 4-*tert*-octylphenol and 17*β*-estradiol (n=3). The mixture was designed based on the reported singel compound effects of the respective chemicals in Petersen and Tollefsen (2011). The concentration response curve was modeled by non-linear regression using a sigmoidal concentration-respons curve. Data not included in the curve fitting due to possible onset of systemic toxicity are shown as open circles (○). The cytotoxicity, measured as metabolic activity is presented by asterics (*). The concentration addition (CA) and independent action (IA) prediction models are presented as grey solid line and grey dotted line respectively.


GFP expression
% of control
17α-ethynylestradiol
17β-estradiol
4-tert-
octylphenol
Estrone
Bisphenol A
% Survival
mol/L
mol/L


GFP expression
% of control
140
120
100
80
60
40
20
0
A
Observed data
CA
IA
10^{-10}
10^{-9}
10^{-8}
mol/L
B
Observed data
CA
IA
10^{-7}
10^{-6}
10^{-5}
mol/L
C
Observed data
CA
IA
10^{-7}
10^{-6}
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mol/L

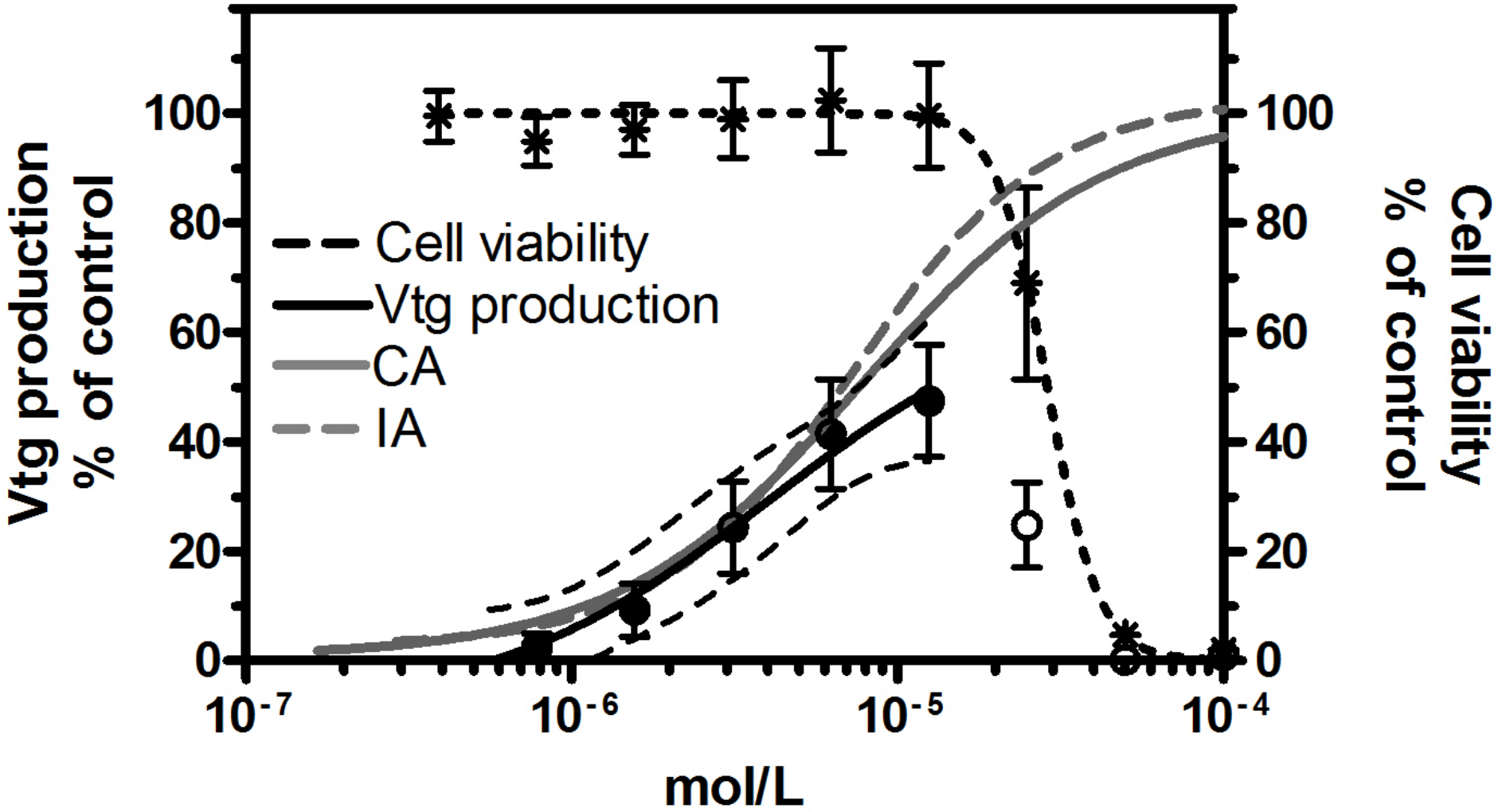
Vtg production
% of control
Cell viability
% of control
Cell viability
Vtg production
CA
IA
100
80
60
40
20
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
mol/L

Table 1. Characteristics of the expression of green fluorescent protein (GFP) in the tg(*cyp19a1b*-GFP) zebrafish strain exposed to different oestrogens and xenoestrogens

Compound	Abbreviation	EC_{50}[a] GFP expression (mol/L)	slope[a]	Goodness of fit (R^2)[a]
17*α*-Ethynylestradiol	EE2	$3.3E^{-11}$ ($2.6E^{-11}$-$4.3E^{-11}$)[b]	2.4	0.90
17*β*-estradiol	E2	$3.4E^{-9}$ ($2.3E^{-9}$-$4.9E^{-9}$)	0.99	0.90
Estrone	E1	$4.4E^{-9}$ ($2.9E^{-9}$-$6.8E^{-9}$)	0.83	0.85
4-*tert*-octylphenol	OP	$6.2E^{-7}$ ($4.8E^{-7}$-$8.0E^{-7}$)	1.2	0.80
Bisphenol A	BPA	$7.4E^{-6}$ ($5.9E^{-6}$-$9.2E^{-6}$)	1.4	0.83

[a]EC_{50}, slope and R^2 values are obtained from the fitted concentration-response curves. [b]Values in brackets show the 95% confidence intervals.

Table 2. Characteristics of the toxicity of 4-*tert*-octylphenol and bisphenol A on the tg(*cyp19a1b*-GFP) zebrafish strain.

Compound	Abbreviation	LC_{50}[a] (mol/L)	slope[a]	Goodness of fit (R^2)[a]
4-*tert*-octylphenol	OP	$2.2E^{-6}$ ($1.8E^{-6}$-$2.8E^{-6}$)[b]	-2.5	0.87
Bisphenol A	BPA	4.7E-5 ($4.1E^{-5}$-$5.4E^{-5}$)	-9.1	0.98

[a]LC_{50}, slope and R^2 values were obtained from the fitted concentration-response curves. [b]Values in brackets show the 95% confidence intervals.

Table 3. Calculated model deviation ratio (MDR) between the predicted and observed effect concentrations (EC_x) obtained after exposure (1 - 5 days post fertilization) of the tg(*cyp19a1b*-GFP) zebrafish embryo to three mixtures of oestrogens and xenoestrogens

Mixture 1 (E2 + EE2 + E1)				Mixture 2 (BPA + OP + E2)				Mixture 3 (BPA + OP + EE2 + E1)			
mol/L	%effect[a]	MDR CA[b]	MDR IA[c]	mol/L	%effect	MDR CA	MDR IA	mol/L	%effect	MDR CA	MDR IA
$2.4E^{-10}$	2.0	n.a.	n.a.	$1.6E^{-07}$	-1.7	n.a.	n.a.	$7.7E^{-08}$	-0.84	n.a.	n.a.
$4.7E^{-10}$	13	0.85	0.92	$3.1E^{-07}$	4.7	0.77	0.92	$1.5E^{-07}$	5.7	0.88	1.1
$9.4E^{-10}$	29	1.3	1.3	$6.3E^{-07}$	16	1.2	1.5	$3.1E^{-07}$	16	1.3	1.7
$1.9E^{-09}$	52	1.5	1.4	$1.3E^{-06}$	32	1.4	1.5	$6.2E^{-07}$	28	1.1	1.4
$3.8E^{-09}$	76	1.8	1.4	$2.5E^{-06}$	53	1.5	1.4	$1.2E^{-06}$	41	0.85	0.97
$7.5E^{-09}$	96	**3.3**	2.0	$5.0E^{-06}$	74	1.6	1.3	$2.5E^{-06}$	51	0.57	0.61
$1.5E^{-08}$	110	n.a.	n.a.	$1.0E^{-05}$	92	**2.4**	1.3	$4.9E^{-06}$	57	**0.34**	**0.35**
$3.0E^{-08}$	120	n.a.	n.a.					$9.9E^{-06}$	61	**0.19**	**0.19**

Mixture 1: 17*β*-estradiol (E2), 17*α*-ethinylestradiol (EE2) and estrone (E1)

Mixture 2: bisphenol A (BPA), 4-*tert*-octylphenol (OP) and E2

Mixture 3: BPA, OP, EE2 and E1

n.a. – not applicable, observed effects were lower or higher than the minimum and maximum predicted effects, respectively.

[a] The % effect was calculated based on the modeled concentrations response curve for the observed effect data, [b] CA – concentration addition, [c] IA – independent action, Concentrations omitted from the curve-fitting due to toxicity are not shown in the table. Bold text indicates where the MDR is larger than a factor of two.

Table 4. Calculated model deviation ratio (MDR) between observed and predicted effect concentrations in rainbow trout hepatocytes exposed to a ternary mixture of bisphenol A, 4-*tert*-octylphenol and 17β-estradiol for 96hours.

mol/L	%effect	MDR CA[b]	MDR IA[c]
$3.9E^{-07}$	-2.8	n.a.	n.a.
$7.8E^{-07}$	3.0	**0.38**	**0.18**
$1.6E^{-06}$	12	0.85	0.99
$3.1E^{-06}$	24	0.91	0.97
$6.3E^{-06}$	38	0.79	0.77
$1.3E^{-05}$	49	0.60	0.53

MDR = predicted EC_X / observed EC_X

n.a. – not applicable, observed effect level was lower than the minimum predicted effect.

[a]The % effect was calculated based on the modeled concentrations response curve for the observed effect data, [b]CA – concentration addition, [c]IA – independent action. Concentrations omitted from the curve-fitting due to toxicity are not shown in the table. Bold text indicates where the MDR is larger than a factor of two.