Mixtures of Estrogenic Chemicals Enhance Vitellogenic Response in Sea Bass

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Background: The potential impact of natural and synthetic estrogens on aquatic ecosystems has attracted considerable attention because it is currently accepted that their joint effects are more severe when they are present in mixtures. Although it is well-known that they occur as mixtures in the marine environment, there is little information about the combined effects of estrogenic chemicals on marine biota.

Objective: In 14-day tests with juvenile sea bass, we analyzed singly and in combination the estrogenic activity of estradiol (E_2), ethynylestradiol (EE_2), and bisphenol A (BPA) using vitellogenin induction as an end point.

Methods: Fish were exposed to each compound, and on the basis of these concentration–response data, we predicted mixture effects by applying the model of concentration addition. The mixtures were tested using a fixed-ratio design, and the resulting mixture effects were compared to the predictions.

Results: EE_2 was the most potent steroid, with an EC_{50} (median effective concentration) of 0.029 μg/L, 3.6 times more potent than E_2 (EC_{50} = 0.104 μg/L); BPA was the least potent chemical, with an EC_{50} of 77.94 μg/L. The comparative assessment yielded a good agreement between observed and predicted mixture effects.

Conclusions: This study demonstrates the potential hazard of these compounds to seawater life by their ability to act together in an additive manner. It provides evidence that concentration addition can be used as a predictive tool for assessing the combined effects of estrogenic chemicals in marine ecosystems.

Key words: bisphenol A, concentration addition, estradiol, ethynylestradiol, mixture effects, sea bass, vitellogenin. *Environ Health Perspect* 115(suppl 1):115–121 (2007). doi:10.1289/ehp.9359 available via *http://dx.doi.org/* [Online 8 June 2007]

It is well established that certain environmental chemicals are able to disrupt endocrine function in humans and wildlife by mimicking or antagonizing the action of hormones. Reproductive disorders in wildlife have also been linked to these endocrine-disrupting chemicals (EDCs). Because of clear indications of endocrine disruption in wild fish (reviewed by Jobling and Tyler 2003), a major concern is the presence of estrogenic chemicals (both steroids and xenoestrogens) in the aquatic environment. Although comprehensive exposure information is lacking for estrogenic chemicals in water bodies, it is expected that each single chemical is present at low or ineffective concentrations. Recent monitoring studies have shown that organisms are exposed to more than one of these chemicals simultaneously (Allen et al. 1999; Petrovic et al. 2002; Quirós et al. 2005). Chemical-related endocrine effects are therefore likely to be the consequence of exposure to a mixture of endocrine chemicals, rather than to a single chemical. To improve the ecologic quality of water bodies, it is essential that tools are available that permit effect assessments for mixtures of chemicals.

The classical concept of concentration addition (CA), also known as dose addition (Loewe and Muischnek 1926), is now accepted as a reasonable expectation for describing the joint toxic effects of chemical mixtures. It is based on the idea of a similar action of mixture components, and the joint effects of chemicals can be described accurately using this concept, provided all mixture components meet the similarity criterion (e.g., Faust et al. 2001; Payne et al. 2001). There are good reasons to assume that CA might also be a reasonable tool to predict joint effects of estrogenic chemicals: they act at an identical molecular binding site, the estrogen receptor, and stimulate a cascade of cellular estrogen-dependent processes on endogenous hormone function. From a mechanistic point of view, the expectation of concentration additivity seems therefore to be justified. This was confirmed by several *in vitro* mixture studies, for example, with the yeast estrogenicity screen assay (YES) and human breast cancer cell proliferation assay (E-SCREEN) (Payne et al. 2001; Silva et al. 2002). However, even if chemicals interact with same target site (i.e. estradiol receptor), it is often unknown whether this similarity will feed through to the final end point of investigation, especially when complex effector chains are involved between the primary site of action and the end point, and when the pharmacokinetics of compounds differ.

Thus, the question arises whether *in vitro* findings of concentration-additive mixture effects can be confirmed through *in vivo* studies, for instance, by using estrogen-responsive precursor protein for egg yolk-vitellogenin (VTG) in fish as the end point. This protein is normally synthesized in the liver of female fish, but males also posses the VTG gene. The induction of VTG by estrogenic chemicals offers a sensitive and integrated measure of estrogenic activity (Sumpter and Jobling 1995). Recent experimental findings with freshwater fish showed conclusively that 17β-estradiol (E_2) in combination with 17α-ethynylestradiol (EE_2), bisphenol A (BPA), nonylphenol (NP), and octylphenol (OP) act additively, and that the VTG response can be predicted very accurately by the CA model (Brian et al. 2005). This is in agreement with outcomes from binary mixtures of E_2 and EE_2 in rainbow trout (Thorpe et al. 2001, 2003). However, evidence that (xeno)estrogens also act additively in marine fish species is lacking. These chemicals are able to induce vitellogenesis in marine fish under experimental conditions (Folmar et al. 2000; Robinson et al. 2003), and they are present in estuaries and harbors. Moreover, wild species (e.g., *Platichthys flesus*) from these areas showed elevated VTG levels in relation to those from reference sites (Allen et al. 1999; Hashimoto et al. 2000).

In experiments that use flow-through systems, lower exposure concentrations are often

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measured with increasing exposure duration, and this usually leads to marked differences between nominal and measured concentrations of test chemicals. Possible reasons are manifold and include low water-flow rates, biodegradation, uptake by fish, and chemical adsorption. This causes a higher uncertainty for the expected measured concentrations and consequently also for any concentration–response analysis. Thus, the question arises as to how experimental conditions in flow-through systems influence the predictability of mixture effects of endocrine chemicals.

In an attempt to improve the scientific basis for a predictive combined effect assessment of (xeno)estrogens in the marine environment, we studied the combined effects of three model (xeno)estrogens (E_2, EE_2, and BPA) on VTG induction in juvenile marine fish. The European sea bass, *Dicentrarchus labrax*, was selected as a test species because it is an economically important fish and amenable to investigations of endocrine-related effects (Navas et al. 2004; Teles et al. 2004). Our empirical approach was based on four steps: *a*) concentration–response studies for three individual chemicals; *b*) prediction of combined effects (VTG induction) for a specific mixture assuming concentration additivity, based on information generated in the previous step; *c*) testing the selected mixture as defined in the previous step; and *d*) assessing the predictive power of the CA model by comparing predicted mixture effects with experimentally determined effects on the basis of measured concentrations in flow-through systems (comparative assessment).

Materials and Methods

Test organisms and chemicals. Sexually undifferentiated juvenile sea bass (approximately 1 year of age) were obtained from Coelho & Castro Lda. (Póvoa do Varzim, Portugal) and were kept in 2,000-L glass fiber tanks (density, 2–4 kg/m^3) supplied with natural seawater (20 ± 1‰) until exposures began. Fish used for studies had an average body weight of 30 ± 10 g (study 1, n = 224) and 32 ± 10 g (study 2, n = 120). E_2 (98% purity), EE_2 (98% purity), and BPA (99% purity) were purchased from Sigma-Aldrich, (St. Louis, MO, USA), and the marine salt was supplied by Sera Premium (Heinsberg, Germany).

All fish were treated humanely and with regard for alleviation of suffering. We followed the principles in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996).

Water supply and test apparatus. Exposure studies were conducted in 60-L glass tanks under flow-through system conditions. Master stock solutions of chemicals were prepared in methanol (analytical grade) and stored at –20°C. Aqueous stock solutions were regularly prepared from aliquots of master solutions and administrated to the tanks using a multichannel peristaltic pump equipped with silicon tubing (Watson-Marlow, Falmouth, Cornwall, UK). Solutions were delivered into mixing vessels that were supplied with seawater. Diluted solution flowed into the experimental tanks, which resulted in a theoretical complete water change every 30 hr. Delivery of test chemical commenced 1 week before the start of each exposure. Dosing started with the simultaneous addition of a required volume of chemical solution directly into the tanks. Methanol concentration in the tanks never exceeded 0.0625 mL/L. Water flowing from tanks was filtered through activated carbon before being delivered into the municipal sewage system. Seawater used in the system was artificially prepared using marine salt diluted in dechlorinated filtered municipal tap water. Dissolved oxygen saturation (> 80%) and total ammonia concentrations (< 0.001–0.2 mg/L) were monitored weekly. Test vessels and tanks were constructed of glass, with a minimum of other materials in contact (e.g., silicon rubber tubing) with the test solutions.

Experimental design. Exposures were carried out at 15°C in artificial seawater (20‰) under a photoperiod of 12 hr light:12 hr dark. Before exposure, animals were allowed to acclimatize for 1 week to experimental test conditions. Eight fish were introduced in each tank and maintained for a 14-day exposure period. Fish were fed food pellets (Aquasoja, Portugal) 1% of body weight per day throughout the exposure period. Each chemical was tested singly in all experiments. Selected concentrations of E_2 and BPA were repeated after a given time lag. Concentrations of methanol served as the solvent control, and 100 ng/L EE_2 was the positive control. Both controls were run in each experiment.

Estrogenicity testing of single compounds (study 1). We planned the concentrations of test chemicals based on range-finding studies, such that the entire effect range was covered between low and maximal effects, allowing statistical estimation of concentration–response functions for effects between 1 and 100%. Each experiment was performed using at least six different concentrations, ranging from 0.04 to 3.7 nM (10–1,000 ng/L) for E_2, 0.03 to 6.8 nM (10–2,000 ng/L) for EE_2, and 0.04 to 7.0 µM (10–1,600 µg/L) for BPA. Selected concentrations of E_2 (0.04, 0.15, 0.37, and 3.7 nM) and BPA (0.04, 0.13, and 0.44 µM) were repeated after 3 months. Data from repeated exposures were then pooled with the data from the earlier studies. Stock solutions of each tested chemical were prepared in methanol (analytical grade) and dosed to glass mixed vessels by means of a peristaltic pump at a rate of 0.1–0.5 mL/min; these solutions were then mixed with the dilution water flowing to mixing vessels at a rate of 32 mL/min, resulting in 1:321–1:65 dilutions.

Estrogenicity testing of the mixture (study 2). To assess whether joint effects of three estrogenic chemicals were additive, we compared the observed effects with the CA predictions for a wide range of different VTG levels. To keep the number of fish to a minimum, the "response-surface" approach (Myers et al. 1989) was deemed unsuitable because of its high testing demands. Instead, we chose the "fixed-ratio" design (Faust et al. 2001). Here, the ratio of concentrations of individual compounds is kept constant, and only the total concentration of the mixture is varied. By using this approach a complete concentration–response relationship for the mixture can be generated, which allows a comparative assessment between observed and predicted EC values for a wide range of effects. We prepared a master stock solution containing each of the chemicals at their nominal EC_{50} (median effective concentrations; equipotent ratio). Dilutions of this mixture were then tested, such that the entire range of VTG responses between 100 and 0% was covered evenly according to the expectation of the CA model. After 2 months, we repeated three selected mixture concentrations in an independent second mixture study.

Analytical chemistry. We determined actual exposure concentrations at three time points during the study. The first set of water samples was collected 1 week after the chemical dosing of the tanks began (t_0). After this first sampling, the fish were placed into the tanks. The second set (t_7) was taken after 2 weeks of dosing, and the third set (t_{14}) was taken after the third and final week, on the day that the experiment was terminated. The sample bottles were silylated before use. All samples were solid-phase extracted on a DVB Speedisk (Baker, Deventer, the Netherlands).

We analyzed BPA using two different methods. For the single chemical exposures as well as the t_0 sampling points of the mixture exposures, we used an isocratic HPLC method (adapted from Belfroid et al. 1999) coupled to diode array detection (Varian model 9065; Palo Alto, CA, USA). Separation was done using a mobile phase consisting of methanol/water (60/40, vol/vol). For the t_7 and t_{14} sampling points of the mixture experiments, the same method was used as for E_2 and EE_2, consisting of gas chromatography coupled to ion trap detection (GC-ITD) using a Saturn 2200 ion trap detector (Varian model 9095), which had the advantage of requiring a smaller sample volume because of the higher sensitivity of the method.

For the analyses of E_2, EE_2, and BPA using GC-ITD, we added a deuterated standard containing E_2-d4, EE_2-d4, or BPA-d6 to the samples prior to solid phase extraction (adapted

from Belfroid et al. 1999 and Houtman et al. 2004). Following the cleanup of the extracts with C18 cartridges, derivatization was carried out using silyl reagent before analysis.

Unless stated otherwise, concentration–response data given in this article refer to the arithmetic mean of measured concentrations in the three periods of sampling (t_0, t_7, t_{14}), as proposed by the Organisation for Economic Co-operation and Development (OECD 2000).

Vitellogenin in blood plasma. Fish were anesthetized and blood was extracted from the caudal vein using a heparinized syringe. Plasma was obtained after blood centrifugation (6,000 × *g* for 7 min at 5°C) in heparinized tubes containing phenylmethylsulfonyl fluoride (1 mM) and stored at –20°C until required for VTG analysis. Plasma VTG levels (n = 8/treatment) were determined using a competitive ELISA assay, as described by Maňanós et al. (1994a) with minor modifications. Sea bass VTG used for coating ELISA plates and as a standard was isolated as described by Maňanós et al. (1994b). The antibody against sea bass VTG was raised in rabbits. The secondary antibody (goat anti-rabbit antibody IgG) and the tetramethyl benzidine peroxidase substrate kit were obtained from BIO-RAD (Hercules, CA, USA). The range of the standard VTG curve was 2–100 ng/mL, corresponding to 80% and 20% of binding, with 50% of binding around 15 ng/mL. Juvenile plasma samples were tested at a dilution of 1:10 or higher in order to place all measurements within the confidence range of standard curve; intraassay and interassay coefficients of variation (CVs) were similar to those described by Maňanós et al. (1994a).

Calculation of concentration–response relationships. We accounted for slight differences in absolute control effects between studies by standardizing absolute effect scales to relative effects: the mean VTG concentration in fish from negative-control and positive-control tanks were used as the minimum and maximum responses, respectively, in order to standardize individual VTG measurements to values between zero (i.e., no VTG induction) and one (i.e., VTG level in positive control). Scaling to relative effects was carried out after individual VTG effect data were $\log_{10}$-transformed (i.e., an EC_{50} corresponds to the concentration that produces a $\log_{10}$-transformed VTG induction), a value that is the median in relation to negative and positive controls.

We performed statistical concentration–response analyses in the same way for all compounds and for the mixture by applying a best-fit procedure: various nonlinear regression models were fitted independently to the same data set, and we selected the best fit on the basis of statistical criteria. If data from repeated studies were obtained, the pooled data was used; to account for intra- and inter-experimental variability associated with this nested data scenario, we adopted the generalized nonlinear mixed modeling approach [see Brian et al. (2005) for details]. Otherwise, the regression models were fitted to the data as described by Scholze et al. (2001).

Effect concentrations (ECx) were calculated from the functional inverse of the best fitting model. Statistical uncertainties for estimated effect concentrations were expressed as 95% confidence intervals (CIs) and approximately determined by applying the bootstrap method (Efron and Tibshirani 1993). Additionally, we derived no observed effect concentrations (NOECs) using the likelihood ratio test under total order restriction (Bretz and Hothorn 2003). Calculations for statistical inference are based on absolute VTG values, and all approaches were implemented using SAS statistical software (SAS 2001).

Calculation of predicted mixture effects. Based on the best-fit regression functions of single compounds, we calculated expected effect concentrations for the ternary mixture in definite ratios. Quantitative relations between effects of single substances and the mixture are described by the concept of CA. For a multicomponent mixture of n components, it is defined by

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1}, \qquad [1]$$

where ECx_i denotes the effect concentrations of single compounds 1 to n (i.e., those concentrations that alone would produce the same quantitative effect x as the mixture), ECx_{mix} is the mixture concentration that induces an overall effect x, and p_i is the proportion of the ith component in the mixture. Individual effect concentrations, ECx_i, are derived from individual concentration response functions, F_i, by using their inverse functional form. Graphs of predicted concentration response curves were obtained by calculating numerous ECx_{mix} values, with x varying from 1% to 99% in steps of ≤ 1%. All individual effect concentrations, ECx_i, are estimates and are therefore subject to stochastic variability, which means that the predicted mixture effect concentration also has a measure of statistical uncertainty. This was achieved using the bootstrap method (Efron and Tibshirani 1993), which enabled 95% CIs to be derived for predicted mean effect. Predicted and observed effect concentrations were deemed to be statistically significantly different when the 95% CI of predicted and observed effects did not overlap.

The analytical determination of each mixture concentration often necessitated adjustments of the nominal mixture ratios, with relative proportions, p_i, of the compounds being different for each measured mixture exposure. This still enabled the calculation of expected mixture concentrations because all the information required is available as demanded by Equation 1. In a strict quantitative sense, however, this can be done only for the tested mixture concentrations; because extrapolation methods are unsuitable, the mixture data cannot be figured out using the same concentration scale. However, if the variation of the individual mixture ratios follows a random process, then smoothing techniques can be applied in order to estimate an "average" mixture ratio, which also enables the usual concentration–response relationships to be constructed for the mixture. We determined the mean mixture ratio by calculating the average fraction for each compound within the total mixture using data for the six highest tested mixture concentrations.

Results

Analytical determination of exposure concentrations. Analytical data ascertaining nominal concentrations for single exposures and for the mixture are given in Table 1. For the sake of simplicity, only average values of data from t_0, t_7, and t_{14} from the first mixture testing are shown (the second mixture study yielded nearly identical mixture ratios). The average variation of the individual BPA measurements between the sampling days was relatively low (CV = 18%), and the exposures were constant over the testing period. We found relatively good agreement between nominal and measured concentrations, with an average recovery rate of around 95% for single exposures and for within the mixture. For single EE_2 and E_2 exposures, data variation between the sampling days was higher, with an average CV of 70% for E_2 and 80% for EE_2, and 27% for all positive controls (100 ng/L EE_2). This higher variation mainly occurs because recovery rates at t_0 (90%) were higher, but they decreased after 1 week of testing to around 20–40%, resulting in an overall mean recovery rate of 47.0% for EE_2 and 46.7% for E_2 (Table 1). These findings were confirmed by repeated studies; thus, we exclude laboratory accident as the reason for these rates. Within the mixture, the recovery rates for EE_2 were slightly higher, with an overall average recovery of 55.2%. For E_2, recovery rates were similar to those for the individual studies.

The mixture ratio was originally conceived to be proportional to nominal EC_{50} values of the individual compounds. Because of the lower recovery rates for both steroids, it is obvious that the mixture ratio differed when based on measured concentrations. As a result, the fraction of BPA in the mixture was higher than planned, with an average 99.76% instead of

99.52% of the total concentration (Table 2). For the effective exposure ranges of the mixture (3.84–38.4 mg/L, nominal), recovery rates for all three compounds were relatively stable and of low variation, indicating that the corresponding mixture ratios for each mixture concentration were nearly identical. Thus, the use of a common average mixture ratio for all mixture exposures appears justified, and observed and predicted mixture effects can be compared for untested concentration ranges by interpolation.

Estrogenicity of E_2, EE_2, and BPA. Figure 1 shows concentration–response data for each tested chemical and their best-fit regression curves. The corresponding functions, model parameters, and statistical estimates of estrogenic potencies are given in Table 2. Each of the tested chemicals produced a clear concentration–effect pattern in juvenile sea bass. Fish mortality was not observed. Because of the relatively low data variability (the CV was 2.5–5% for the negative controls and 2.5–7% for the positive controls), the statistical power was sufficiently large to detect responses down to 5%; this allowed precise regression estimations of mean VTG responses. The effect concentrations for median and low VTG induction (EC_{50}, EC_{10}) are given in Table 2; the corresponding 95% CIs are relatively low for all compounds. Using the median effect level as reference, we found that EE_2 was the most potent steroid tested, with an EC_{50} of 0.029 µg/L, 3.6 times more potent than E_2 (EC_{50} = 0.104 µg/L). As expected, BPA was the least potent of the chemicals, with an EC_{50} of 77.94 µg/L. All tested BPA concentrations produced statistically significant responses, with 10 µg/L being the lowest

Table 1. Nominal and measured concentrations for individual compounds and mixtures over 14-day exposures in fish.

	EE_2 (ng/L)			E_2 (ng/L)			BPA (µg/L)		
Mixture nominal	Nominal	Measured	Recovery (%)	Nominal	Measured	Recovery (%)	Nominal	Measured	Recovery (%)
Mixture study									
57.6 mg/L	62	32	51.2	217	81	37.5	57	48	83.7
38.4 mg/L	41	26	62.3	145	76	52.5	38	35.7	93.3
19.2 mg/L	21	12	56.9	72	33	45.6	19	19	99.4
5.76 mg/L	6.2	3.1	50.7	22	11	46.4	5	5.6	98.3
3.84 mg/L	4.1	2.3	55.0	14	7.1	49.1	3.8	4.2	109.9
1.92 mg/L	2.1	0.8	38.8	7.2	2.5	34.6	1.9	1.5	78.5
0.576 mg/L	0.6	0.7	110.5	2.2	1.0	46.1	0.6	0.6	110.5
Average[a]			55.2			46.2			96.9
Single-substance studies									
	10	4.7	47.0	10	4.7	47.0	10	10	100
	32	14.9	46.6	40	16.6[b]	41.5	30	34	113.3
	100	48.3	48.3	100	46.7	46.7	100	84.5[b]	84.5
	320	137.7	43.0	110	56.7	51.6	200	167	83.5
	1,000	470	47.0	300	140	46.7	400	400	100
	2,000	1,002	50.1	500	233	46.6	800	760	95
				1,000	467	46.7	1,600	1,606	100.4
Average[a]			47.0			46.7			95.2

Measured concentrations are expressed as arithmetic mean from data from t_0, t_7, and t_{14}.
[a]Average recovery rate calculated from data from the five highest tested mixture concentrations. [b]Concentrations are from two independent studies.

Table 2. Statistical concentrations of VTG descriptors for single and mixture exposures of E_2, EE_2, and BPA in juvenile sea bass.

	Fraction in	Concentration–response function[a]						EC_{50}	EC_{10}	NOEC
Substance	mixture[b]	RM	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	$\hat{\theta}_{min}$	$\hat{\theta}_{max}$	[µg/L (95% CI)][c]	[µg/L (95% CI)][c]	(µg/L)
E_2	0.001780	Weibull	2.88	3.48	—	0	1.13	0.104 (0.091–0.117)	0.031 (0.014–0.041)	0.0145
EE_2	0.000603	Logit	13.84	9.24	—	0	1.18	0.029 (0.027–0.033)	0.017 (0.015–0.022)	0.0047
BPA	0.997617	Glogit	6.76	0.97	5,925	0	1.51	77.94 (66.99–94.86)	9.12 (3.09–13.46)	< 10.0
Mixture		Weibull	–3.97	2.66	—	0	1.22	17.77 (15.30–20.62)	3.68 (2.43–5.34)	0.635

Abbreviations: EC_{10}, concentration effective for 10% increase; Glogit, generalized logit; max, maximum; min, minimum; RM, regression model.
[a]Functions as defined by Scholze et al. (2001): θ_1, θ_2, θ_3, and θ_{max} are statistical estimates of model parameters given for concentrations expressed in micrograms per liter (rounded values); $\hat{\theta}_{min}$ was set always to zero. [b]Ratio of the concentration of each compound to total mixture concentration, derived from Table 1. [c]EC_{50} and EC_{10} for normalized VTG increase, calculated from the given concentration–response function (using nonrounded parameter values).

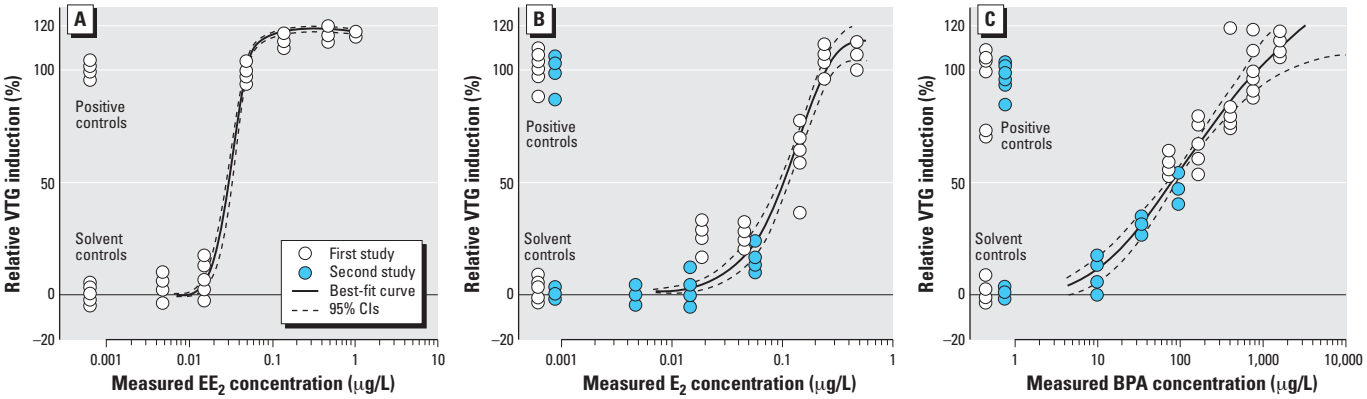

Figure 1. Pooled concentration–response data of plasma VTG induction (%) and best-fit regression curves for EE_2 (*A*), E_2 (*B*), and BPA (*C*) in juvenile sea bass.

tested exposure yielding a VTG induction of around 10% (EC_{10} = 9.12 µg/L). The NOECs derived for E_2 and EE_2 were equal to the lowest tested concentrations and were lower than the respective EC_{10}.

A positive control of 100 ng EE_2/L was expected to produce the maximal possible VTG induction in our system. This assumption was based mainly on outcomes from previous range-finding studies; furthermore, this dose is commonly used as positive control in flow-through systems with fish (e.g., Brian et al. 2005). However, our studies demonstrated clearly that higher EE_2 concentrations are able to induce stronger effects, with a maximal induction of around 3×10^8 ng VTG/mL, 6 times higher than observed for 100 ng EE_2/L. Consequently, our assumed maximal control reference represents a slight underestimate, with a corresponding impact on every effect and effect concentration estimation. However, because we used the $\log_{10}$-transformed VTG scale for the assessment, the differences were negligible and did not change the statistical estimates. For example, the estimated EC_{50} for EE_2 might in reality refer not to a 50% effect, but to 49.3%. To guarantee comparability between studies, we did not change the positive control concentration.

Estrogenicity of mixtures. The mixture was prepared from a master stock solution containing E_2, EE_2, and BPA at a ratio of their nominal EC_{50} values (Table 2); dilutions of 100, 66, 33, 10, 6.7, 3.3, and 1% were tested, corresponding to expected VTG responses between 100% and 0%. This experiment was repeated for three mixture concentrations (corresponding dilutions of 66, 33, and 10%), and the VTG data for both studies agreed excellently (Figure 2). Figure 3 shows the individual and mean VTG responses from the first mixture study normalized to the negative and positive controls, and based on measured concentrations. There was excellent agreement with the CA-predicted concentration–response curve, which was generated based on the concentration–response functions shown in Table 2. We did not detect a statistically significant deviation from predictions for either of the selected effect levels (Table 3). The predicted EC values were based on a common, average mixture ratio. When we used the exact mixture ratios measured for each mixture concentration, the resulting mixture effects were almost identical to those in Figure 2 and did not fall outside the 95% CI of the prediction curve (Figure 3).

Figure 4 shows a comparison of the observed mixture effects with the expected effects of the individual compounds that would occur if they were present alone at their respective levels in the mixture. All individual curves are based on the regression fits of the compounds tested alone (Table 2). Figure 4 shows that a single compound alone cannot explain the observed mixture effect. Thus, a joint VTG response is apparent. Furthermore, Figure 4 shows that concentrations of the compounds without statistically significant effects can still produce a detectable mixture effect when they are present together. For example, the comparison between the median VTG response for the nominal 19.2 mg/L mixture and the corresponding individual effects of the compounds demonstrates how strongly low-steroidal exposures can enhance a weak VTG induction by BPA; for BPA alone, we observed a 20% VTG response, but the presence of 12 ng/L EE_2 and 33 ng/L E_2 increased the VTG induction to 50%.

Discussion

Current knowledge about the sensitivity of marine fish to estrogenic environmental chemicals is still limited, and our study fills these gaps by providing data about VTG induction in sea bass. The VTG responses observed here are comparable to the sensitivity rank orders reported for freshwater species: In our study, EE_2 was the most potent inducer of VTG, an order that was also found in rainbow trout and zebra fish, in which the lowest observed effect concentration (LOEC) for EE_2 was 4-fold lower than that for E_2 (20 ng/L) (Van den Belt et al. 2003). However, for fathead minnow and rainbow trout, up to 25-fold higher potency differences between EE_2 and E_2 have been reported (Brian et al. 2005; Thorpe et al. 2003). The estimated EC_{10} values for VTG induction, 17 ng/L for EE_2 and 31 ng/L for E_2, were of the same magnitude as values shown to induce endocrine disruption in freshwater and seawater fish (e.g., Folmar et al. 2001; Panter et al. 1998). Exposure of 6 ng/L EE_2 to sand goby induced impaired male maturation and reproductive behavior (Robinson et al. 2003). Länge et al. (2001) reported a lack of sexual differentiation in the male fathead minnow after exposure to 4 ng EE_2/L.

The sensitivity of the sea bass to BPA (EC_{10} = 9 µg/L) is comparable to that of the fathead minnow (EC_{10} = 50 µg/L) (Sohoni et al. 2001), but it seems to be higher in comparison to other freshwater species; that is, the EC_{10} was about two orders of magnitude lower than the LOEC for juvenile rainbow trout (LOEC = 1,000 µg/L), and the EC_{50}

Table 3. Statistical uncertainty of predicted and observed effect concentrations [mean (95% CI)] for the mixture.

Effect level	Mixture concentration (µg/L) Observed [mean (95% CI)]	Predicted by CA [mean (95% CI)]
10%	3.68 (2.43–5.34)	4.96 (1.56–6.68)
30%	10.35 (8.47–12.44)	12.10 (10.33–14.38)
50%	17.77 (15.30–20.62)	19.84 (18.93–23.28)
70%	26.92 (23.23–30.64)	28.65 (27.25–33.38)
90%	39.71 (35.51–45.47)	39.88 (37.76–45.40)

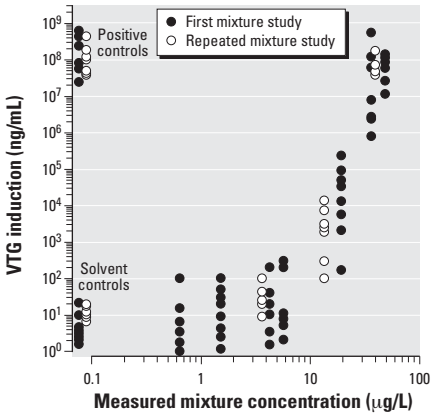

Figure 2. Pooled concentration–response data of plasma VTG induction (ng/mL) for the mixture (EE_2, E_2, and BPA), solvent control (methanol), and positive control (100 ng EE_2/L) in juvenile sea bass. Each point indicates an individual VTG response.

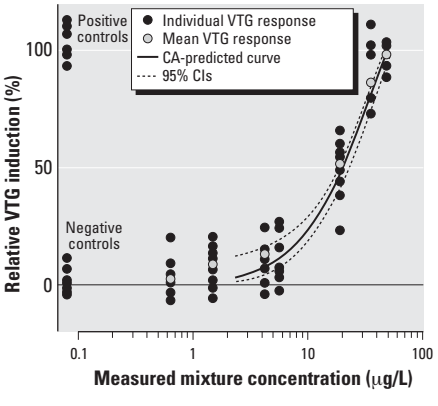

Figure 3. Comparison between the observed and CA-predicted effects of the mixture of estrogenic chemicals in juvenile sea bass. The individual and mean VTG responses are from the first mixture study. The responses are normalized to the negative and positive controls and based on measured concentrations.

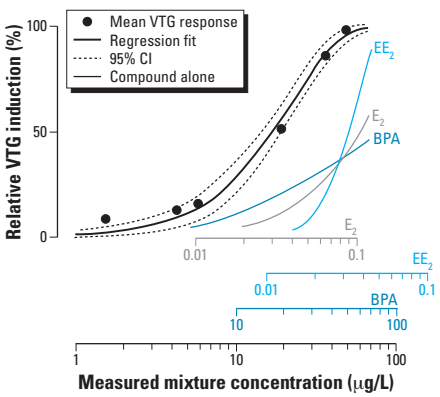

Figure 4. The observed mixture effects, characterized by mean VTG levels and regression fit, compared with the expected concentration VTG curves of the individual compounds at the concentrations present in the mixture. For clarity, their concentration scales are shown.

(78 µg/L) was nearly 2-fold lower than for fathead minnow (EC_{50} = 158 µg/L) (Van den Belt et al. 2003). It is unclear whether this is due to the high stability of the compound in our test system or to differences in metabolism.

The relevance of exposure to E_2, EE_2, and BPA in estuaries and coastal zones is increasingly recognized. This is not confined only to surface waters, but also occurs in sediments and marine biota. BPA can be found in seawater at concentrations of up to 2 µg/L and in seafood at levels of up to 213 µg/g (Basheer et al. 2004). Because both steroidal estrogens are poorly removed in coastal treatment plants (Braga et al. 2005a), they could be detected in sediments in the proximity of a coastal primary sewage treatment plant in ranges of 0.22–2.48 ng/g (E_2) and up to 0.5 ng/g (EE_2) (Braga et al. 2005b). However, it is more likely that the measured environmental exposures, at which we observed significant VTG responses in juvenile sea bass under controlled experimental conditions, reflect the levels encountered at highly exposed point sources rather than the average diffuse environmental burden, which is likely to be much lower. Although a realistic exposure assessment for EDCs in the marine environment is still missing, it is conceivable that even very low concentrations of these compounds can contribute to an overall significant mixture effect, as confirmed in this and other mixture studies (Brian et al. 2005; Silva et al. 2002; Thorpe et al. 2003, 2006). Thus, it cannot be excluded *a priori* that the complex mixture scenario of the EDCs in the marine environment contributes significantly to adverse health effects in fish. If a sufficient number of EDCs is present, theoretically significant VTG inductions can occur when the individual compounds are below the technical detection limit. Because of the difficulties of trace analysis in seawater matrix, the detection limits for estrogenic chemicals are quite high. Therefore this scenario is not unrealistic.

In our studies, each tank (30 L) was supplied with a relatively low water exchange rate (30 L/day), mainly to minimize the relatively high costs of artificial seawater. Because of these testing limitations, we detected strong differences between nominal and measured concentrations, in particular for E_2 and EE_2, where the measured exposures were remarkably lower at the middle and the end of the study (t_7, t_{14}) than shortly before the placement of fish (t_0). This can be explained by active microbial degradation (methanol solvent influence), adsorption to various surfaces such as organic matter (fish feces) in the water and glassware, or the uptake of the chemicals by fish. In contrast, BPA was very stable in seawater (95.2% average recovery), which is in good agreement with the recent findings of Kang and Kondo (2005).

In theory, measured concentrations of the test chemicals in the water should provide quite accurate reflections of the actual exposure conditions. However, it is not clear whether analytical data give a valid estimation for exposures that are responsible for the observed VTG induction after 14 days. Even when recovery rates are extremely low, we cannot always assume that the fish were exposed to small amounts. If we exclude the possibility that technical issues were responsible for a lower chemical in flux (which was proven not to be the case in our study), then low recovery rates could be the result of large uptake by fish, leading to low concentrations. In this case, the sole use of measured levels would give underestimations of the actual exposure. The resulting uncertainties in terms of effective concentrations are difficult to model statistically, and the use of arithmetic means of all measured concentrations may be a poor reflection of biologically active concentrations. However, because of the unknown complex relationship between intake, uptake, technical testing environment, and the observed biological effect at the end of the study, more valid approaches are not available. This general uncertainty explains some of the differences in potency when concentration–response results for the same compound and species are compared from laboratories using flow-through systems with different flow rates. The question arises whether these technical issues influence the outcomes of mixture studies, especially when they are aiming to investigate the predictability of mixture effects. The *in vivo* mixture studies by Brian et al. (2005) and Thorpe et al. (2001, 2003) did not demonstrate relevant differences between nominal and measured exposures over the whole exposure period, both for the single exposures and mixtures. Thus, the experimental design of their mixture studies put the assessment of the predictive power of the CA model on a sound footing. The high replacement rates of the test chemicals, together with relatively large tanks and small fish, were largely responsible for the small variations between nominal and actual concentrations in these studies.

If the recovery patterns between nominal and measured concentrations are reproducible for each compound over the whole testing period, then the important prerequisite of comparability for the CA model is fulfilled and the observed and predicted mixture effects allow a comparative assessment. This applies to each possible difference pattern between nominal and measured concentrations. Moreover, the relative relationship between observed and predicted effects for the mixture remains the same when using nominal instead of measured concentrations, and thus a comparative assessment based on nominal concentrations will come to the same conclusions as when based on measured concentrations.

In the present study, we detected higher recovery rates of EE_2 in the mixture than we observed in the single studies, which were confirmed by the second mixture study. Thus, the reproducibility assumption was violated, and the comparison between observed and predicted mixture effects could only be done on measured concentrations. The detailed information about actual concentrations of the components in the mixture was essential.

A mixture analysis based on the fixed-ratio design can be problematic when the mixture composition varies significantly between the tested mixture concentrations (e.g., when the recovery rate for one individual component is not constant). The observed effect data are not comparable in the sense that they can be analyzed on a common concentration scale, consequently, statistical concentration–effect regression approaches cannot be used for the generation of a fitting curve. Thus, observed and predicted mixture effects can be compared only for the tested mixture concentrations. However, it might be possible to model the variable mixture compositions in function of the single measured exposures (variable mixture ratio). This would also allow a statistical regression analysis for nontested mixture concentrations.

The present study highlights the potential hazard of joint exposure to steroidal estrogens (E_2 and EE_2) and BPA to marine fish. The combined effects of the mixture were greater than those of its individual components. We have shown that the pharmacologic concept of CA very accurately describes the VTG induction of estrogenic chemicals in marine fish. This is in good agreement with the outcomes from previous multicomponent mixture studies in freshwater fish (Brian et al. 2005; Thorpe et al. 2001, 2003, 2006). Taken altogether, these studies provide sufficient evidence that EDCs act dose-additively in inducing VTG, independent of the fish species. If information is available about each compound present in the mixture and if exposure patterns and the effects are reproducible, then CA can be used as a tool to predict accurately the joint effects of EDCs.

Because the effect assessment for EDCs is receiving more and more attention, a proper exposure assessment remains important for assessing the risk. One factor that limits progress in risk assessments for EDC mixtures in marine life is a lack of information on relevant exposure levels. Monitoring programs for most of the known EDCs are not implemented for the marine environment. Moreover, it is unknown how many EDCs are in the field, although new exposure assessment tools, such as the adaptation of the toxicity identification and evaluation for EDCs, might be a promising solution for the future.

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