Relative Potencies and Combination Effects of Steroidal Estrogens in Fish

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The natural steroids estradiol-17β (E2) and estrone (E1) and the synthetic steroid ethynylestradiol-17α (EE2) have frequently been measured in waters receiving domestic effluents. All of these steroids bind to the estrogen receptor(s) and have been shown to elicit a range of estrogenic responses in fish at environmentally relevant concentrations. At present, however, no relative potency estimates have been derived for either the individual steroidal estrogens or their mixtures in vivo. In this study the estrogenic activity of E2, E1, and EE2, and the combination effects of a mixture of E2 and EE2 (equi-potent fixed-ratio mixture), were assessed using vitellogenin induction in a 14-day in vivo juvenile rainbow trout screening assay. Median effective concentrations, relative to E2, for induction of vitellogenin were determined from the concentration–response curves and the relative estrogenic potencies of each of the test chemicals calculated. Median effective concentrations were between 19 and 26 ng L⁻¹ for E2, 60 ng L⁻¹ for E1, and between 0.95 and 1.8 ng L⁻¹ for EE2, implying that EE2 was approximately 11 to 27 times more potent than E2, while E2 was 2.3 to 3.2 times more potent than E1. The median effective concentration, relative to E2, for the binary mixture of E2 and EE2 was 15 ng L⁻¹ (comprising 14.4 ng L⁻¹ E2 and 0.6 ng L⁻¹ EE2). Using the model of concentration addition it was shown that this activity of the binary mixture could be predicted from the activity of the individual chemicals. The ability of each individual steroid to contribute to the overall effect of a mixture, even at individual no-effect concentrations, combined with the high estrogenic potency of the steroids, particularly the synthetic steroid EE2, emphasizes the need to consider the total estrogenic load of these chemicals in our waterways.

Introduction

Wastewater treatment works (WwTWs) effluents are complex mixtures of substances which are known to contain many compounds with endocrine-active properties, notably estrogens (1–3). Studies have shown that exposure of caged and wild fish to effluents from WwTWs results in the induction of the estrogenic biomarker, vitellogenin (VTG) (4–8). Feminization of the reproductive duct has also been observed in early life-stages of roach, Rutilus rutilus, exposed to estrogenic effluent during the period of sexual differentiation (6). Furthermore, intersex (the simultaneous presence of both testicular and ovarian characteristics, believed to be a consequence of exposure to estrogens) has been observed in the gonads of wild populations of roach (9) and gudgeon, Gobio gobio (10), living in UK rivers downstream of WwTW effluent discharges.

Given that the vitellogenic response is an estrogen-dependent process and both formation of oocytes in the testis of males (11, 12) and feminization of the reproductive duct (13) can be induced by exposure to estrogens, attempts to identify the causative agents in effluents have focused on estrogens and estrogen-like chemicals. Toxicity identification and evaluation (TIE) studies, directed at isolating and identifying the major estrogenic chemicals present in WwTW effluents in the UK, have shown that the most active (estrogenic) fraction (>80% of total activity in domestic effluent) contains the natural and synthetic steroidal estrogens (1, 6). In these studies estradiol-17β (E2) was measured at concentrations ranging from 1 to 88 ng L⁻¹, estrone (E1) was present at concentrations ranging from 1 to 220 ng L⁻¹, and 17α-ethynylestradiol (EE2) was found at concentrations ranging from below the limit of detection (<0.2 ng L⁻¹) up to 7.0 ng L⁻¹. Other researchers investigating the presence of natural and synthetic estrogens in aquatic environments receiving domestic effluents have identified similar concentrations. In mainland Europe and Canada, E2 has been measured in effluent at concentrations between 1 and 12 ng L⁻¹ (14, 15), and up to 64 ng L⁻¹ (15), respectively. Concentrations of E1 in Europe and Canada range from below the limit of detection (<1 ng L⁻¹) up to 70 ng L⁻¹ (14, 15). Ethynylestradiol-17α has been detected in WwTWs effluents at concentrations up to 15 ng L⁻¹ in mainland Europe (7, 15), and up to 42 ng L⁻¹ in Canada (15), but more generally is found at concentrations between <0.5 ng L⁻¹ (detection limit for effluent) and 5 ng L⁻¹.

Laboratory studies have demonstrated that the concentrations of E2, E1, and EE2 found in some aquatic environments that receive domestic effluents are sufficient to induce at least some of the feminizing effects reported in caged and wild fish. Reported LOECs for induction of plasma VTG in rainbow trout (exposed for 21 days) are 9 ng L⁻¹, 44 ng L⁻¹, and 0.1 ng L⁻¹ for E2 (16), E1 (17), and EE2 (4), respectively. Similar effective concentrations for VTG induction by steroidal estrogens apply to cyprinid fish species (18). Feminization of the reproductive duct has also been demonstrated in juvenile male zebrafish, Danio rerio, when exposed to E2 (100 ng L⁻¹) from 21 to 42 days post fertilization, during the period of sexual differentiation (19). It has been known for a long time that induction of ovo-testis can result from exposure to pharmaceutical doses of steroid estrogens.

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More recently, however, there are data suggesting that considerably lower exposure concentrations can induce oocyte recruitment in the teleost Japanese medaka, Oryzias latipes, when the exposure occurs during specific windows of early development (20). These data demonstrate that each of the individual steroids is able, individually, to induce an estrogenic response. These steroid estrogens, however, are present as a mixture in the environment and to understand their likely impact in wild fish, information on their relative potencies and combination effects is needed. In this investigation, induction of VTG in juvenile female rainbow trout was used first, to determine the relative potency of E2, E1, and EE2 over a 14-day exposure period, and second, to investigate the effect of a fixed-ratio binary mixture of E2 and EE2 (in which each chemical was present at an equal potency).

Materials and Methods

Test Organisms. Female juvenile rainbow trout were obtained from West Country Trout, Trafalgar Farm, Cornwall, UK (expt I) and from Houghton Springs Fish Farm, Dorset, UK (expt II). The body weight of the fish used was 10.47 ± 0.71 g (mean ± SEM; n = 24) in expt Ia, 6.51 ± 0.48 g (n = 24) in expt Ib, 7.89 ± 0.22 g (n = 24) in expt II. In all experiments, fish were maintained for 14 days under flow-through conditions in de-chlorinated water at 15.0 ± 1 °C, with a 16 h:8 h light/dark photoperiod, with 20 min dawn and dusk transition periods. Prior to the start of each experiment, fish were acclimated in the same conditions for a minimum of 10 days. Throughout the exposures, fish were fed 1% of body weight per day of Keystart Hatchery 1200 fish food pellets (BOC Pauls Limited, Renfrew, UK).

Test Chemicals. Estradiol-17β (98% purity; Lot 46H1146), E1 (99% purity; Lot 125H0223), and EE2 (98% purity; Lot 45H0716) were purchased from Sigma, Poole, Dorset, UK.

Water Supply and Test Apparatus. The supply of de-chlorinated water to the laboratory dosing system was monitored daily for conductivity, hardness, and free chlorine, and was tested for alkalinity and total ammonia twice weekly. The conductivity of the test water ranged from 197 to 236 μS cm⁻¹, the hardness ranged from 41.0 to 52.0 mg L⁻¹ (as CaCO₃), and free chlorine was present at < 2 μg L⁻¹. Alkalinity ranged from 12.8 to 28.2 mg L⁻¹ and ammonia (as N-NH₃) was < 0.05 mg L⁻¹. Dissolved oxygen concentrations and pH levels were determined in the individual tanks on days 0 and 1 and then twice weekly throughout the exposure period. In all experiments, the dissolved oxygen concentration remained > 80% of the air saturation value throughout the exposures and pH values ranged from 7.0 to 7.5. Water temperatures were monitored constantly throughout the exposure period and ranged between 15.1 and 15.6 °C. Dilution water and test chemical flow rates were checked at least three times per week. The flow rate provided a 99% replacement time in approximately 7 h. The test vessels had a working volume of approximately 45 L and were constructed of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions.

Analytical Chemistry. The actual concentrations of the reference chemicals were monitored throughout all experiments. Water samples were collected from each tank into solvent-cleaned flasks on days 0 and 14 of the exposures in expt I and days 0, 7, and 14 of the exposures in expt II.

For measurement of E2 and EE2 in expt I, 500 mL water samples were extracted under vacuum (50 mL min⁻¹) onto preconditioned solid-phase extraction columns. Estradiol-17β and EE2 were eluted from the columns using 5 mL of methanol, the solvent was then removed under a stream of nitrogen, and the extracts were resuspended in ethanol. The extracts were analyzed using established radioimmunoassays for E2 (21) and EE2 (22). The limits of detection for the two radioimmunoassays were 1.0 ng L⁻¹ for E2 and 0.1 ng L⁻¹ for EE2.

For measurement of E1, 2.5 L water samples were extracted under vacuum (50 mL min⁻¹) onto preconditioned 47 mm Envi-disks (Supelco). Estrone was eluted from the disks using 30 mL of methanol, the residual solvent was removed under a stream of nitrogen, and the extract was resuspended in 1.1 methanol/water. A Jasco AS-851 auto-sampler was used to inject 200 μL samples onto a 100 mm × 4.6 mm (i.d.) ACU 049 column, fused silica with Genesis 4 μm C₁₈ (Jones Chromatography) stationary phase. Estrone was eluted isocratically using 65:35 methanol/water at 1.5 mL min⁻¹, delivered from a Jasco PU-980 pump. An ultraviolet detector (UV-975, Jasco) set at 210 nm was used for the detection of E1. The limit of detection was 0.2 μg L⁻¹.

For the binary mixtures experiment (expt II), it was decided not to use a radioimmunoassay to analyze concentrations of E2 and EE2 in the mixtures tanks (where E2 is present at a concentration 30-fold higher than that of EE2) due to the small potential for cross-reaction of E2 in the EE2 RIA (E2 has < 0.003% cross reaction with antibodies raised against EE2). Concentrations of E2 and EE2 were, therefore, verified using gas chromatography mass spectrophotometry (GCMS). Water samples were spiked with deuterated-E2 and then extracted under vacuum (50 mL min⁻¹) onto preconditioned 47 mm C₁₈ Envi-disks (Supelco). Estradiol-17β and EE2 were eluted from the disks using 30 mL of methanol, and residual solvent was removed under a stream of nitrogen. The extracts were derivatized by heating at 120 °C for 30 min with 200 μL of pyridine and 300 μL of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA). After cooling of the extracts, 300 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added and the vial was heated to 60 °C for 20 min. The reagents were removed under nitrogen and the extracts resuspended in 250 μL of dichloromethane (1000 μL for the 750 ng L⁻¹). The derivatized samples were analyzed on a Polaris ion trap GCMS (Thermoquest). The analysis conditions were as follows: sample volume, 2.5 μL; GC column, HP-5MS 30 m × 0.25 mm (i.d.) fused silica with 0.25 μm stationary phase film thickness (Hewlett-Packard) using helium as the carrier gas at 1 mL min⁻¹; injector temperature, 300 °C; column program, (1) 50 °C for 10 min, (2) increase to 300 °C at 8 °C min⁻¹, (3) isothermal at 300 °C for 10 min. The MS was operated in the electron ionization mode (70 eV) and set up to carry out M⁺ - M⁻57 ions. Precursor (parent) ions m/z 458 and m/z 460 (trimethylsilyl (TMS)/tert-butyldimethylsilyl (TBDMS) derivative of E2 and its deuterated analogue, respectively) and m/z 410 and m/z 482 (TBDMS derivative of EE2 and TMS/TBDMS derivative of EE2, respectively) were stored in the ion trap, fragmented, and then scanned out to give daughter ion spectra for each compound. The major daughter ion for each compound was the [M + H₂O-57]⁺, i.e., m/z 419 and m/z 421 (TMS/TBDMS derivative of E2 and its deuterated analogue, respectively) and m/z 371 and m/z 467 (TBDMS derivative of EE2 and TMS/TBDMS derivative of EE2, respectively). These ions were profiled, and the resulting peaks were integrated to give peak areas, which were used for the calculations, using deuterated E2 as the internal standard. The limits of detection were 0.2 ng L⁻¹ for E2 and 0.4 ng L⁻¹ for EE2.

Experimental Design. Experiment I - Relative Potencies of E2, EE2, and E1. Groups of 12 juvenile female rainbow trout were exposed for 14 days to a dilution water control (DWC), methanol solvent control (SC), and nominal concentrations of E2 at 1.0, 3.2, 10, 32, 100, and 320 ng L⁻¹; EE2 at 0.10, 0.32, 1.0, 3.2, 10, and 32 ng L⁻¹; and E1 at 1.0, 3.2, 10, 32, 100, and 320 ng L⁻¹. The E2 and EE2 exposures were run simultaneously.
and, therefore, shared a common group of control fish (expt Ia); the E1 exposure was run separately, with an independent control group (expt Ib).

Stock solutions of each chemical were prepared weekly in HPLC grade methanol (Fisher Scientific) and dosed to glass mixing vessels by means of a peristaltic pump, at a rate of 0.04 mL min⁻¹, to mix with the dilution water flowing to the mixing vessels at a rate of 400 mL min⁻¹. The SC vessel received the same rate of addition of methanol, such that the water in all test vessels, except the DWC, contained 0.1 mL of methanol per liter.

Experiment II—Combination Effects of E2 and EE2. Groups of 12 juvenile female rainbow trout were exposed for 14 days to a DWC, methanol SC and nominal concentrations of E2 at 1.0, 10, 25, 75, and 750 ng L⁻¹ and EE2 at 0.040, 0.40, 1.0, 5.0, and 30 ng L⁻¹, and to fixed-ratio binary mixtures of E2 + EE2 (25:1) at concentrations of 5.0 ng L⁻¹ + 0.20 ng L⁻¹, 12.5 ng L⁻¹ + 0.50 ng L⁻¹, and 87.5 ng L⁻¹ + 3.5 ng L⁻¹, respectively. The fixed-ratio of 25:1 was calculated from the ratios of the median effect concentrations derived for E2 and EE2 in expt I.

Stock solutions of each chemical were prepared weekly in HPLC grade methanol (Fisher Scientific) and dosed to glass mixing vessels by means of a peristaltic pump, at a rate of 0.04 mL min⁻¹, to mix with the dilution water flowing to the mixing vessels at a rate of 400 mL min⁻¹. From each primary mixing vessel the test solution flowed into a second mixing vessel to produce the binary mixtures, and then into the exposure tanks. The SC vessel received the same rate of addition of methanol, such that the water in all test vessels, except the DWC, contained 0.1 mL of methanol per liter.

Fish Sampling. In all experiments, a subgroup of fish (n = 24) was sampled at the outset (day 0) of the experiment and then all exposed fish were sampled on day 14. Fish were sacrificed in a lethal dose (200 mg L⁻¹) and then all exposed fish were sampled on day 14. Fish were sacrificed in a lethal dose (200 mg L⁻¹) and then all exposed fish were sampled on day 14. Fish were sampled at the outset (day 0) of the experiment and then all exposed fish were sampled on day 14. Fish were sacrificed in a lethal dose (200 mg L⁻¹) and then all exposed fish were sampled on day 14.

Mathematical Modeling and Statistical Analyses. For the description of the concentration-effect relationships for the individual test compounds and for the binary mixtures, a 4-parameter logit regression model was used

\[
f(x) = \begin{cases} \frac{\theta_{\max} - \theta_{\min}}{1 - \exp(-\theta_1 - \theta_2 \log_{10}(x))} & \text{for } x > 0 \\ \theta_{\min} & \text{for } x = 0 \end{cases}
\]

where \( x \) = concentration and \( f(x) \) = mean effect. The model parameter \( \theta_{\min} \) describes the minimal mean effect (control response), \( \theta_{\max} \) is the asymptotical maximal effect, \( \theta_1 \) is termed the “location” parameter, and \( \theta_2 \) characterizes the “steepness” of the concentration-response relationship. Because of heterogeneous nonrandom variabilities in the replicated data (heteroscedasticity), each model was fitted using the estimation method of generalized least squares (24). To fulfill the statistical prerequisite of symmetrically distributed effect data for this estimation method, the plasma VTG concentrations were log-transformed. The mean effect, \( f(x) \) in eq 1, therefore, always corresponds to the log-transformed VTG concentration. Effect concentrations were determined on the basis of the estimated regression eq 1 by its functional inverse as

\[
x = \text{POW} \left( -\frac{\theta_1 - K}{\theta_2} \right) \text{ with } K = \log_e \left( \frac{\theta_{\max} - \theta_{\min}}{Y - \theta_{\min}} - 1 \right)
\]

where \( Y \) is a given effect (i.e., log-transformed VTG concentration), \( \text{POW}(t) \) is 10 raised to the power \( t \), and \( \theta_{\min} \), \( \theta_{\max} \), \( \theta_1 \), and \( \theta_2 \) are the estimates of the unknown model parameters \( \theta_{\min} \), \( \theta_{\max} \), \( \theta_1 \), and \( \theta_2 \). The median effect is defined as the average between the mean control and the mean effect produced by the highest tested concentration of E2 using the log-transformed VTG scale, resulting in a VTG concentration of approximately 60 μg mL⁻¹. LOECs were determined using a nonparametric Wilcoxon’s rank sum test (25).

Given that the vitellogenic response is an estrogen-dependent process, the model of concentration addition (CA) was used to model the theoretical concentration-response relationship for the fixed-ratio binary mixture of E2 and EE2 (26). The model of CA is based on the assumption that chemicals act via a similar mechanism to elicit an effect, such that one chemical acts as a dilution of the other and can be substituted at a constant proportion for the other. This model is usually defined for a binary mixture of substances 1 and 2 by eq 3

\[
\frac{c_1}{EC_{X1}} + \frac{c_2}{EC_{X2}} = 1
\]

where \( c_1 \) and \( c_2 \) are the individual concentrations of the substances 1 and 2 constituting the mixture that produces an effect \( x \) and \( EC_{X1} \) and \( EC_{X2} \) denote the equivalent effect concentrations of the single substances 1 and 2 that alone would produce the same effect \( x \) as the mixture. The sum of \( c_1 \) and \( c_2 \) equals the total concentration that produces the combined effect \( x \), i.e., \( EC_{X \text{ Mixture}} \) therefore the individual concentrations \( c_1 \) and \( c_2 \) can be expressed as proportions \( p_1 \) and \( p_2 \) of the total concentration, i.e., \( p_1 = c_1/EC_{X \text{ Mixture}} \) and \( p_2 = c_2/EC_{X \text{ Mixture}} \). Eq 3 can, therefore, be rearranged as

\[
EC_{X \text{ Mixture}} = \left( \frac{p_1}{EC_{X1}} + \frac{p_2}{EC_{X2}} \right)^{-1}
\]

The individual effect concentrations \( EC_{X1} \) and \( EC_{X2} \) can be derived from eq 2 on the basis of the estimated regression functions (eq 1). Holding the ratio \( p_1/p_2 \) fixed, the calculations can be performed for different given combined effects \( x \) (assuming that the corresponding effect concentrations of the individual components exist) leading to a graph of the concentration-effect curve for the mixture. This estimated concentration-response relationship is then compared with the observed concentration-response for the experimental mixture.

A special case arises when calculating the maximal effect of the mixture: eq 4 assumes that the concentration for an expected mixture effect \( x \), can be calculated only when each of the individual components is able to produce the same level of effect when applied singly. When considering components with different asymptotical maximal effects (estimated as \( \theta_{\max} \) according to eq 1 on a logarithmic effect scale), it is not possible to calculate the mixture concentrations for all effects between (and above) these two individual maximal effects (from a modeling aspect, this also holds true for all effects between (and below) both estimated asymtotical minimal effect levels, but as these effect levels are usually very similar, this is less relevant). Moreover, the concentration-effect curve of the mixture has to approach the lower estimated asymtotical maximal effect level of both individual components. Whether this is only the result of a weakness of the mathematical formula is so far unknown, and its examination is not the purpose of this paper; therefore, the expected mixture effects have only been calculated up to the beginning of the calculable maximal effect.

The individual effect concentrations \( EC_{X1} \) and \( EC_{X2} \) are subject to a stochastic variability, therefore, the calculated

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TABLE 1. Experiment I: Mean Measured Tank Concentrations of Estradiol-17b (E2), Estrone (E1), and Ethynylestradiol-17a (EE2) Over the 14-Day Exposures

<table>
<thead>
<tr>
<th>nominal (ng L⁻¹)</th>
<th>E2 (ng L⁻¹)</th>
<th>E1 (ng L⁻¹)</th>
<th>EE2 (ng L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>nt</td>
<td>0.13 ± 0.1</td>
<td>0.21 ± 0.1</td>
</tr>
<tr>
<td>0.32</td>
<td>nt</td>
<td>0.33 ± 0.3</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>1.0</td>
<td>2.3 ± 0.8</td>
<td>0.74 ± 0.4</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>3.2</td>
<td>4.8 ± 1.5</td>
<td>3.3 ± 0.1</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>14 ± 4.0</td>
<td>11 ± 0.6</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>32</td>
<td>47 ± 14.1</td>
<td>28 ± 4.7</td>
<td>26 ± 4.7</td>
</tr>
<tr>
<td>100</td>
<td>99 ± 12.1</td>
<td>86 ± 4.9</td>
<td>nt</td>
</tr>
<tr>
<td>320</td>
<td>463 ± 155.9</td>
<td>319 ± 15.3</td>
<td>nt</td>
</tr>
</tbody>
</table>

*The test chemicals were not detected in the dilution water or solvent controls. Data as means ± SD (n = 2). nt = not tested, these concentrations were not included in the experimental design.

TABLE 2. Experiment II: Mean Measured Tank Concentrations of Estradiol-17b (E2) and Ethynylestradiol-17a (EE2) in the Individual and Binary Mixture Tanks Over the 14-Day Exposures

<table>
<thead>
<tr>
<th>mean measured conc.</th>
<th>E2 (ng L⁻¹)</th>
<th>EE2 (ng L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nominal individual mixture</td>
<td>0.04 ± 0.00</td>
<td>nt</td>
</tr>
<tr>
<td>5.0</td>
<td>0.4 ± 0.04</td>
<td>0.2 ± 0.00</td>
</tr>
<tr>
<td>10.0</td>
<td>0.4 ± 0.33</td>
<td>nt</td>
</tr>
<tr>
<td>12.5</td>
<td>nt</td>
<td>0.6 ± 0.07</td>
</tr>
<tr>
<td>25.0</td>
<td>nt</td>
<td>1.0 ± 0.07</td>
</tr>
<tr>
<td>87.5</td>
<td>nt</td>
<td>3.5 ± 0.00</td>
</tr>
<tr>
<td>175.0</td>
<td>nt</td>
<td>7.0 ± 0.23</td>
</tr>
<tr>
<td>750.0</td>
<td>nt</td>
<td>30 ± 0.33</td>
</tr>
</tbody>
</table>

*The test chemicals were not detected in the dilution water or solvent controls. Data as means ± SEM (n = 3). nt = not tested, these concentrations were not included in the experimental design.

Results and Discussion

Mean measured concentrations of the individual chemicals and their binary mixtures are given in Tables 1 and 2, respectively. All exposure concentrations are described using means of the actual measured concentrations. In expt I, the mean measured concentrations of E2 were between 100 and 150% of nominal, with the exception of the lowest concentration of E2, 1.0 ng L⁻¹, where the mean measured concentration was 230% of nominal. This lowest concentration of E2 was at the limit of detection for the analytical method and thus likely to be less accurate than for the higher E2 concentrations. For EE2, the mean measured concentrations were between 45 and 130% of nominal and for E1 they were between 74 and 108% of nominal. In expt II, the mean measured concentrations were between 76 and 100% of nominal for E2 and between 100 and 114% of nominal for EE2. Differences that were observed between nominal and actual measured concentrations were likely to arise as a consequence of small inaccuracies in the preparation of the stock solutions and/or the dosing to the test vessels, as well as loss of the test chemical through absorption to surfaces and degradation. This highlights the importance of using analytical methods to verify nominal exposure concentrations when assessing the potential environmental hazard of a chemical. It should be noted, however, that discrepancies between nominal and measured concentrations of the test chemical may also occur as a consequence of discrepancies in the analytical procedure.

The concentrations of VTG in the plasma of juvenile female fish at the onset of the experiments were 370 ± 50 ng mL⁻¹, 330 ± 60 ng mL⁻¹, and 440 ± 220 ng mL⁻¹ for expts Ia, Ib, and II, respectively. These concentrations are similar to those established previously in juvenile female rainbow trout of this weight and age (16). Holding the fish on a maintenance ration (1% body weight/day) resulted in no detectable increases in plasma VTG concentrations in either the DWC or in the SC fish after the 14-day exposure period in any of the experiments (p > 0.05).

There were no mortalities in fish exposed to any of the test chemicals in any of the experiments. Each of the individual test chemicals produced concentration-dependent increases in plasma VTG (Figures 1 and 2). In expt I, NOEC and LOEC values were 4.8 and 14 ng L⁻¹ for E2, 0.74 and 3.3 ng L⁻¹ for EE2, respectively (Table 3). In expt II, NOEC and LOEC values were 9.6 and 14 ng L⁻¹ for E2 and 1.13 and 7.6 ng L⁻¹ for EE2, respectively (Table 3). The observed LOECs for induction of VTG by these steroid estrogens are consistent with those determined in other investigations. Reported LOECs for E2 range from 10 ng L⁻¹ in adult rainbow trout exposed for 21 days (16, 17), to 50 ng L⁻¹ in fathead minnows exposed from 24 h post-fertilization to 30 days post-hatch (27), up to 100 ng L⁻¹ in male roach exposed for 21 days (17). The LOECs for E1 range from 31.8 ng L⁻¹ in male fathead minnows exposed for 21 days (28), to 44 ng L⁻¹ in male rainbow trout exposed for 21 days (17), and 66 ng L⁻¹ in golden orfe, Leuciscus idus, exposed for 7 days (29). For EE2, reported LOECs vary from 0.1 to 1.79 ng L⁻¹ in adult male rainbow trout exposed for 10 and 21 days, respectively (4, 30), to 1.67 ng L⁻¹ in adult zebrafish exposed for 21 days (31). Although the published values compare favorably with one another (and with the data in this study), it is difficult to use the available data to derive relative estrogenic potencies for the steroid estrogens. This is due to the variety of test species and life-stages used in these studies and the different test conditions employed (i.e., duration of exposure). Ideally, chemicals should be tested in the same system under identical exposure conditions to ensure that differences in the biological response are due to the potency of the test chemical and not a result of differences in the exposure conditions. It should also be emphasized that there are difficulties in deriving relative estrogenic potencies using LOEC values. A LOEC is a statistically derived test value used simply to define the lowest concentration of a test chemical that has a significant effect, and as such will depend on how closely the test concentrations are spaced in an experiment, the number of test organisms employed, and the observed variability in the effect data. LOECs can be
used reliably for determination of relative estrogenic potencies only when comparable concentrations series are employed and all test conditions are identical. Ideally, relative potencies should be determined by estimating (through interpolative smoothing, or biometrical modeling) the concentration of each chemical expected to produce a predetermined level of effect.

In the present study, the estrogenic potencies of the test chemicals were determined using the median effective concentrations (EC50s), for each of the test chemicals, relative to E2. The EC50 values, determined via the functional inverse of the estimated concentration—response function (see eq 2), were 26, 60, and 0.95 ng L\(^{-1}\) for E2, E1, and EE2, respectively, in expt I, and 19 and 1.8 ng L\(^{-1}\) for E2 and EE2, respectively, in expt II (Table 3). The observed differences in the EC50 values between the two experiments for both E2 and EE2 are most likely due to the natural variability one might expect for differences in the vitellogenic responses between different batches of fish. Ethynylestradiol-17\(\alpha\) was consistently the most potent steroid tested, with an estrogenic potency between 11 and 27 times greater than that of E2 and between 33 and 66 times greater than that of E1 (E2 was 2.3 to 3.2 times more potent than E1). These relative estrogenic potency estimates for EE2 and E1, compared with E2 for induction of VTG, are consistent with those indicated in other investigations. Ethynylestradiol-17\(\alpha\) has been reported to be approximately 10-fold more potent than E2 in inducing VTG in male sheepshead minnow (exposed for 16 days (32)), and E1 has been reported to be approximately 2–5 (17) and 3–10 (28) fold less potent than E2, in inducing VTG in male rainbow trout and fathead minnows, respectively (exposed for 3 weeks). Interestingly, in both our study and that conducted by Panter et al. (28), the concentration—response curve obtained for E1 was not as steep as that obtained for E2, and E1 was observed to have a lower LOEC than E2, even though the response curve for E1 is clearly displaced to the right of the curve obtained for E2 (i.e., E1 is indeed less potent than E2 when considering the full concentration range; Figure 1). This highlights the importance of considering the full concentration—response curve when determining potency.

To date, only one other study has compared the relative potencies of these three steroidal estrogens in a single experimental system (20). In that study, the induction of testis-ova was assessed in Japanese medaka following exposure during early life-stages, and the observed LOECs were used to determine relative potencies (20). The reported LOECs for E2, E1, and EE2 were 10, 10, and 0.1 ng L\(^{-1}\), respectively, indicating that E2 and E1 were equipotent, but EE2 was approximately 100 times more potent than E2 or E1. Together, these data clearly illustrate that EE2 is considerably more potent in inducing VTG and disrupting gonadal development than either E2 or E1 in fish. This high potency of EE2, combined with its persistence in the environment compared with the natural steroids (33, 34), and its capacity to bioaccumulate (10 000-fold in the bile after a 3-week exposure in rainbow trout (7)), suggests that EE2 is likely to be of considerable importance as an environmental estrogen.

To date, investigations into the in vivo effects of these environmental steroids have focused on the effects of the individual chemicals, despite the fact that they are often present in combination in the aquatic environment. In this investigation, a single binary mixtures experiment was conducted to investigate the in vivo combination effects of a natural and synthetic steroid estrogen and in turn assess whether the total estrogenic activity of a steroid estrogen mixture could be determined from the activity of the individual chemicals. The mixture of E2 and EE2, at a fixed 25:1 ratio, produced concentration-dependent increases in plasma VTG (in a manner similar to the individual steroid estrogens). All mixture concentrations of E2 and EE2 tested, including the lowest (5.3 ng of E2 L\(^{-1}\) and 0.2 ng of EE2 L\(^{-1}\)), induced a significant increase in the plasma VTG concentration (\(p < 0.05\)). When comparing the observed VTG induction data for the mixtures with the expected mixture effects, using the model of CA, the mixtures of E2 and EE2 were only shown to act in an additive manner, at low effect levels (VTG concentrations below 10 000 ng L\(^{-1}\)) (Figure 2). The 95% confidence belt for the observed mixture data overlapped with the 95% confidence bootstrap belt for the calculated mean of CA. With increasing level of effect (increased VTG

FIGURE 1. Experiment I. Plasma vitellogenin (VTG) concentrations in female juvenile rainbow trout exposed to (●) estradiol-17\(\beta\) \((n = 12)\), (○) ethynylestradiol-17\(\alpha\) \((n = 12)\), and (×) estrone \((n = 12)\) in expt I. In some cases the VTG concentrations were very similar between fish within a treatment, therefore not all data points are visible. For each of these exposures the 95% confidence belts of the fitted concentration—response relationships are shown.
concentrations above 10,000 ng L\(^{-1}\)), there was a divergence between the concentration–response relationship for the observed mixture data and the calculated mean of CA, with no overlap of the 95% confidence bootstrap belt. This divergence from expectation may be due to the binary mixture of E2 and EE2 acting in a less than additive manner at high concentrations, but is more likely a result of the limitations of the experimental design and modeling procedures employed. The statistical confidence belts describe the precision of the estimated regression model for the observed mixture effects and are based on three important assumptions: (1) all experimental conditions for the single substance experiments are the same as for the mixture experiment, (2) the selection of an appropriate regression model to describe the concentration–effect relationship, and (3) the experimental design allows a valid fitting of the regression model to the observed effect data. The first assumption was easily resolved by testing the individual chemicals and their binary mixtures simultaneously, using shared stock solutions, to ensure that the experimental conditions were identical and to remove any possible bias. The logit regression model selected for these experiments was flexible enough to describe very different sigmoid, monotone increasing concentration–effect relationships, and alternative models such as the 4-parameter probit, -weibull, or langmuir resulted in no improvements. Uncertainties arise when considering the final assumption. Because of the complexities involved in conducting mixture experiments using flow-through conditions it was only

![FIGURE 2. Experiment II. Plasma vitellogenin (VTG) concentrations in female juvenile rainbow trout exposed (A) to (●) estradiol-17β (E2; \(n = 12\)) and to (○) ethynylestradiol-17α (EE2; \(n = 12\)) and (B) to fixed-ratio binary mixtures (nominally 25:1) of E2 and EE2 (●) \((n = 12)\). In some cases the VTG concentrations were very similar between fish within a treatment, therefore not all data points are visible. For each of these exposures the concentration–effect relationships, estimated using a 4-parameter logit regression model, are shown as solid lines (A, black (EE2) or gray (E2); B, gray) with 95% confidence belts. The expected VTG response for the binary mixture, calculated using the model of concentration addition, is shown (B) as a solid black line with a 95% confidence bootstrap belt.](image-url)
possible to test three exposure concentrations for the mixture, along with each of the individual chemicals, and the appropriate controls. Although the data for each of the individual chemicals (each tested at five concentrations) could be described adequately enough to allow an accurate estimation of the complete concentration—effect curve, responses for only three exposure concentrations allow for very different possible concentration—effect curves. It is, therefore, conceivable that the estimated curve for the observed mixture may not be as accurate as ideally required and the true curve could be steeper than assumed, particularly if the observed mixture effects of the highest mixture concentration already correspond to the maximal effect level. Such a curve would show less deviation from the predicted curve. An additional problem arises when considering the curve for the prediction of concentration addition (Figure 2B). In these experiments the maximum of the prediction for concentration addition could not be defined on the basis of the maxima of the single compounds. This problem arises first because induction of VTG is dependent not only on the experimental conditions employed, but also on the individual test compounds, with different chemicals resulting in different maximal responses. As a single maximal response could not be clearly defined, the single substance models were fitted independently to the experimental data sets, resulting in independent maximal effects. Second, the definition of the maximal effect, the delta max model parameter, describes an asymptote, which means that it is not possible to decide which concentration has (or has not) produced a maximal effect. Finally, the equation for the model of CA assumes that the maximal response for the mixture is identical to the lower of the two maximal effects obtained for the single components. However, it is just as likely that the maximal response for the combination would be identical to the higher of the two maximal effects obtained for the single components. These issues serve to illustrate the complexity of analyzing relatively simple binary mixtures of estrogens. Analysis of effects of more complex mixtures of endocrine-active chemicals (with a range of mechanisms of action) in vivo will be a formidable challenge, especially given the practical limitations of both the mathematical modeling procedures and conducting large scale in vivo experiments where organisms are exposed simultaneously to the appropriate number of exposure concentrations of both the individual chemicals and the mixtures.

Irrespective of the limitations of the experimental design and the modeling procedure employed in these investigations, the data demonstrate that E2 and EE2 are each able to contribute to the overall effect of the mixture, producing a mixture that is more potent than either of the individual chemicals. Given the high potency of the natural and synthetic steroidal estrogens, particularly EE2, and their presence in combination in a number of rivers at biologically active concentrations, the issue of estrogenic mixtures is an important one, especially as there are now known to be many other chemicals in effluents with estrogenic activity. This is of concern considering the ability of some chemicals to interact in an additive manner, in vivo, at concentrations below their individual LOECs (35). It is, therefore, important to consider the total estrogenic load of our waterways and not only those individual chemicals present at biologically active concentrations when assessing the potential effects of a mixture.

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Literature Cited


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