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Assessing the Biological Potency of Binary Mixtures of Environmental Estrogens using Vitellogenin Induction in Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

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Experiments were conducted to assess the in vivo potency of binary mixtures of estrogenic chemicals using plasma vitellogenin (VTG) concentrations in juvenile rainbow trout (*Oncorhynchus mykiss*) as the endpoint. The estrogenic potencies of estradiol-17β (E2), 4-*tert*-nonylphenol (NP), and methoxychlor (MXC) were determined following 14 day exposures to the individual chemicals and binary mixtures of these chemicals. E2, NP, and MXC all induced concentration dependent increases in plasma VTG, with lowest observed effect concentrations of 4.7 and 7.9 ng L^{-1} for E2, 6.1 and 6.4 μg L^{-1} for NP, and 4.4 and 6.5 μg L^{-1} for MXC. Concentration−response curves for fixed ratio binary mixtures of E2 and NP (1:1000), E2 and MXC (1:1000), and NP and MXC (1:1) were compared to those obtained for the individual chemicals, using the model of concentration addition. Mixtures of E2 and NP were additive at the concentrations tested, but mixtures of E2 and MXC were less than additive. This suggests that while NP probably acts via the same mechanism as E2 in inducing VTG synthesis, MXC may be acting via a different mechanism-(s), possibly as a result of its conversion to HPTE which is an estrogen receptor α agonist and an estrogen receptor β antagonist. It was not possible to determine whether mixtures of MXC and NP were additive using VTG induction, because the toxicity of MXC restricted the effect range for which the expected response curve for the binary mixture could be calculated. The data presented illustrate that the model of concentration addition can accurately predict effects on VTG induction, where we know that both chemicals act via the same mechanism in mediating a vitellogenic response.

Introduction

A large number of natural and synthetic chemicals have been labeled as endocrine active, due to their ability to mimic endogenous hormones. Endocrine active chemicals mediate their effects by binding to hormone receptors as agonists or antagonists, by inhibiting the enzymes responsible for steroid hormone biosynthesis, and/or by inducing the enzymes responsible for steroid metabolism (*1*). Particular focus has fallen on chemicals which mimic estradiol-17β (E2) by binding to the estrogen receptor(s) (ERs) to elicit agonist and/or antagonistic responses (*2, 3*). These chemicals are collectively described as xenoestrogens. With the exception of the synthetic steroids, the xenoestrogens discovered so far are only weakly active when compared with endogenous steroids. However, evidence for reproductive abnormalities that are indicative of sex hormone disruption in wild fish populations, supported by in situ monitoring studies with caged fish, implies that some aquatic environments contain xenoestrogens at concentrations high enough to be of concern to wildlife (*4*−*6*). These "field" observations are supported by in vivo laboratory studies, where exposure to some xenoestrogens induces estrogenic effects in fish at environmentally relevant concentrations (*7*−*9*).

The aquatic environment receives a large influx of natural and synthetic chemicals from agricultural, industrial, and domestic sources. In Europe, natural estrogens such as E2 and estrone, synthetic steroids including, 17α-ethynylestradiol, and other nonsteroidal chemicals known to have estrogenic effects (such as alkylphenols), have been detected in effluents that discharge into rivers (*10, 11*). This wide range of xenoestrogens in the aquatic environment highlights the importance of improving our understanding of combination effects of these chemicals in organisms as well as their individual effects. In many cases xenoestrogens are present at concentrations too low to be considered of concern individually, but the presence of mixtures of these chemicals means there is a potential for additive and/or interactive effects. The existence of interactive effects implies that the estrogenic effect of a mixture somehow deviates from what is expected, on the basis of the estrogenic effects of the single agents. There are two main analytical models for defining the expected effects of a mixture: the model of concentration addition (CA), which assumes that the compounds act via a similar mechanism in producing an effect (*12*), and the model of response addition, which assumes that the compounds act via independent pathways (*13*). If a mixture of xenoestrogens is more potent than would be expected, the combination effect is described as more than additive (synergistic), and if it is less effective, the combination effect is described as less than additive (antagonistic). Such deviations from expectation should be demonstrated at more than one concentration of the mixture.

In this study, the estrogenic activities of three environmental estrogens, namely E2, 4-*tert*-nonylphenol [NP], and methoxychlor [MXC], were assessed individually and in binary mixtures (to investigate possible interactive effects) using plasma vitellogenin (VTG) concentrations in juvenile female rainbow trout (*9*), as the response. VTG induction in fish is specifically an estrogen-dependent process, normally restricted to mature females. During reproductive development in female fish, the hypothalamic-pituitary−gonadal axis stimulates the ovary to produce E2, which is released into the bloodstream and transported to the liver. Here it diffuses passively into hepatocytes and binds to the ER, stimulating transcription of the VTG gene(s). The VTG synthesized is then transported to the ovary and sequestered

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by the developing oocytes, to be stored as yolk for the subsequent embryo (*14*). Although VTG synthesis is normally restricted to maturing females, immature females and male fish possess the machinery for VTG production, and exposure to estrogens (and their mimics) can trigger VTG synthesis via the ER. Given that all the available evidence shows that steroid estrogens and their mimics act via the ER to induce VTG synthesis, the model of CA was used to assess the estrogenic activity of binary mixtures of the test chemicals. A simulation technique termed "bootstrap" (*15*) was used with the model of CA to construct a 95% confidence belt around the line of prediction. This provided a statistical basis to determine whether deviations from expectation were significant (those that fall outside the 95% confidence of the predicted curve) or simply due to natural variation in the biological response (those that fall within the 95% confidence of the predicted curve).

Materials and Methods

Test Organisms. Female juvenile rainbow trout (approximately three months old) were obtained from West Country Trout, Trafalgar Farm, Cornwall, U.K. (experiments I and II) and from Houghton Springs Fish Farm, Dorset, U.K. (experiment III). The body weight of the fish used was 10.47 ± 0.71 g (mean $\pm$ SEM; $n = 24$) in experiment I; 6.51 ± 0.48 g ($n = 24$) in experiment II; and 7.89 ± 0.22 g ($n = 24$) in experiment III. 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 light:8 h 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 provided with a feeding ration of 1% of body weight per day of Keystart Hatchery 1200 fish food pellets (BOCM Pauls Limited, Renfrew, U.K.).

Test Chemicals. Methoxychlor (99% purity) was purchased from ChemService, Greyhound, Birkenhead, U.K. (Lot 180-80A), technical grade NP (99% purity) was purchased from Acros, Fisher Scientific, Loughborough, U.K. (Lot A010020701), and E2 (98% purity) was purchased from Sigma, Poole, Dorset, U.K. (Lot 46H1146).

Water Supply and Test Apparatus. The supply of dechlorinated 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 204 to 238 μS cm^{-1}, the hardness from 41.3 to 47.7 mg L^{-1} (as $CaCO_3$), and free chlorine was < 2 μg L^{-1}. Alkalinity ranged from 21.2 to 27.8 mg L^{-1}, and ammonia (as $N-NH_3$) was <0.02 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 7.04 to 7.54. Water temperatures were monitored constantly throughout the exposure period and ranged from 15.2 to 15.8 °C. Dilution water and test chemical flow rates were checked at least three times per week. The flow-rate provided a 99% replacement time of approximately 7 h.

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, 7, and 14 of the exposures.

For measurement of E2, 2.5 L water samples were spiked with 5 ng L^{-1} deuterated-E2 and then extracted under vacuum (50 mL min^{-1}) onto preconditioned 47 mm C_{18} Envi-disks (Supelco). E2 was eluted from the disks using 30 mL of methanol, and the residual solvent was removed under a stream of nitrogen. The extracts were derivatized by heating to 120 °C for 20 min with 200 μL of pyridine and 300 μL of *N*-(*tert*-butyldimethylsilyl)-*N*-methylthrifluoroacetamide with 1% *tert*-butyldimethylchlorosilane (MTBSTFA + 1% TBDMCS). After cooling, 300 μL of bis(trimethysilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) was added, and the vial was heated to 60 °C for 20 min. The reagents were removed under nitrogen, and the extracts were resuspended in 250 μL of dichloromethane. The derivatized samples were analyzed on a Hewlett-Packard 6890 gas chromatograph (GC) coupled to a Hewlett-Packard 5973 mass spectrometer (MS), using helium as the carrier gas at 1 mL min^{-1}. The analysis conditions were as follows; sample volume, 1 μL; GC column, HP-5MS 30 m $\times$ 0.25 mm (id) fused silica with 0.25 μm film thickness; 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 impact ionization mode (70 eV) with selected ion monitoring (SIM) of the M-57 ions for the TBDMS/TMS derivative of E2 and its deuterated analogue, ions m/z 401 and 403, respectively.

For measurement of NP, 1 L water samples were extracted under vacuum (50 mL min^{-1}) onto preconditioned 47 mm C_{18} Empore disks (3M). NP was eluted from the disk using 10 mL of methanol, and the extract was diluted 1:1 with HPLC grade water. Extracts were analyzed on a HPLC, using a Jasco PU980 LC pump with a mobile phase composition of 80:20 methanol:water at 2 mL min^{-1}. The analysis conditions were as follows; sample volume, 50 μL; HPLC column, Hypersil H5ODS 150 mm $\times$ 4.6 mm (id) (Hichrom); Jasco FP920 fluorescence spectrometer detection at 230 nm (Ex) and 302 nm (Em).

For measurement of MXC, water samples were liquid/liquid extracted using hexane; extraction ratios for sample:hexane were 80:1 (controls), 10:1 (2.4−7.5 μg L^{-1} treatments), or 1:1 (13.5−24.0 μg L^{-1} treatment). Extracts were analyzed on a Varian 3400 GC, using nitrogen as the carrier gas at 35 mL min^{-1}. The analysis conditions were as follows: sample volume, 1 μL; GC column, 1 m $\times$ 2 mm (i.d.) column packed with 3% OV17 on 100/120 mesh (Phase Separations); injector temperature, 255 °C; column isothermal at 235 °C; electron capture detection at 300 °C.

Experimental Design. Fish were exposed to five concentrations of each of the individual chemicals and three concentrations of each binary mixture. Each experiment included a dilution water control (DWC) and a solvent control (SC). In all experiments, each treatment consisted of a single replicate containing 12 fish. The test vessels had a working volume of 45 L and were constructed of glass, with a minimum of other materials (silicon rubber tubing and adhesive) in contact with the test solutions.

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 0.040 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 0.01 mL methanol per liter.

For each experiment a "fixed ratio" design was used for the binary mixtures, in which the ratio of the two test chemicals was kept constant, while the total concentration of the mixture was varied. The ratio used for each experiment was selected on the basis of earlier work in which E2 was found to be approximately 1000-fold more potent than NP and MXC (*9*).

Experiment I − E2 + NP. Groups of 12 juvenile female rainbow trout were exposed for 14 days to nominal concentrations of E2 at 2.4, 4.2, 7.5, 13.5, and 24.0 ng L^{-1} and NP at 2.4, 4.2, 7.5, 13.5, and 24.0 μg L^{-1} and to binary mixtures

TABLE 1. Mean Measured Tank Concentrations of Test Chemicals, in the Individual and Binary Mixture Exposures over the 14 Days[a]

Experiment I. 17β-Estradiol and 4-*tert*-Nonylphenol

mean measured concn estradiol (ng/L)			mean measured concn nonylphenol (μg/L)		
nominal	individual	mixture	nominal	individual	mixture
2.4	2.3 ± 0.0		2.4	1.8 ± 0.4	
4.2	4.7 ± 0.2	4.9 ± 0.3	4.2	3.6 ± 0.3	3.3 ± 0.4
7.5	7.2 ± 0.2	7.4 ± 0.2	7.5	6.1 ± 0.7	6.1 ± 1.1
13.5	13.3 ± 0.4	12.6 ± 0.2	13.5	10.2 ± 0.6	10.2 ± 1.1
24.0	21.3 ± 0.3		24.0	12.2 ± 3.8	

Experiment II. 17β-Estradiol and Methoxychlor

mean measured concn estradiol (ng/L)			mean measured concn methoxychlor (μg/L)		
nominal	individual	mixture	nominal	individual	mixture
2.4	4.6 ± 1.2		2.4	1.4 ± 0.1	
4.2	4.2 ± 0.0	4.3 ± 0.0	4.2	4.4 ± 0.3	3.8 ± 0.2
7.5	7.9 ± 0.4	7.2 ± 0.1	7.5	4.6 ± 0.3	5.7 ± 0.4
13.5	12.6 ± 0.1	12.8 ± 0.0	13.5	13.0 ± 0.3	13.2 ± 0.8
24.0	23.0 ± 0.2				

Experiment III. 4-*tert*-Nonylphenol and Methoxychlor

mean measured concn nonylphenol (μg/L)			mean measured concn methoxychlor (μg/L)		
nominal	individual	mixture	nominal	individual	mixture
2.4	1.8 ± 0.1		2.4	2.0 ± 0.1	
4.2	2.9 ± 0.2	3.0 ± 0.3	4.2	4.3 ± 0.1	3.5 ± 0.2
7.5	6.4 ± 0.4	6.3 ± 0.6	7.5	6.5 ± 0.2	6.8 ± 0.5
13.5	11.0 ± 0.7	11.9 ± 0.7	13.5	14.3 ± 2.1	11.4 ± 1.2
24.0	16.3 ± 2.0		24.0	20.2 ± 0.7	

[a] Data as means ± SEM (n = 3).

of E2 + NP at concentrations of 4.2 ng L^{-1} + 4.2 μg L^{-1}, 7.5 ng L^{-1} + 7.5 μg L^{-1}, and 13.5 ng L^{-1} + 13.5 μg L^{-1}, respectively.

Experiment II − E2 + MXC. Groups of 12 juvenile female rainbow trout were exposed for 14 days to nominal concentrations of E2 at 2.4, 4.2, 7.5, 13.5, and 24.0 ng L^{-1} and MXC at 2.4, 4.2, 7.5, 13.5, and 24.0 μg L^{-1} and to binary mixtures of E2 + MXC at concentrations of 4.2 ng L^{-1} + 4.2 μg L^{-1}, 7.5 ng L^{-1} + 7.5 μg L^{-1}, and 13.5 ng L^{-1} + 13.5 μg L^{-1}, respectively.

Experiment III − MXC + NP. Groups of 12 juvenile female rainbow trout were exposed for 14 days to nominal concentrations of MXC at 2.4, 4.2, 7.5, 13.5, and 24.0 μg L^{-1} and 4-NP at 2.4, 4.2, 7.5, 13.5, and 24.0 μg L^{-1} and to binary mixtures of MXC + NP at concentrations of 4.2 μg L^{-1} + 4.2 μg L^{-1}, 7.5 μg L^{-1} + 7.5 μg L^{-1}, and 13.5 μg L^{-1} + 13.5 μg L^{-1}, respectively.

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}) of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) (Sigma), buffered with 1 M NaOH to pH 7.3. Blood was collected by cardiac puncture, using a heparinized syringe (5000 Units heparin mL^{-1}) and centrifuged (7000g; 5 min, 15 °C), and the plasma was removed and stored at −80 °C until required for VTG analysis. Plasma samples were assayed for VTG using an established homologous rainbow trout RIA (*16*).

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

$$f(x) = \theta_{min} + \frac{\theta_{max} - \theta_{min}}{1 + \exp(-\theta_1 - \theta_2 * \log_{10}(x))} \text{ if } x > 0$$

where x = concentration and $f(x)$ = mean effect.

The model parameter θ_{min} describes the minimal mean effect (control response), θ_{max} is the asymptotical maximal effect, θ_1 is termed the "location" parameter, and θ_2 characterizes the "steepness" of the concentration response relationship. The experiments were not designed to determine maximal effects, so estimation of θ_{max} contains a high degree of statistical uncertainty. Due to heterogeneous nonrandom variabilities in the replicated data (heteroscedasticity), each model was fitted using the estimation method of generalized least squares (*15*). To fulfill the statistical prerequisite of symmetrically distributed effect data for this estimation method, the plasma VTG concentrations were log10-transformed. LOECs were determined using a nonparametric Wilcoxon's rank sum test (*17*).

The expected concentration−response relationships of the binary mixtures were determined using the model of CA (*12*). The model can only be used to calculate mixture effects for the same effect range observed for the individual components of the mixture, and if these effects are quantitatively describable in a statistically valid way. Therefore, the expected curve of the binary mixtures can only be calculated up to an effect range which is determined by the minimum of the two estimated model parameter θ_{max} of both compounds. The concentration−response curve for VTG induction is very steep, covering several orders of magnitude (ng/mL to mg/mL) and often results in a high variability in the vitellogenic response between individuals in a treatment. Such an inherent variability can complicate any analysis on exposure to individual or mixtures of chemicals. For this reason the "bootstrap" methodology (*15*) was employed with the model of CA, to determine the statistical accuracy of the predicted combined effects.

Results and Discussion

Measured Concentrations of the Test Chemicals. Mean measured tank concentrations of the individual chemicals

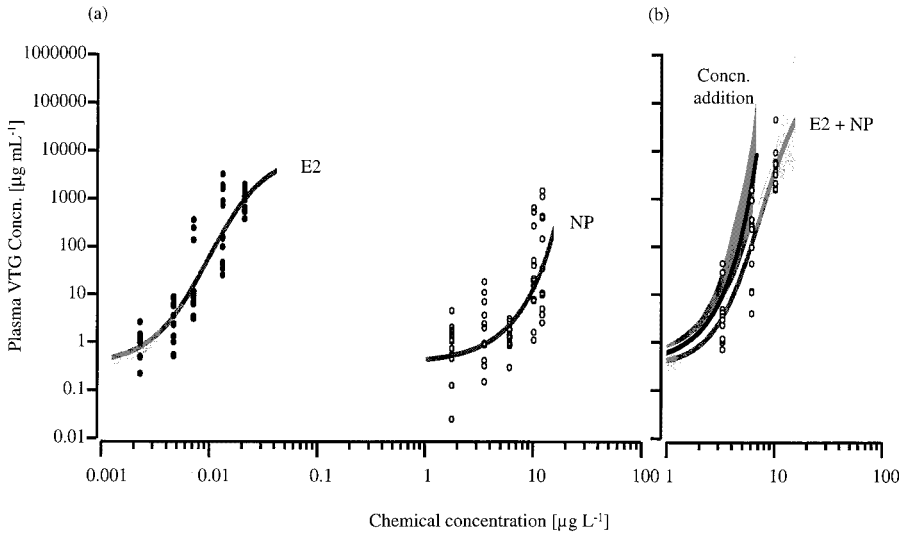

FIGURE 1. Plasma vitellogenin (VTG) concentrations in female juvenile rainbow trout exposed to (a) estradiol-17β (E2) (closed circles) and 4-*tert*-nonylphenol (NP) (open circles) and (b) fixed ratio binary mixtures (1:1000) of E2 and NP (open circles). In some cases the plasma VTG concentrations were very similar between fish within a treatment; therefore, not all data points are visible. For each of these exposures the 95% confidence belts (light gray shaded regions) of the fitted concentration−response relationships (gray lines) are shown. The expected vitellogenic response for the binary mixture, calculated using the model of concentration addition, is shown as a black line (b) with a 95% confidence bootstrap belt (dark gray shaded region).

and their binary mixtures are given in Table 1. All exposure concentrations are described using means of the actual measured concentrations. For most chemical exposures the mean measured concentrations were between 70 and 110% of nominal. However, for the highest concentration of NP, 24 μg L^{-1}, the mean measured concentration was between 51% and 68% of nominal. In experiment II (E2 + MXC), the mean measured concentration of E2 was 190% of nominal for the lowest test concentration (2.4 ng L^{-1}), and the mean measured concentrations of MXC were between 60% and 105% of nominal.

Plasma Concentrations of VTG in Control Fish. The concentrations of VTG in the plasma of juvenile female fish at the onset of the experiments were 500 ± 80 ng mL^{-1}, 310 ± 50 ng mL^{-1}, and 540 ± 80 ng mL^{-1} for experiments I, II, and III, respectively. There were 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 ($p > 0.05$).

Mixtures of E2 and NP. There were no mortalities in fish exposed to E2 and NP or in fish exposed to binary mixtures of these chemicals. Estradiol-17β (concentrations ranging from 2.3 to 21.3 ng L^{-1}) and NP (concentrations ranging from 1.8 to 12.2 μg L^{-1}) produced concentration-dependent increases in plasma VTG (Figure 1), with LOECs of 4.7 ng L^{-1} (plasma VTG concentration of 4100 ± 940 ng mL^{-1}, $p < 0.05$) and 6.1 μg L^{-1} (plasma VTG concentration of 1450 ± 230 ng mL^{-1}, $p < 0.05$) for E2 and NP, respectively. The mixture of E2 and NP, at a fixed 1:1000 ratio, also produced a concentration-dependent increase in plasma VTG, with the lowest mixture concentration tested (4.9 ng L^{-1} E2 and 3.3 μg L^{-1} NP) inducing a 17-fold increase in VTG concentration (8720 ± 4370 ng mL^{-1}, $p < 0.05$). The observed VTG induction data for the mixture of E2 and NP was close to that predicted by the model of CA (Figure 1). The 95% confidence belt of the fitted concentration−response relationship for the observed mixture data overlapped with the 95% confidence bootstrap belt for the calculated mean of CA, for the whole effect range tested.

For many estrogen mimics the pathways by which they alter estrogen-sensitive pathways have yet to be fully characterized. NP has been shown to have multiple mechanisms of action on the endocrine system; it acts as both an estrogen and as an antiandrogen in male fathead minnows (*18*) and in recombinant yeast reporter gene assays (*3*), and it also affects some of the cytochrome p450 mixed function oxidase enzymes (*19*). Some estrogenic activity of NP may also result from alterations it can cause in concentrations of endogenous E2, rather than by effects mediated via the ER directly (*20*). The concentration additive behavior of NP with E2 in these experiments, however, together with the ability of NP to bind the ER (but not the AR) in rainbow trout liver and brain cells (*21*), suggests that the VTG response induced by NP is mediated via the ER alone. This may also be true for the related alkylphenolic chemical, octylphenol, which has been shown to act in an additive manner with E2, in inducing VTG synthesis in rainbow trout (*22*).

Mixtures of E2 + MXC. For fish exposed to 13.0 μg L^{-1} MXC there was a 58% mortality by day 14, and for fish exposed to the mixture of 13.2 μg L^{-1} MXC + 12.8 ng L^{-1} E2 there was a 25% mortality. In all other chemical exposures, 100% of the fish were alive at the end of the experiment. A previous study reported a 96 h median lethal concentration for MXC of 31.2 μg L^{-1} in juvenile rainbow trout (*23*). The toxicity of MXC has been demonstrated to increase with duration of exposure, and this may account for the apparent greater toxicity of MXC observed in this study, compared with Heming et al. (*23*).

In experiment II, E2 (concentrations ranging from 4.2 to 23.0 ng L^{-1}) and MXC (concentrations ranging from 1.4 to 13.0 μg L^{-1}) produced concentration-dependent increases in plasma VTG (Figure 2), with LOECs of 7.9 ng L^{-1} for E2 (plasma VTG concentration of 22 460 ± 8660 ng mL^{-1}, $p < 0.05$) and 4.4 μg L^{-1} for MXC (plasma VTG concentration of 9340 ± 6590 ng mL^{-1}, $p < 0.05$). The mixture of E2 and MXC, at a fixed 1:1000 ratio, also produced a concentration-dependent increase in plasma VTG (Figure 2), with the lowest mixture concentration tested (4.3 ng L^{-1} E2 and 3.8 μg L^{-1} MXC) inducing a plasma VTG concentration of 18340 ± 7680 ng mL^{-1} ($p < 0.05$). When comparing the measured VTG induction that occurred for the mixture, with the expected mixture effects, calculated according to the model of concentration addition, mixtures of E2 and MXC were shown to act in a less than additive manner (Figure 2). The concentration−response curve for the experimental mixture of E2 and MXC is displaced to the right of the predicted

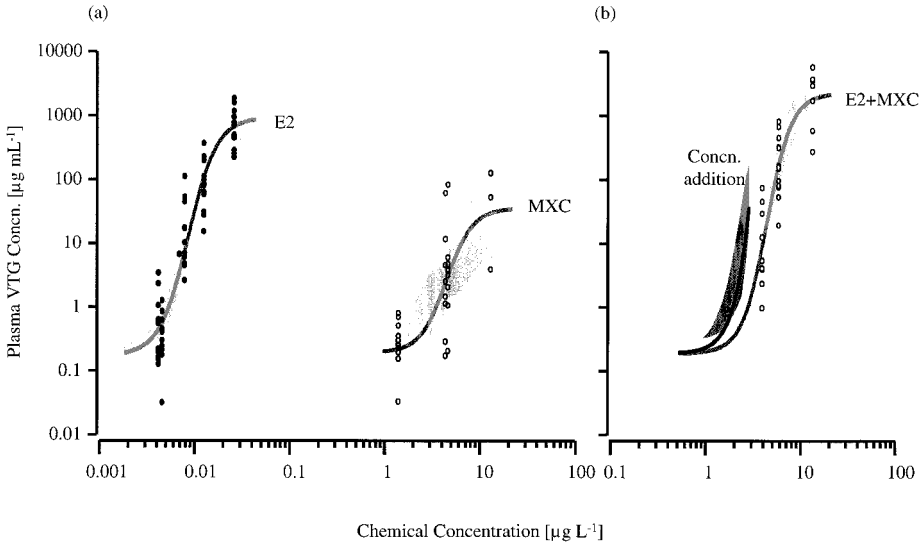

FIGURE 2. Plasma vitellogenin (VTG) concentrations in female juvenile rainbow trout exposed to (a) estradiol-17β (E2) (closed circles) and methoxychlor (MXC) (open circles) and (b) fixed ratio binary mixtures (1:1000) of E2 and MXC (open circles). In some cases the VTG concentrations were very similar between fish within a treatment; therefore, not all data points are visible. For each of these exposures the 95% confidence belts (light gray shaded regions) of the fitted concentration−response relationships (gray lines) are shown. The expected vitellogenic response for the binary mixture, calculated using the model of concentration addition, is shown as a black line (b) with a 95% confidence bootstrap belt (dark gray shaded region).

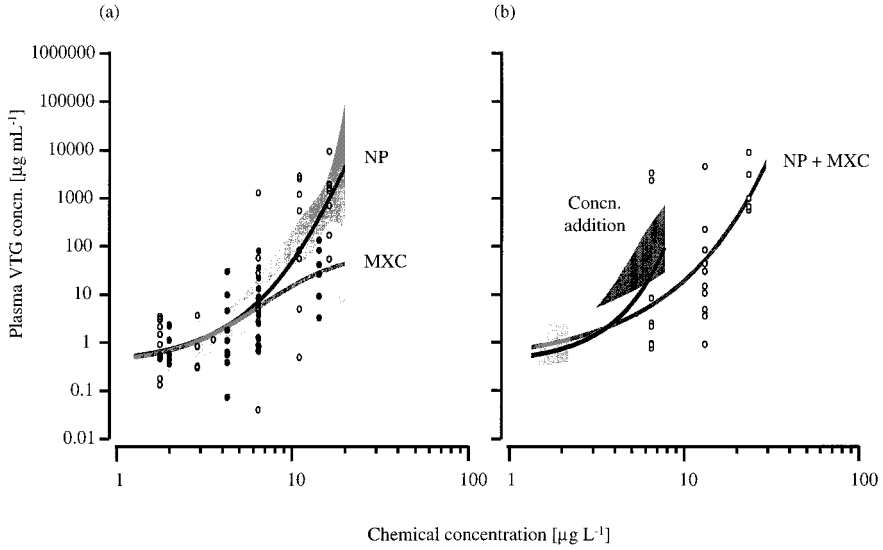

FIGURE 3. Plasma vitellogenin (VTG) concentrations in female juvenile rainbow trout exposed to (a) methoxychlor (MXC) (closed circles) and 4-*tert*-nonylphenol (NP) (open circles) and (b) fixed ratio binary mixtures (1:1) of MXC and NP (open circles). In some cases the VTG concentrations were very similar between fish within a treatment; therefore, not all data points are visible. For each of these exposures the 95% confidence belts (light gray shaded regions) of the fitted concentration−response relationships (gray lines) are shown. The expected vitellogenic response for the binary mixture, calculated using the model of concentration addition, is shown as a black line (b) with a 95% confidence bootstrap belt (dark gray shaded region).

concentration−response curve, and there is no overlap between the two 95% confidence belts for the whole effect range tested.

The model of CA is based on the assumption that the components of a mixture mediate their effects by similar modes of action. The small but significant deviation from additivity for the mixture of E2 and MXC suggests that MXC mediated a vitellogenic response via a different mechanism (or pathway) to E2. This complies with reports in the literature that MXC does not interact directly with the ER, in the same way as E2. The estrogenic activity of MXC is thought to result from its primary metabolite, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is produced in the liver (by O-demethylation) (*24*). In mammals, HPTE has been found to act as an agonist for ERα but as an antagonist for ERβ (*25*). Although, ERβ has not yet been identified in the rainbow trout, it has been identified in another species of teleost, the seabream (*26*). In the seabream, ERα and ERβ are coexpressed in the liver, although whether they are both involved in the regulation of vitellogenesis has still to be investigated. If, however, both ERα and ERβ are present in the liver of rainbow trout and both play a role in the regulation of vitellogenesis, then the mixed agonist/antagonist activity of HPTE, in the presence of E2, might well account for the observed deviation from additivity in this study.

Mixtures of NP + MXC. There was 100% mortality of fish exposed to MXC at a concentration of 20.2 μg L^{-1} after 6 days and 50% mortality in fish exposed to 14.3 μg MXC L^{-1} after 14 days. In fish exposed to the binary mixture of 11.4 μg L^{-1} MXC + 11.9 μg L^{-1} NP, there was a 58% mortality after 14

days, but in all other chemical exposures all of the fish were alive at the end of the experiment.

NP (concentrations ranging from 1.8 to 16.3 $\mu g\ L^{-1}$) and MXC (concentrations ranging from 2.0 to 20.2 $\mu g\ L^{-1}$) produced concentration-dependent increases in plasma VTG (Figure 3), with LOECs of 6.4 $\mu g\ L^{-1}$ for NP (plasma VTG concentration of 128 760 ± 117 540 ng mL^{-1}, $p < 0.05$) and 6.5 $\mu g\ L^{-1}$ for MXC (plasma VTG concentration of 16 390 ± 7160 ng mL^{-1}, $p < 0.05$). The mixture of NP and MXC, at a fixed 1:1 ratio, produced a concentration-dependent increase in plasma VTG, with the lowest mixture concentration tested (3.0 $\mu g\ L^{-1}$ NP and 3.5 $\mu g\ L^{-1}$ MXC) inducing a plasma VTG concentration of 723 055 ± 481 814 ng mL^{-1} ($p < 0.05$). Determination of the predicted effects of the binary mixture on the basis of concentration addition depends on the concentration−response curves derived for the individual compounds. Due to the toxicity of MXC in our study, only a partial concentration−response curve was obtained, restricting the effect range for which the expected response curve for the binary mixture could be calculated (Figure 3). At the lowest mixture concentration tested (4.2 $\mu g\ L^{-1}$ NP + 4.2 $\mu g\ L^{-1}$ MXC), the 95% confidence belt of the fitted concentration−response relationship for the observed mixed data overlapped with the 95% confidence bootstrap belt for the calculated mean of CA, suggesting that NP and MXC were acting in an additive manner. However, it was not possible to confirm or negate this for mixtures of NP and MXC at the higher concentrations tested (because of fish mortality). On the basis of the findings for the mixture of E2 and MXC, the mixture of NP and MXC, would be expected to be less than additive. Having said this, the effects of NP on cytochrome P450 mixed function oxidase enzymes (*19*) might affect the conversion of MXC to HPTE and so affect the estrogenic activity of the mixture of NP and MXC.

In each of the experiments, VTG induction occurred at the lowest mixtures concentrations tested, even when the components of the mixtures were present at concentrations below their individual LOECs. These binary mixture concentrations, at which effects on plasma VTG occurred, reflect environmentally relevant concentrations of the chemicals in some aquatic environments. Surveys of domestic effluents in the UK have detected concentrations of E2 ranging from 1 to 88 ng L^{-1} (*10*, *27*, *28*) and concentrations of NP ranging from 1 to 5 $\mu g\ L^{-1}$ (*11*, *28*). These effluents discharge directly into rivers, where measured concentrations range from 1 to 5 ng L^{-1} for E2 (*11*) and from 1 to 2.3 $\mu g\ L^{-1}$ for NP (*11*). MXC is no longer used in Europe, but it is still used extensively in the USA and Canada as well as in many third world countries, to control biting fly larvae. Due to the large number of these and other oestrogenic chemicals present in the aquatic environment, additivity at concentrations below the LOECs for the individual chemicals needs to be considered when assessing the possible implications of endocrine active chemicals in the aquatic environment. The estrogenic potency of mixtures of chemicals discharged into the environment is, therefore, important in any risk assessment strategy. Although the applicability of the concept of CA has so far not been applied to the field of endocrine disruption, the data from these experiments suggest that it is suitable for the prediction of combination effects, where the components of the mixture act through the same mechanism of action on a clearly defined endpoint. The model accurately predicted the combination effects on VTG induction for the mixture of E2 and NP, where we know that both chemicals act via the ER in mediating a vitellogenic response. For the mixture of E2 and MXC the model of CA overpredicted the combination effect, but this is perhaps not surprising given the complex mechanism by which MXC has been reported to elicit an estrogenic response (*24*, *25*). Our studies further highlight the difficulties in assessing combination effects of even simple mixtures of environmental estrogens, on a clearly defined endpoint with a known mechanism of action.

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