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Identification of Estrogenic Chemicals in STW Effluent. 2. In Vivo Responses in Trout and Roach

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The occurrence of certain natural and synthetic steroidal estrogens in the final effluent from STW has been demonstrated. 17β-Estradiol and estrone were present at concentrations in the tens of nanograms per liter range, and the synthetic estrogen 17α-ethynylestradiol was also identified, albeit in the low nanogram per liter range. The findings from subsequent in vivo tank trial experiments, in which adult male rainbow trout (*Oncorhynchus mykiss*) and adult roach (*Rutilus rutilus*) were exposed for 21 days via the water to environmentally relevant concentrations of 17β-estradiol and estrone are presented. In addition, the response of adult male and female roach following exposure to 17β-estradiol (1, 10, and 100 ng/L) was compared to the response to the alkylphenolic xenoestrogen, 4-*tert*-octylphenol (1, 10 and 100 μg/L). Plasma levels of vitellogenin were determined using previously validated radioimmunoassays in order to measure the estrogenic response of the fish to the varying concentrations of the compounds tested. The results indicate that environmentally relevant concentrations of natural steroidal estrogens are sufficient to account for the levels of vitellogenin synthesis observed in caged male fish placed downstream of certain STW effluent discharges in British rivers.

Introduction

Advances in civilization coupled with rising population levels have resulted in an increasing need to treat and recycle available water resources. It is estimated that 30% of all U.K. water is of a recycled nature (*1*). Following reuse, the water is returned to the aquatic environment, usually via sewage-treatment works (STWs) of varying processes and performance, which improve its quality, but it may be abstracted again further downstream. In the U.K. and other countries with a high population density, the volume of effluent discharged from STWs can be considerable, sometimes contributing up to 50% of the flow of a river, a figure that can rise as high as 90% in periods of low rainfall.

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The consequence of modern water management strategies (with the possibility of enrichment of certain compounds in the water, e.g., alkylphenols and steroids) to both consumers and the wildlife has not been established. However, casual observations by anglers in the mid-1980s of hermaphrodite roach (*Rutilus rutilus*) inhabiting STW lagoons, which were subsequently confirmed by a follow-up survey, stimulated a number of studies using caged rainbow trout that identified effluent from STWs as being estrogenic (*2*). In these studies, caged male rainbow trout were maintained in STW discharges, and the effluent was tested for its ability to stimulate the production of the female egg yolk protein vitellogenin (VTG) in these exposed fish.

Vitellogenin production in male fish is a sensitive biomarker to estrogenic contamination (*3*), and the presence of high levels of VTG in male fish exposed to domestic STW effluents was indicative of an environmental source of estrogen exposure. British rivers receiving STW effluent are not unique in terms of their ability to stimulate VTG production in male fish (*4, 5*), as similar findings have been reported in wild populations of male carp (*Cyprinus carpio*) captured near a major urban sewage treatment plant in the United States (*6*).

Until recently, informed speculation has brought about much of the stimulus for research into the likely chemicals implicated in contributing to the observed estrogenic activity of STW effluent. Domestic sewage and industrial effluents are highly complex mixtures in terms of their chemical composition. Despite this, the main active component of the oral contraceptive pill (17α-ethynylestradiol), as well as alkylphenols and their short-chain polyethoxylates (final biodegradation products of nonionic surfactants), were implicated as potential contributors (*2, 7*).

We now report (in an accompanying paper) the findings from the analysis of seven domestic effluents that were chemically fractionated and screened for biological activity using an estrogen screen based on recombinant yeast (*8*). 17β-Estradiol (E2) and estrone (E1) were identified at concentrations in the tens of nanograms per liter (from approximately 4 to 48 ng of E2/L and 1 to 76 ng of E1/L), and the synthetic estrogen 17α-ethynylestradiol (EE2) was also identified, albeit at the low nanogram per liter range (from nondetectable to approximately 7 ng of EE2/L). The presence of steroids and steroid-like compounds in water has also been reported elsewhere. For example, analysis of municipal wastewater-digested sludges from the Lyon and Paris areas (France) identified the fecal sterol coprostanol (formed by bacterial reduction of cholesterol in the intestine of higher animals) at concentrations ranging from 2.28 to 4.05 mg/g (*9*). Moreover, the presence of synthetic (EE2 and diethylstilbestrol) and steroidal estrogens (17β-estradiol, estrone, and estriol) in STW effluent, river water, and/or drinking water has been reported to occur at the low nanogram up to the tens of nanograms per liter range (*10*−*13*).

Relatively little is known about the sensitivity of any fish species to estrogens (both natural and synthetic), particularly when the chemical is administered via the water, which is the most realistic and environmentally relevant route of exposure. Until now, the preferred route of chemical exposure in fish has usually been by injection, as the exposure is relatively easy to define, without the need for complex analytical chemistry. On the basis of the current literature, it was impossible to determine whether the concentrations of 17β-estradiol and estrone identified in the previous study (*8*) would be estrogenic to fish. In addition, little is known about species differences in sensitivity to estrogen exposure.

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To address these issues and to put the results of the fractionation studies into an environmental context, laboratory studies were conducted in which rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) were exposed to low concentrations of estrogenic chemicals. In addition, the response of adult male and female roach following exposure to 17β-estradiol was compared to the response to the alkylphenolic xenoestrogen, 4-*tert*-octylphenol. In this paper, we demonstrate that environmentally relevant concentrations of natural steroidal estrogens, administered via the water, are sufficient to stimulate vitellogenin synthesis in both species of fish and may account for the biological activity observed in caged male trout exposed to certain domestic STW effluents.

Materials and Methods

The data presented are the product of two separate experiments. The objective of the first experiment was to compare the relative sensitivities of the rainbow trout (an introduced species from North America) and the roach (an indigenous species) to the main natural estrogen, 17β-Estradiol, and the alkylphenolic xenoestrogen, 4-*tert*-octylphenol. The main objective of the second experiment was to determine the sensitivity of trout to estrone. A secondary objective was to assess whether interactive effects could occur if the trout were exposed simultaneously to 17β-estradiol and estrone (as would occur when the fish were exposed to effluent).

General Experimental Approach. Groups of adult male rainbow trout (*Oncorhynchus mykiss*) ($n = 10$) and/or adult roach (*Rutilus rutilus*) ($n = 20-30$) were held in large glass tanks (500 L) that received a continuous flow-through of borehole water, reconstituted with a standard addition of salts (*14*) after reverse osmosis filtration. The larger sample number of roach was included in order to obtain at least 10 males, as these fish cannot be sexed by external features alone unless they are sexually mature. For each concentration and treatment, both fish species were maintained in a single tank (although physically separated) and therefore received an identical exposure, which was necessary if a valid comparison of their sensitivities was to be obtained. Separation was necessary because rainbow trout may be aggressive toward roach. Throughout the experiments, the fish were fed daily to satiation. The trout were fed with BP Nutrition size 40 expanded pellets, and the roach were fed with uncolored blow fly larvae. Before the fish were introduced, the experimental aquaria were allowed a period to equilibrate in order for the concentrations of the chemicals in the tanks to stabilize, and samples were periodically taken to ensure that the concentrations were as expected. Appropriate control tanks (water only, water plus the methanol used to dissolve the compounds) were also included.

17β-Estradiol (E2), estrone (E1), and 4-*tert*-octylphenol (OP) were >99% pure and were purchased from Sigma Chemical Company (Dorset, U.K.). All stock solutions were prepared in methanol, and these were then added to a glass mixing vessel by means of a peristaltic pump at a flow rate of 0.1 mL/min where they were combined with reconstituted borehole water flowing into the tanks at a rate of 3 L/min. The final methanol concentration in the tanks was below 0.05 mL/L.

Determination of Steroid Levels in Tank Water. Where possible, the levels of the test compounds were monitored both before and throughout all the experiments using GC-MS analysis. The 2-L volumes (for nominal steroid concentrations $\geq$ 25 ng/L) or 20-L volumes (for nominal steroid concentrations < 25 ng/L) of tank water were spiked with a d_2-17α-ethynylestradiol internal standard (25 ng/L). HPLC grade methanol was also added to the water (to a final concentration of 0.5%) to aid extraction of the steroids onto a preconditioned C18 SPE cartridge (5 g). A glass wool filter was attached upstream of the C18 cartridge to remove particulates. The sample vessels were pressurized using compressed air in order to force the sample through the cartridge at a flow rate of 10 mL/min. After extraction, the cartridge was dried by drawing through a stream of air for 15 min, wrapped in solvent-rinsed (HPLC grade ethyl acetate) aluminum foil, and stored at −20 °C prior to analysis.

The 5-g C18 cartridge was allowed to defrost for 2 h at room temperature in a fume hood, after which it was rinsed with 5 mL of 25:75 methanol:water to remove polar contaminants from the cartridge. The components on the cartridge were then eluted with 2 × 10 mL of dichloromethane (DCM). The DCM sample was dried with anhydrous sodium sulfate and reduced in volume (by rotary evaporation, followed by a gentle blow down under a stream of nitrogen) to 200 μL. Extracts were analyzed by GC−MS in full scan mode, and steroids were quantified using the masses described previously (*8*). In additon to the samples, a series of response factor samples, blank samples, and QC samples were run to assist in sample quantitation and method validation.

Determination of Octylphenol Levels in Tank Water. A 200-mL sample of water was collected from the tank water into a 1-L glass bottle. Butylphenol (internal standard) was then added to the sample at a final concentration of 20 ng/L. One milliliter of methanol (HPLC grade) was then added to the sample to aid solid-phase extraction. Following equilibriation (30 min), the sample was extracted using solid-phase extraction onto 500 mg of C18(EC) IST Isolute columns (Jones Chromatography, Hengoed, Wales, U.K.). The cartridge was dried for 10 min by drawing through air, wrapped in solvent-rinsed aluminum foil (HPLC grade ethyl acetate), and stored at −20 °C prior to analysis.

The 500-mg C18 cartridge was defrosted (1 h at room temperature in a fume hood) prior to elution with 2.5 mL of ethyl acetate, followed by 2.5 mL of DCM. The elutriate was dried with anhydrous sodium sulfate and reduced in volume (by rotary evaporation, followed by a gentle blow down under a stream of nitrogen) to 200 μL. Extracts were analyzed by GC−MS in full scan mode using a previously validated procedure (*15*). In addition to the samples, a series of response factor samples, blank samples, and QC samples were run to assist in sample quantitation and method validation.

Blood Sampling and Radioimmunoassays. Blood samples were taken by caudal venipuncture from all the rainbow trout, both initially and at the end of the 3-week period of exposure, but the roach were considered to be too small to withstand multiple blood sampling. Therefore, no initial blood samples were taken from the roach, but after 21 days, all the exposed fish were blood sampled. In addition, a single group of roach and trout were blood sampled prior to the commencement of the experiment to establish initial values for the parameters measured. Blood plasma was assayed for vitellogenin (VTG) content using previously validated radioimmunoassays (*16, 17*). The Gonadosomatic Index (GSI; gonad weight expressed as a percentage of the total body weight) was also determined in both trout and roach.

Experimental Design. In the first experiment, both species of fish were exposed for 21 days to either E2, at nominal concentrations of 1, 10, and 100 ng/L, or to the xenoestrogen OP, at nominal concentrations of 1, 10, and 100 μg/L. In the second experiment, rainbow trout were exposed for 21 days to E1 at nominal concentrations of 6.25, 12.5, 25, 50, and 100 ng/L, E2 alone, and a combination of E1 and E2, all at nominal concentrations of 25 ng/L. A group of fish exposed to 25 ng of E2/L was also included to allow a comparison of the potency between E1 and E2, while the inclusion of a group of fish exposed to both natural estrogens

TABLE 1. Concentrations of Octylphenol and 17β-Estradiol in Tank Water Samples (First Experiment)[a]

tank tested	nominal concentration	measured concn 19/2/96	11/3/96	18/3/96	25/3/96*	1/4/96	8/4/96
1	0	0	< 0.2 μg/L				
5	1 μg of OP/L	1.4 μg/L	0.6 μg/L				
2	10 ng of E2/L						
4	1 ng of E2/L						
7	10 μg of OP/L	11.3 μg/L	6 μg/L				
3	100 μg of OP/L, inlet		105 μg/L	125 μg/L	74 μg/L	106 μg/L	145 μg/L
3	100 μg of OP/L outlet		149 μg/L	145 μg/L	49 μg/L	90 μg/L	114 μg/L
8	100 ng of E2/L		45 ng/L				

[a] An asterisk (*) denotes when the fish were added to the tanks.

TABLE 2. Concentration of Estrone and 17β-Estradiol in Tank Water Samples (Second Experiment)[a]

tank tested	nominal concn	measured concn 3/6/96	6/6/96	14/6/96
control	0	0	0	0
8	100 ng of estrone/L	96 ng/L	60 ng/L	44 ng/L
7	50 ng of estrone/L	48 ng/L	51 ng/L	46 ng/L
3	25 ng of estrone/L	36 ng/L	26 ng/L	24 ng/L
2	25 ng of estrone/L	18 ng/L	17 ng/L	
	+	+	+	
	25 ng of 17β-estradiol/L	*	13 ng/L	
4	25 ng of 17β-estradiol/L	14 ng/L	17 ng/L	
5	12.5 ng of estrone/L	*	14 ng/L	
6	6.25 ng of estrone/L	*	4 ng/L	

[a] An asterisk (*) indicates no recovery.

was intended to assess whether additivity or synergism could occur between these two steroids.

Statistical Analyses. All statistical analysis of the data was performed using STATVIEW 4.1 (Abacus Concepts, Inc, USA). Statistical analysis of the normalized data was performed using a one-way analysis of variance to assess the effects of different treatments on vitellogenin concentration. This was followed by Scheffe's test for multiple comparisons. A paired Students' *t*-test was used to compare pre- and post-samples within a single treatment (trout only).

Results

Actual Concentrations of OP, E2, and E1 in the Test Aquaria. Table 1 presents the actual concentrations of OP and E2 that were measured in the aquaria both before and during the first experiment. In all test aquaria, the actual concentrations achieved were close to the anticipated nominal concentrations and remained stable throughout the duration of the experiment. Table 2 presents the data on the actual concentrations of E1 and/or E2 in the test aquaria during the second experiment. In nearly all cases, the actual concentrations measured were close to the nominal concentrations with the largest disparity occurring in tank 8, in which the actual concentration of E1 (44 ng/L) when the fish were introduced was only half the nominal concentration (100 ng/L).

Exposure of Rainbow Trout to 17β-Estradiol. Figure 1 illustrates that exposure of male rainbow trout (mean weight and GSI ± SEM; 249.9 ± 9.7 g and 0.644 ± 0.062, respectively) to varying doses of E2 produced a clear dose-related increase in vitellogenin production. As expected, the initial (preexposure) VTG levels were very low, averaging around 50 ng/mL. Only the highest concentration of E2 (100 ng/L) produced a response (mean ± SEM; 2.6 ± 0.5 mg/mL) that was significantly above ($p < 0.0001$) the methanol control postexposure group. Although the 10 ng of E2/L exposure

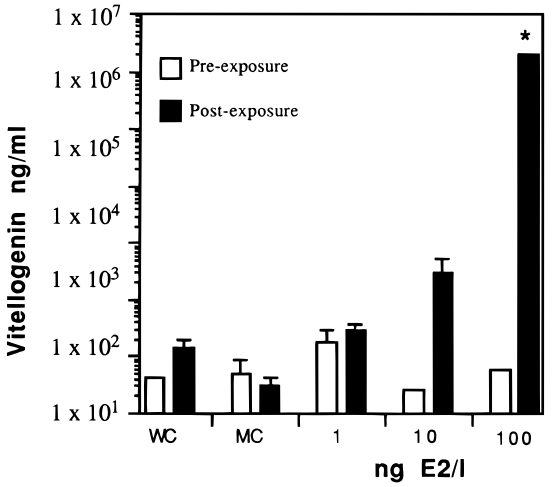

FIGURE 1. Effect of different concentrations of 17β-estradiol (E2) on vitellogenin synthesis in rainbow trout exposed for 3 weeks. Values shown are the mean concentrations ($n = 10$) of vitellogenin in the pre- and postexposure blood plasmas. The error bars represent the standard errors of the mean (± SEM). Also included are the water control (WC) and the methanol control (MC). An asterisk (*) denotes significant differences from the methanol postexposure group at $p < 0.0001$. Note that in all the figures the vitellogenin concentrations are plotted on a log scale due to the great range of values measured.

group also appears to have produced a response greater than the methanol control postexposure group, this response was not statistically significant despite identical sample sizes. This was due to the large variability in the vitellogenic response (ranging from around 30 to approximately 5000 ng/mL), which occurred at this threshold concentration. In the 10 ng of E2/L exposure group, only three fish reponded (of which two fish produced vitellogenin levels >1000 ng/mL) with the remaining seven maintaining basal VTG levels of around 30 ng/mL. Despite this, the magnitude of the variability in response (illustrated by the error bars) appears to be small due to the use of a logarithmic scale on the vertical axis. Nevertheless, when the pre- and postexposure samples of each fish were compared within each group, the 10 ng/L postexposure group (1.4 ± 0.8 μg of VTG/mL) was also significantly elevated ($p < 0.05$) above the preexposure level (22 ± 5 ng of VTG/mL), indicating that for a 3-week exposure the estimated threshold response probably occurred at a concentration between 1 and 10 ng of E2/L.

Exposure of Male and Female Roach to 17β-Estradiol. Figure 2 illustrates that exposure of male roach (mean weight and GSI ± SEM; 13.4 ± 0.3 g and 1.022 ± 0.06, respectively) to varying doses of E2 also produced a clear dose-related increase in VTG production. As expected, the initial (pre-exposure) VTG levels in the male roach were low, averaging around 80 ± 10 ng/mL (mean ± SEM). The magnitude of

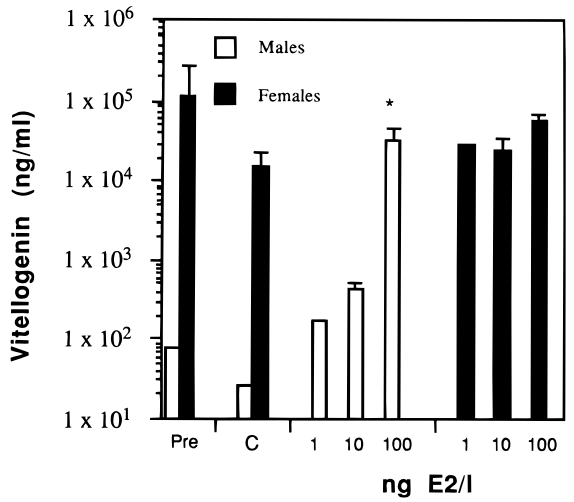

FIGURE 2. Effect of different concentrations of 17β-estradiol (E2) on vitellogenin synthesis in male and female roach exposed for 3 weeks as compared to the control (C). Values shown are the mean concentrations of vitellogenin in the blood plasma samples. The error bars represent the standard error of the mean (± SEM). Also included are the vitellogenin levels from a single group of roach that were blood sampled prior to the onset of the experiment (Pre) in order to establish initial vitellogenin levels. An asterisk (*) denotes significant differences from the control postexposure group at $p <$ 0.0001.

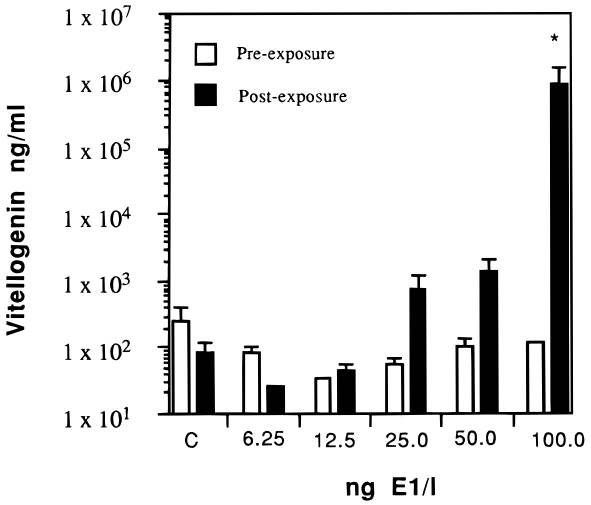

FIGURE 3. Effect of different concentrations of estrone (E1) on vitellogenin synthesis in rainbow trout exposed for 3 weeks as compared to the control (C). Values shown are the mean concentrations ($n = 10$) of vitellogenin in the pre- and postexposure blood plasmas. The error bars represent the standard error of the mean (± SEM). An asterisk (*) denotes significant differences from the control postexposure group at $p <$ 0.0001.

the vitellogenic response in male roach was smaller than that observed in rainbow trout. For example, after a 3-week exposure to 100 ng of E2/L, the plasma VTG levels in male rainbow trout were around 2 mg of VTG/mL, whereas at the same dose the roach had levels of around 60 μg/mL; that is, the rainbow trout produced about 30 times more VTG at the same exposure. Only the roach exposed to the highest concentration of E2 (100 ng/L) produced an elevated response (32 ± 12 μg/mL) that was significantly ($p < 0.0001$) above the methanol control group. The vitellogenin concentration in the 1 ng of E2/L ($n = 13$) and 10 ng of E2/L ($n = 12$) dose also appeared to be elevated above the vitellogenin concentration in the control group, but this effect was not significant, probably due in part to the small sample size of the control group ($n = 4$) caused by initial difficulties in blood sampling these small fish.

The female roach (mean initial weight and GSI ± SEM; 12.6 ± 0.8 g and 11.4 ± 1.0, respectively) contained high initial VTG concentrations (115 ± 160 μg/mL) at the beginning of the experiment ($n = 12$). This was expected, as the experiment was conducted during March and April, when these fish were close to full sexual maturity (*18*). Comparison of the VTG concentrations in the female roach from the presample group (Pre) and the postexposure control group (C) show that VTG levels decreased about 10-fold during the course of the experiment. This could be due to stress and/or to the VTG production ceasing naturally as the fish approached full sexual maturity and ovulation. The latter explanation seems to be the most likely as the GSI was found to decrease from 11.4 ± 1.0 at the beginning of the experiment down to 3.6 ± 0.4 at the termination of the experiment. Unlike the male roach, exposure of the female roach to the varying doses of E2 did not significantly elevate plasma VTG levels above the levels observed at the end of the experiment in the control group (15 ± 8 μg/mL). It is possible that the highest concentration of E2 (100 ng/L) elevated the female plasma VTG levels slightly (to 56 ± 12 μg/mL), but this effect was not significant, possibly due to the small sample size of both the exposed ($n = 9$) and control groups ($n = 3$).

Exposure of Rainbow Trout to Estrone. Figure 3 illustrates that exposure of male rainbow trout (mean weight and GSI ± SEM; 328.2 ± 9.4 g and 0.217 ± 0.009, respectively) to varying concentrations of E1 produced a clear dose-related increase in VTG production. In agreement with the results presented in Figure 1, the initial (preexposure) VTG levels were low, averaging around 100 ng/mL. Only the highest concentration of E1 (100 ng/L) produced a response (890 ± 600 μg/mL) that was significantly ($p < 0.0001$) elevated as compared to the methanol control postexposure group (83 ± 40 ng/mL). However, when the pre- and postsamples were compared within each group, the 50 ng/L postexposure group (1.5 ± 0.8 μg/mL) was also significantly ($p < 0.01$) elevated as compared to the preexposure level (105 ± 34 ng/mL), indicating that for a 3-week exposure the estimated threshold response probably occurred at a concentration between 25 and 50 ng of E1/L.

Exposure of Rainbow Trout to Estrone and 17β-Estradiol, both Alone and in Combination. Figure 4 depicts the response of male rainbow trout to a 3-week exposure to E1 (25 ng/L) and E2 (25 ng/L) both alone and in combination. The single concentration of E2 was included primarily to provide an estimate of its relative potency compared to E1 (Figure 3). The concentration of 25 ng of E2/L was chosen because it was expected (based on the results presented in Figure 1) to induce a response, albeit a small one, thus providing scope for the combination (25 ng of E1 + 25 ng of E2/L) to produce an additive (or synergistic) response, should one occur, that was still within the dose−response relationship. Figure 4 illustrates that a 3-week exposure to either 25 ng of E1/L or 25 ng of E2/L was not sufficient to significantly elevate plasma VTG concentrations, although in both cases the postexposure vitellogenin concentration was greater than the preexposure concentration (by 10- and 7-fold, respectively). In contrast, the fish exposed to a combination of 25 ng of E1/L and 25 ng of E2/L had significantly elevated ($p < 0.0001$) VTG concentrations (17.4 ± 6 mg/mL). In this treatment group, the plasma VTG levels increased about 150 000-fold during the 3-week exposure to reach a level close to the maximal obtainable response of 50 mg/mL. The response to 25 ng of E1/L plus 25 ng of E2/L was significantly greater than the response to 50 ng of E1/L ($p < 0.0001$) and was also greater (but not significantly) than the response to 100 ng of E1/L (Figure 3).

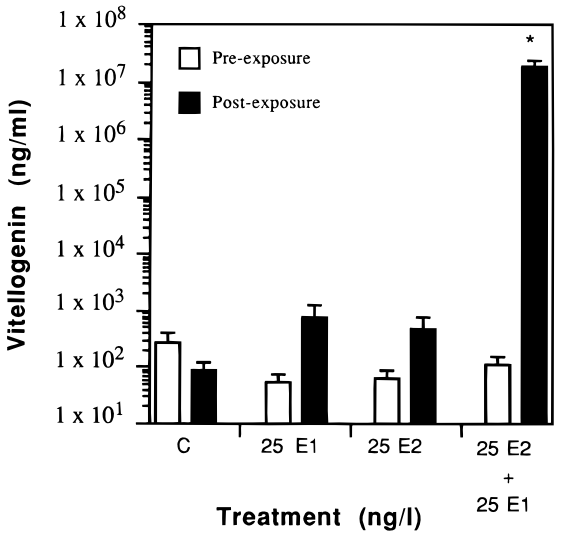

FIGURE 4. Effect of estrone (25 ng/L) and 17β-estradiol (25 ng/L) alone and in combination on vitellogenin synthesis in rainbow trout exposed for 3 weeks as compared to the control (C). Values shown are the mean concentrations ($n = 10$) of vitellogenin in the pre- and postexposure blood plasmas. The error bars represent the standard error of the mean (± SEM). An asterisk (*) denotes significant differences from the control post-sample at $p < 0.0001$.

Exposure of Male Trout and Roach (Both Sexes) to 4-*tert*-Octylphenol. Figure 5A illustrates that exposure of male rainbow trout to varying doses of OP produced a clear dose-related increase in vitellogenin production. As expected, the initial (preexposure) VTG levels were very low, averaging around 50 ng/mL. Both the 10 and 100 μg/L concentration of OP produced responses (mean ± SEM; 240 ± 140 μg/mL and 42 ± 6 mg/mL) that were significantly above ($p < 0.0001$) the methanol control postexposure group, indicating that for a 3-week exposure the estimated threshold response probably occurred at a concentration between 1 and 10 μg of OP/L.

Figure 5B illustrates that exposure of male roach to varying concentrations of OP produced a dose-related increase in VTG production. The initial (preexposure) VTG levels in the male roach were low, averaging around 80 ± 10 ng/mL. Only exposure to the highest dose of OP (100 μg/L) ($n = 11$) produced an elevated response (116 ± 42 μg/mL) that was significantly above the VTG concentration of the control group ($p < 0.0001$). The VTG concentration in the 10 μg/L ($n = 12$) and 1 μg/L ($n = 16$) concentrations also appeared to be elevated above the control group, but this was not significant, probably due in part to the small sample size of the control group ($n = 4$). The fact that exposure to 10 μg of OP/L did not elevate plasma VTG concentrations in the male roach (Figure 5), whereas it did in the trout (Figure 5a), may also indicate that the trout were more sensitive to OP exposure.

The female roach contained high VTG concentrations (115 ± 160 μg/mL) at the beginning of the experiment, although the levels decreased slightly during the course of the experiment (even in the fish that were maintained in clean water). However, unlike the female roach exposed to E2 (see Figure 2), the group exposed to the highest concentration (100 μg of OP/L) contained plasma VTG levels (215 ± 49 μg/mL) that were significantly elevated ($p < 0.001$) above those in the groups exposed to the lower OP doses, indicating that VTG synthesis was further stimulated in these fish.

Discussion

In this study, the ability of environmentally relevant concentrations of 17β-estradiol (E2) and estrone(E1), determined

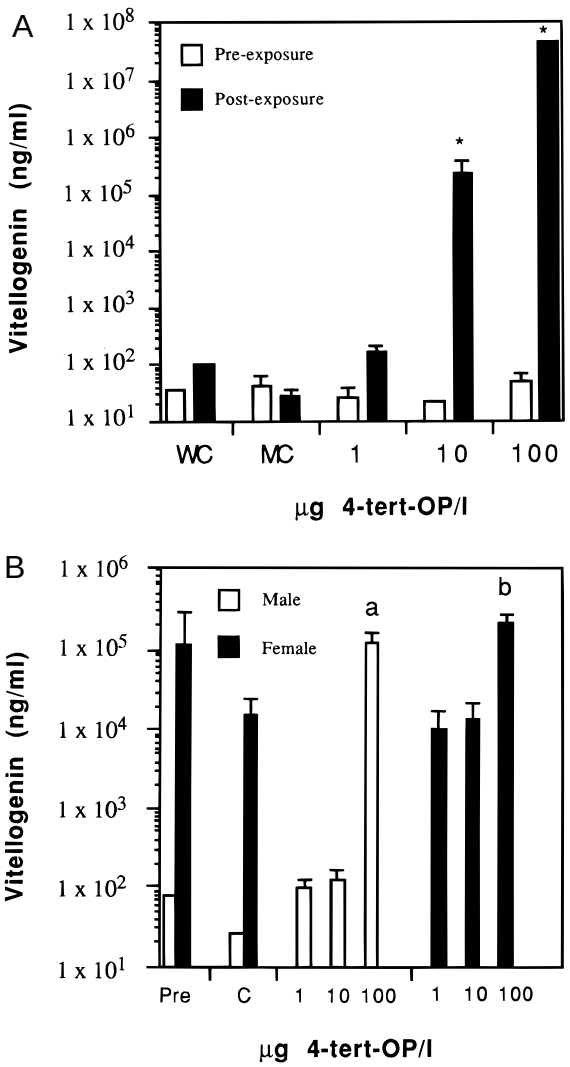

FIGURE 5. Effect of different concentrations of 4-*tert*-octylphenol (OP) on vitellogenin synthesis in trout and roach exposed for 3 weeks. Values shown are the mean concentration of vitellogenin in the blood plasma samples. The error bars represent the standard error of the mean (± SEM). (A) Response in male trout. Also included are the water control (WC) and the methanol control (MC). An asterisk (*) denotes significantly different from the methanol control at $p < 0.0001$. (B) Response in male and female roach. Also included are vitellogenin levels from a single group of roach that were blood sampled prior to the onset of the experiment (Pre) in order to establish initial vitellogenin levels. 'a' denotes significantly different from the control post-sample (C) at $p < 0.0001$. 'b' denotes significantly different from the female 1 and 10 μg 4-t-OP/L post-sample groups at $p < 0.001$.

previously (*8*), to induce vitellogenin production in male rainbow trout and roach (both sexes) was determined. The results clearly demonstrate that male roach and trout were extremely sensitive to E2 and E1 at the concentrations tested and responded by producing high plasma levels of vitellogenin (VTG) during the 3-week exposure period. In rainbow trout, the threshold concentration for a response to E2 was between 1 and 10 ng/L (Figure 1). The magnitude of the vitellogenic response was smaller in the male roach (Figure 2) than in the rainbow trout (which produced about 30 times more VTG at the same exposure), possibly due to physiological considerations. For example, female rainbow trout normally produce plasma VTG concentrations as high as 50 mg/mL (*19*) as compared to roach and other cyprinids that peak at around 1 mg/mL (*20*). This may be a consequence of differences in egg size, which are typically larger in

salmonids such as rainbow trout as compared to cyprinids. As the 10 ng of E2/L exposure elevated plasma vitellogenin levels in the trout (Figure 1) but not in the male roach (Figure 2), this may be indicative of species differences in sensitivity toward estrogen exposure. Similar results were obtained with 4-*tert*-octylphenol (OP). Exposure of rainbow trout to both 10 and 100 μg of OP/L produced significant elevations ($p < 0.0001$) in plasma VTG concentrations as compared to the control (Figure 5A), with the threshold response occurring between 1 and 10 μg/L. This was consistent with previous findings (*21*). However, in the male roach, the threshold for a response to OP occurred between 10 and 100 μg/L (Figure 5B). Although we were not able to accurately quantify this based on the experimental data, the species difference in sensitivity does not appear to be large and may be less than a factor of 10. However, the reported seasonal changes in the affinity and abundance of the oestrogen receptor in fish (*22*) means that an accurate comparison of the sensitivity between different species would require an identical exposure at equivalent stages in their sexual cycle. In addition, exposure to 100 μg of OP/L produced a greater response than the 100 ng of E2/L exposure in both the male roach (Figures 2 and 5B) and the trout (Figures 1 and 5A), indicating that the relative potency of OP was between 1/100 and 1/1000 the potency of E2 in both species.

As the male fish were able to respond to extremely low concentrations of steroidal estrogens present in the water, it is likely that normal physiological levels of E2 in these fish were even lower (probably below 10 pg of E2/mL in trout). However, estrogen receptors were presumably already expressed, enabling a rapid response to the low levels of steroid in the water. In rainbow trout, the threshold concentration for a response to E1 was between 25 and 50 ng/L (Figure 3), which was between 2 and 5 times higher than the concentration of E2 required to produce a similar response (Figure 1), indicating that E1 was slightly less potent than E2.

As the roach used consisted of both males and females, we were able to compare sex differences in their response to E2 and OP. Figure 2 illustrates that at the beginning of the experiment the female roach had high VTG levels, as expected, because the experiment was conducted during March and April when these fish were close to full sexual maturity (*18*). In all posttreatment groups, VTG concentrations were equivalent to the control levels (Figures 2 and 5), except in the group exposed to 100 μg OP/L (Figure 5), where further stimulation produced additional VTG on top of the high initial levels. This indicated that the xenoestrogen could modulate VTG production in the mature females. The 100 ng of E2/L exposure was not capable of elevating VTG levels in the female roach. This may have been anticipated as natural physiological levels of E2 in these fish were probably around 5 ng/mL (*23*), which is 50 times higher than the concentration present in the water. Moreover, as E2 is the main natural estrogen, we would expect the fish to have a greater physiological ability to control its fate in vivo, through metabolism (conjugation and excretion) or binding to sex hormone-binding globulin (SHBG). In in vitro assays, OP is about 1/1000th the potency of E2 (*24, 25*). However, the results presented here and in Jobling et al. (*21*) indicate that OP is more potent *in vivo* (between 1/100th and 1/1000th the potency of E2). This enhanced potency, which may be up to 10-fold higher than predicted from in vitro tests, may be a consequence of bioaccumulation of OP in vivo.

In the real world, fish are unlikely to be exposed to just one estrogenic chemical, but instead will live in an environment in which they are challenged by many different chemicals simultaneously. This scenario particularly applies to fish living in rivers receiving sewage effluent, which is a very heterogeneous mixture of chemicals. As E1 and E2 were detected in all the effluents tested (*8*), we exposed male rainbow trout to these chemicals, both alone and in combination, at a concentration of 25 ng/L. Figure 4 illustrates that a 3-week exposure to either 25 ng of E1/L or 25 ng of E2/L was not sufficient to significantly elevate plasma VTG concentrations. However, the response to 25 ng of E1/L plus 25 ng of E2/L was significantly greater ($p < 0.0001$) than the response to 50 ng of E1/L and was also greater, but not significantly so, than the response to 100 ng of E1/L (Figure 3). Thus, exposure of male trout to E1 (25 ng/L) plus E2 (25 ng/L) stimulated a response that was significantly larger than that produced by the individual steroids alone (25 ng/L). This may have been anticipated, given that our results indicated that E2 was between 2 and 5 times more potent than E1. On this basis, a 25 ng of E2/L concentration would be equivalent, in theory, to between 50 and 125 ng E1/L with the mixture (25 ng of E1/L plus 25 ng of E2/L) containing a potency equivalent to between 75 and 150 ng of E1/L (see Figure 3). This indicates that any estimations of the estrogenic activity of an effluent based on steroid levels should be considered as a whole rather than by its individual components. On the basis of this preliminary information, we were not able to determine whether the effect was synergistic, but it was certainly additive.

Purdom et al. reported the vitellogenic response of male trout to a 10-day immersion exposure to 17α-ethynylestradiol (EE2) at doses ranging from 0.1 to 10 ng/L (*2*). In that study, EE2 was shown to be a potent inducer of vitellogenesis, far exceeding the effect of E2. The potency of EE2 in vivo depends on the 17α-ethynyl group, which increases its longevity in vivo by reducing the rate of metabolism at carbon positions 16 and 17 of the steroid as compared to endogenous steroids (*26*). A concentration of 10 ng of EE2/L produced a response similar in magnitude to those observed in trout exposed for 3 weeks to a range of STW effluents (*2*). Moreover, concentrations as low as 0.1 ng of EE2/L were shown to significantly elevate plasma VTG levels in trout (*2, 27*). These findings suggests that EE2, when present, could also be a major contributor to the estrogenic response observed in caged male fish exposed to domestic effluent, even though its concentration was approximately 10-fold lower than E1 and E2 in domestic effluents (*8*).

The consequence of steroids in river water to aquatic organisms are unknown, and the physiological significance of unnatural vitellogenin production, particularly in male fish, remains unclear. However, it has been shown that synthesis of unnaturally high concentrations in response to pharmacological doses of E2 leads to failure of vital organs and death (*28*). Moreover, exposure to exogenous estrogens can cause feminization of male salmonid fishes if exposure occurs during a critical window spanning about 10 days either side of when the eggs hatch (*29*). Similar effects (induction of hermaphroditism or complete feminisation) were also observed when juvenile carp and juvenile Japanese medaka were exposed, via the water, to 4-*tert*-pentylphenol and *p*-nonylphenol, respectively (*30, 31*), both of which are alkylphenolic compounds reported to be estrogenic in vitro (*25*). An extensive field survey is now currently underway to determine whether the incidence of hermaphrodite roach in the U.K. is correlated to water quality in rivers receiving different STW effluents.

To summarize, these results confirm that steroidal estrogens identified in domestic sewage effluent are present in sufficient quantity to cause synthesis of vitellogenin in fish in vivo, and their effects are additive. The earlier observations of estrogenic activity in domestic effluents (*2*) and some rivers (*4, 5*) can be attributed to the presence of these steroids. We can only speculate as to whether the concentrations of steroids in effluent are sufficient to produce effects in fish living in rivers receiving those effluent

discharges. However, in some rivers where the effluent contributes to a large volume of the flow, it is possible that aquatic organisms may be exposed to concentrations of estrogenic chemicals sufficient to produce biological responses.

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