# Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractioning and In Vitro Biological Screening

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# Identification of Estrogenic Chemicals in STW Effluent. 1. Chemical Fractionation and in Vitro Biological Screening

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A fractionation system, combined with an in vitro assay for detecting estrogenic activity, was developed in order to isolate and identify the major estrogenic chemicals present in seven sewage-treatment works (STW) effluents, receiving primarily domestic effluent, discharging into British rivers. Three sterols were isolated from estrogenic fractions of sewage extracts; these were the natural hormones 17$\beta$-estradiol and estrone and the synthetic hormone 17$\alpha$-ethynylestradiol. 17$\beta$-Estradiol and estrone were present in all the effluents at measured concentrations ranging from 1 ng/L to almost 50 and 80 ng/L, respectively. The concentration of 17$\alpha$-ethynylestradiol was generally below the limit of detection but was positively identified in three of the effluent samples at concentrations ranging from 0.2 to 7.0 ng/L. These data suggest that natural and synthetic hormones may be responsible for the observed induction of vitellogenin synthesis in male fish placed downstream of effluent discharges from STWs that receive mainly domestic inputs.

## Introduction

In 1994, Purdom and colleagues reported that sewage-treatment work (STW) effluent was estrogenic to fish (*1*). The effluents tested were mainly domestic (rather than industrial) in source, indicating that the estrogenic component(s) were likely to be domestic in origin and were probably common to most of them. For this reason, this research program concentrated on effluents receiving primarily domestic input, with limited industrial inputs. At present, no specific examples have been reported where an estrogenic effect on wild fish has been conclusively linked to a particular chemical emanating from STW effluents, although many chemicals, including 17$\alpha$-ethynylestradiol (the main component of the oral contraceptive pill) and alkylphenolic chemicals (breakdown products of one group of nonionic surfactants), have been implicated (*1, 2*). STWs receiving domestic and industrial waste release a complex (and ill-defined) mixture of natural and synthetic chemicals into the aquatic environment, due to their partial or complete resistance to biodegradation during the treatment process. Although effluents have been tested for their toxicity to aquatic organisms, usually in order to determine safe discharge levels, few chemicals within an effluent have been tested for toxicity or hormone disrupting activity on an individual basis, either in vivo or in vitro. As it was clearly not practical to identify, quantify, and test all the individual substances present in effluent, a bioassay-directed fractionation procedure was adopted in which STW effluent was chemically separated into fractions of decreasing complexity. Each fraction was analyzed for estrogenic activity using a yeast-based estrogen screen (*3*). Fractions identified as active in the bioassay were separated further until they could be analyzed by gas chromatography−mass spectrometry (GC−MS), leading to identification of the active chemicals. This procedure, through a series of steps involving separation and resolution, simultaneously eliminates inactive compounds and isolates chemicals that are biologically active without using any preconceived ideas about the identity of the compounds responsible for the activity in the mixture.

Several schemes have been developed in order to isolate and identify toxins from complex mixtures based on a Toxicity Identification and Evaluation (TIE) approach (*4*). Such procedures have been successfully used for the analysis of pollution-related fish mortalities (*5*), industrial effluents (*6, 7*), urban runoff (*8*), STW effluents (*9*), and sediments (*10, 11*). To assess the biological activity (or toxicity) of a complex mixture, a selective end point must be established. In toxicity studies, bioassays based on the life cycle of the cladoceran *Daphnia* are commonly used. In such studies, the *Daphnia* were first exposed to the whole sample to evaluate the toxicity of the mixture, which was then fractionated using a series of chemical separation techniques. At each separation stage, the toxicity of the individual fractions was re-assessed using the bioindicator organism, and the results obtained were used to direct the fractionation procedure toward the most toxic components. This was repeated until an extract was generated containing a small number of toxins. These fractionation procedures are time-consuming and have a number of drawbacks. For example, an increase in toxicity could result with each chemical manipulation of the sample, due to alterations in the bioavailability of toxins. Conversely, interactive effects may be reduced as the mixtures were resolved into single components.

The fine details of each TIE procedure vary, depending on the characteristics of the target toxins, but solid phase extraction (SPE) columns containing reverse-phase C18 have been shown to reversibly bind a broad range of organic compounds, which may be subsequently eluted with a range of solvents according to their polarity. The C18 step is not universally successful, however, as certain compounds are either poorly retained or are retained with such great affinity to the C18 phase or to the plastic components of the cartridge (e.g., tributyltin and some polycyclic aromatic hydrocarbons) that they are difficult to remove subsequently (*12, 13*). Finer fractionation of the C18 SPE eluent is usually performed using high-performance liquid chromatography (HPLC), with a C18 stationary phase (*4*). At each stage, fractions collected are tested for toxicity, and the active fractions are analyzed using

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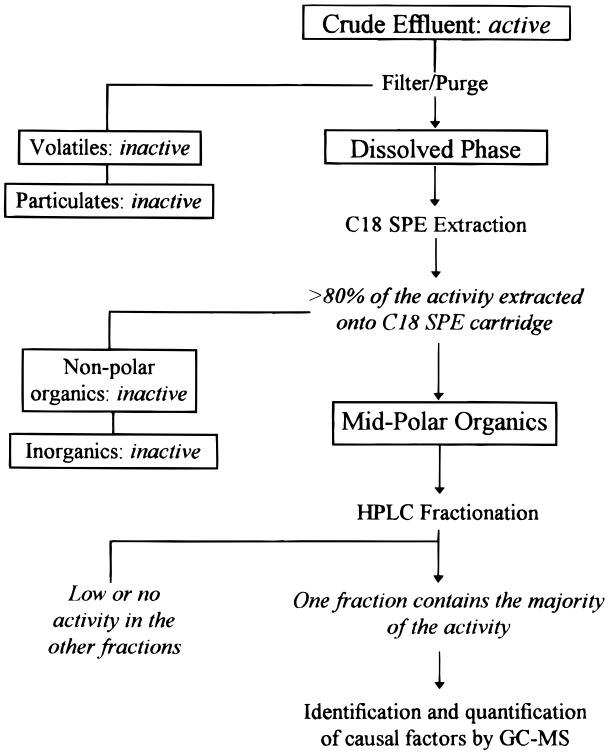


**FIGURE 1. Overview of the scheme used for fractionating domestic sewage effluent together with the findings from preliminary studies.**

an unambiguous technique such as GC−MS or HPLC−MS. We have employed the TIE approach, modified for our specific needs, to identify and quantify the main estrogenic chemicals in domestic STW effluent.

## Materials and Methods

The final methodology employed for the detection and quantification of estrogenic compounds in domestic STW effluent was based on the procedures developed and refined throughout the research program, using various volumes of Southend STW effluent. A summary of the overall fractionation scheme for domestic STW effluent, which also shows the findings from the in vitro bioassay for estrogenic activity, is illustrated in Figure 1.

**Site Selection.** The data presented here were the product of the analysis of seven STW effluents. Sites were selected to provide a range of treatment processes (i.e., primary, secondary, or tertiary treated effluents containing largely domestic waste rather than effluent containing large amounts of industrial waste), convenience of sample collection, continuity with previous sampling programs, and known alkylphenol content. Effluents from the River Lea catchment (Harpenden STW, Rye Meads STW, Deephams STW) as well as Horsham STW (discharges into River Arun) were selected as these effluents had been previously shown to be estrogenic to fish using the in vivo caged male trout bioassay (*2, 14*). Billing STW (discharges into the River Nene) and Naburn STW (discharges into the River Ouse, Yorkshire) were also analyzed. The sites chosen for investigation are summarized in Table 1. Southend STW effluent was used for the majority of the method development because a preliminary assay showed that it contained relatively high levels of estrogenic activity as compared to other effluents and therefore provided a readily measurable response to enable isolation of the estrogenic fraction(s).

**Sampling Procedure.** Discrete samples were taken from each of the STWs in preference to a bulked composite sample (taken throughout a 24-h cycle), as the stabilities of the estrogenic compounds at this stage were unknown. Large numbers of effluent samples were not processed because the methodology was too time-consuming. However, the consistency of the estrogenic activity in each effluent sample was determined by assay on three separate occasions. All the sampling equipment was treated with a weak bleach solution (for disinfection), which was then removed using distilled water. The equipment was rinsed with methanol prior to use to remove any organic contaminants. A 20-L sample of effluent was collected upstream of the point of discharge into the receiving river water using a stainless steel bucket, funnel, and aluminum churn (or stainless steel pressure vessel). A total of 100 mL of methanol was added to the effluent sample on return to the laboratory to prevent bacterial growth and to aid subsequent solid-phase extraction.

**Assay for Estrogenic Activity.** A yeast-based screen for estrogenic activity was employed; full validation of the assay was published previously (*3*). The bioassay detects "real" estrogens (such as 17$\beta$-estradiol, estrone, and estriol) as well as all known xenoestrogens (such as alkylphenolic chemicals, Bisphenol A, *o,p′*-DDT, diethylstilbestrol, phytoestrogens); thus, we expected the bioassay to produce quantitative measurements of estrogenic compounds regardless of the identity of the chemical (or chemicals) responsible for the estrogenic activity of STW effluent.

In this system, the human estrogen receptor is expressed in yeast in a form capable of binding to estrogen-responsive sequences (ERE) situated within a strong promoter sequence on expression plasmids. Upon binding an active ligand, the occupied receptor now binds to the EREs and interacts with transcription factors and other transcriptional components to modulate gene transcription. This causes expression of the reporter gene *Lac-Z*, and the enzyme produced ($\beta$-galactosidase) is secreted into the medium where it metabolizes the chromogenic substrate, chlorophenol red-$\beta$-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance at 540 nm (*3*). Preliminary crude effluent samples (whole, glass wool (GW) filtered, purged, GW filtered and purged, after C18 extraction) were also filter sterilized through 0.2 $\mu$m pore size Whatman Puradisc filters before testing. A volume of approximately 4 mL of the each crude effluent fraction was passed through the 0.2-$\mu$m filter prior to the collection of a separate sample used for testing. Varying amounts of this resulting aqueous filtrate (for example 10, 25, and 50 $\mu$L) were then added to wells on a microtiter plate to which 200 $\mu$L of seeded assay medium (assay medium plus yeast) was added. Methanol and LC fractions from the subsequent rounds of effluent fractionation were added directly to the assay plates (without the need to filter sterilize) and were allowed to evaporate to dryness before adding the seeded assay medium. The plates were incubated at 32 °C for 4 days or until a response was resolved above the background absorbance of the medium, after which the final absorbance of the medium was read at 540 nm.

**SPE Extraction of Effluent Samples.** A 20-L effluent sample was placed inside a container, which was then pressurized in order force the effluent through a Teflon filter cartridge packed with glass wool (previously rinsed with acetone and dried) at a flow rate of 10 mL/min. A 5-g (20 mL volume) and a 2-g (6 mL volume) octadecylsilane (C18) solid-phase extraction (SPE) cartridge (IST, Hengoed), previously conditioned with 15 mL of methanol followed by 15 mL of water, were placed in series after the glass wool filter. The function of the second cartridge was to collect any compounds 'breaking through' the first cartridge. The C18 cartridges were disconnected from the extraction apparatus after all the effluent had passed through. The cartridges were then dried under vacuum with air, using a VacMaster system

**TABLE 1. Details of the Sites Used in the Effluent Fractionation Project Including Population Numbers, Daily Flow through Sewage-Treatment Plant, and the Type of Treatment Process**

| site | population equiv | daily flow ($m^3$) | treatment type |
|---|---|---|---|
| Southend STW | 197 749 | 45 000 | primary settlement treatment (from April to October, 50% of the high flow receives Vitox treatment) |
| Harpenden STW | 31 200 | 8 250 | percolating filters and sand filters |
| Rye Meads STW | 357 000 | 88 500 | diffused air-activated sludge, final settlement and tertiary lagoons |
| Deephams STW | 796 000 | 160 000 | diffused-air activated sludge |
| Naburn STW | 388 000 | 20 000 | screening, primary settlement, biological filtration, secondary humus settlement |
| Horsham STW | 107 250 | 18 000 | biological filtration and settlement lagoons |
| Billing STW | 285 959 | 60 000 | extended aeration |

(IST, Hengoed). When all the water had been dispelled, the cartridges were wrapped in hexane-rinsed foil and were stored at −20 °C until required. For the control, the same procedure was carried out using distilled deionized water. This was termed the 'procedural blank', which was analyzed alongside the effluent fraction at each stage.

**Elution of Material from SPE Cartridges.** After thawing for 2 h in a fume hood at room temperature, the C18 cartridges were connected to the Vacmaster system for elution. Initial fractionation of the dissolved phase was performed using a method based on the U.S. EPA toxicity-based fractionation procedure developed by Mount and Anderson-Carnahan (*4*). SPE columns containing C18 reversibly bind a broad range of compounds. These can then be eluted according to their polarity, enabling the selective removal and concentration of organic compounds from complex mixtures. By controlling the elution sequence of the compounds retained on the C18 matrix, using solvents of varying polarities, the biologically active components can be isolated within discrete fractions appropriate for higher resolution procedures. The compounds retained on the C18 cartridges from the effluent sample were eluted from the column using a series of 5 mL (5-g cartridge) or 2.5 mL (2-g cartridge) volumes of methanol/water mixtures (0%, 25%, 50%, 75%, 80%, 85%, 90%, 95%, and 100% methanol), which were collected in separate vials. The same SPE cartridges were then sequentially eluted with solvents of low polarity to nonpolar solvents (diethyl ether, 50/50 diethyl ether/hexane, and finally hexane) to elute compounds of the widest polarity range that had not been removed previously by the 100% methanol. These samples were blown down to incipient dryness under nitrogen and made up in methanol. All these samples were subsequently assayed for estrogenic activity.

**Reconcentration of Active Fractions.** Following their assessment for estrogenic activity, the active fractions were combined and reconcentrated prior to the next fractionation stage using HPLC. Active SPE fractions were combined in a clean, solvent-rinsed glass Winchester bottle (2.7-L capacity). The sample vials were then rinsed twice with 1 mL of Nanopure water, which was also added to the bottle. The bottle was then filled with 2.5 L of Nanopure water to ensure that the methanol from the samples was diluted sufficiently to allow re-extraction onto the new C18 cartridge. This procedure was necessary because of the difficulty in blowing down large volumes of methanol containing trace amounts of water. However, this method of concentration also reduced the risk of evaporative loss of the semivolatile components.

The diluted sample was re-extracted under vacuum (maximum flow 10 mL/min), and the cartridge was dried as described previously. The sample was eluted from the cartridge into a beaker using 2 × 15 mL of hot methanol (60 °C). The sample was then transferred to a round-bottomed flask along with the contents of the beaker following a 2 × 1 mL methanol rinse. The sample, concentrated by evaporation to a volume of approximately 1 mL using a rotary evaporator, was then transferred to a graduated test tube together with the methanol used to rinse the flask (2 × 0.5 mL of methanol). The test tube sample was then blown down to a volume of 1.5 mL at room temperature under nitrogen, and the sample was placed in a sealed 2-mL vial and stored at 4 °C until required.

**Fine Fractionation by Semipreparative HPLC.** Half of the concentrated 1.5-mL sample was chromatographed by injecting 150 μL (5 times) through a 25 cm × 10 mm × 5 μm Spherisorb ODS2 C18 semipreparative HPLC column (Fisher Scientific, Loughborough, U.K.) at a flow rate of 5 mL/min, with a UV detector (210 nm) using HPLC-grade water and methanol as mobile phase solvents.

Gradient elution was employed using a water and methanol mixture. The standard gradient started with 40% methanol for the first 3 min following the injection onto the column (40%, 0−3 min), after which the polarity was gradually decreased to 100% methanol by 30 min (40−100%, 3−30 min). The carrying solvent was maintained at 100% methanol for a further 10 min (100%, 30−40 min), after which time it was returned to 40% methanol (100−40%, 40−41 min), which was maintained for the remaining duration of the run (40%, 41−45 min).

As the sample components were eluted from the separating column by the carrying solvent, they were monitored through an ultraviolet (UV) detector, and discrete fractions were collected at 1.5- or 1-min intervals (30 × 7.5 mL fractions collected in 45 min or 45 × 5 mL fractions collected in 45 min, respectively) into vials. All the fractions produced were then assessed for estrogenic activity.

**Further Separation of the Active Fraction Using a Shallow HPLC Gradient**. A finer fractionation procedure based on reversed phase HPLC was also developed in order to further purify (separate) the estrogenic fraction already isolated from the first HPLC run. An identical HPLC column was used, but a 'shallower' gradient of solvent mixtures (methanol/water) was employed. The shallow gradient started with 55% methanol for the first 3 min after the injection onto the column (55%, 0−3 min) and was then increased to 60% methanol by 30 min (55−60%, 3−30 min), after which time it was gradually returned to 55% methanol (60−55%, 30−40 min) for the remaining duration of the run (55%, 40−45 min).

**Extraction of Estrogenic HPLC Fractions into Dichloromethane.** The active fractions from each final HPLC run were combined in a 500-mL separating funnel (together with the Nanopure water used to rinse out the sample vials), and the contents of the separating funnel were then diluted to a volume of 300 mL using Nanopure water. A 500-μL aliquot of a 2 μg/mL $d_2$-17β-estradiol internal standard (dissolved in dichloromethane) was also added to the sample. The combined sample was then liquid/liquid extracted three times, using 50 mL of dichloromethane (DCM). The DCM extracts were then dried using anhydrous sodium sulfate and rotary evaporated to a volume of 1 mL. The sample was then re-

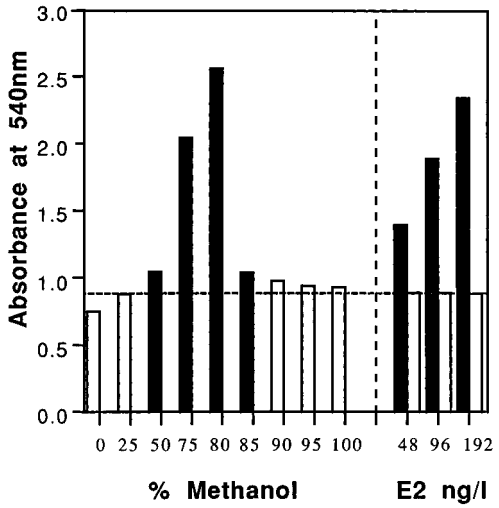


**FIGURE 2. Typical estrogenicity profile obtained from a 20-L sample of domestic effluent following concentration and elution from C18 SPE cartridges using methanol/water mixtures of varying polarity. The bars depict the absorbance of the yeast screen medium following incubation with a 20-$\mu$L aliquot of sample: an absorbance of ~0.8 indicate no estrogenic activity, whereas an absorbance value above this (up to a maximum of 2.8) indicates estrogenic activity. In each case, the activity was eluted from the column using 50−85% methanol. For comparison, the results obtained with various concentrations of 17$\beta$-estradiol (the positive control) are shown.**

duced in volume using a gentle stream of nitrogen to 150 $\mu$L. The fractions eluting immediately before and after the active fractions (bracketing fractions) were prepared in the same way.

**Analysis of Active Fractions by GC−MS.** All the samples as well as the full procedural blank and reference standards were analyzed in triplicate by GC−MS using a Finnigan MAT Magnum ion trap mass spectrometer run in E1 mode at 70 eV with a Varian GC system and Finnigan MAT A200S autosampler. The ions selected for quantitation were those that produced the greatest signal-to-noise ratio. These were 213 270 (estrone), 213 272 (17$\beta$-estradiol), 215 274 ($d_2$-17$\beta$-estradiol internal standard) and 213, 296 (17$\alpha$-ethynylestradiol).

**Determination of Extraction Efficiencies and Detection Limits.** A 20-L tap water sample was spiked with 200 ng of 17$\beta$-estradiol, estrone, 17$\alpha$-ethynylestradiol, and $d_2$-17$\beta$-estradiol, so that a final concentration of 10 ng/L of each steroid was present in the sample. This sample was extracted as before using two preconditioned 5-g C18 cartridges (arranged in series), which were then rinsed with 40 mL of 25% methanol solution (discarded), followed by 2 × 50 mL of 85% methanol solution. The 85% methanol eluate was diluted in 1 L of Nanopure water and reconcentrated using a preconditioned 5-g C18 cartridge. The cartridge was eluted using 20 mL of 100% methanol, which was blown down to a final volume of 1.5 mL. Half of this sample was fractionated by semipreparative scale HPLC, and the resulting fractions were tested for estrogenicity. Active fractions were combined, diluted in water, and liquid/liquid extracted using DCM (3 × 25 mL of DCM). The extracts were dried and concentrated to 150 $\mu$L before analysis by GC−MS.

## Results

**Preliminary Findings.** During initial studies, effluent was filtered through glass wool to remove particulates, purged with nitrogen to remove volatiles, and filtered and purged together. The same procedure was performed on the procedural blank (distilled deionized water). The 'whole' effluent sample was found to be estrogenic (results not shown).

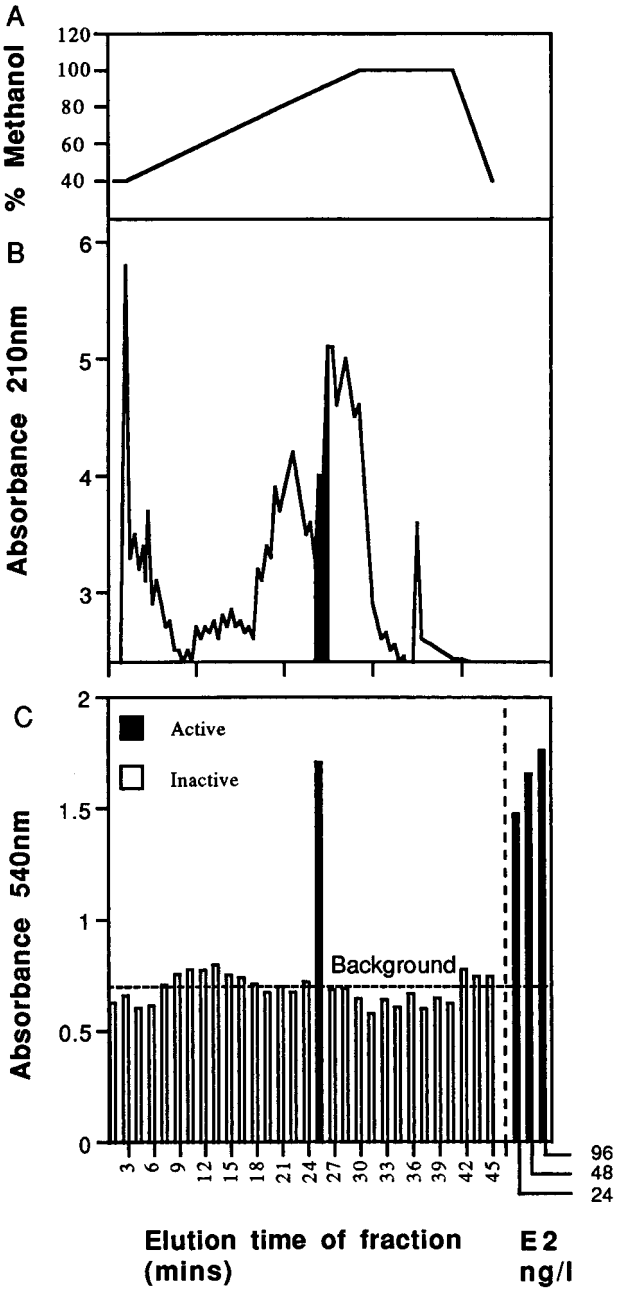


**FIGURE 3. Fine fractionation of Southend effluent using reverse-phase HPLC: (A) methanol gradient, (B) UV absorbance profile, and (C) estrogenic activity in the yeast screen following incubation of a 10-$\mu$L aliquot of each HPLC fraction. See legend for Figure 2 for a fuller explanation of the results obtained from the yeast-based estrogen screen. The figure shows that the estrogenic activity appeared as a single fraction, which was eluted between 24 and 25.5 min after injection of the sample. The shaded section on the UV profile depicts the position where the activity was eluted.**

Samples of effluent were filtered using glass fiber filters, and the material removed by the filters was extracted sequentially using a series of solvents of increasing polarity. These extracts and the filtered effluent were assessed for estrogenic activity in the yeast screen. The biological activity was not removed by filtering (results not shown), and the extracts of the particulate matter were also devoid of estrogenic activity (results not shown). Therefore, the estrogenic component(s) were not retained by the filters but were present in the dissolved phase of the effluent samples. Furthermore, the biological activity was not removed by purging, indicating that the estrogenic component was

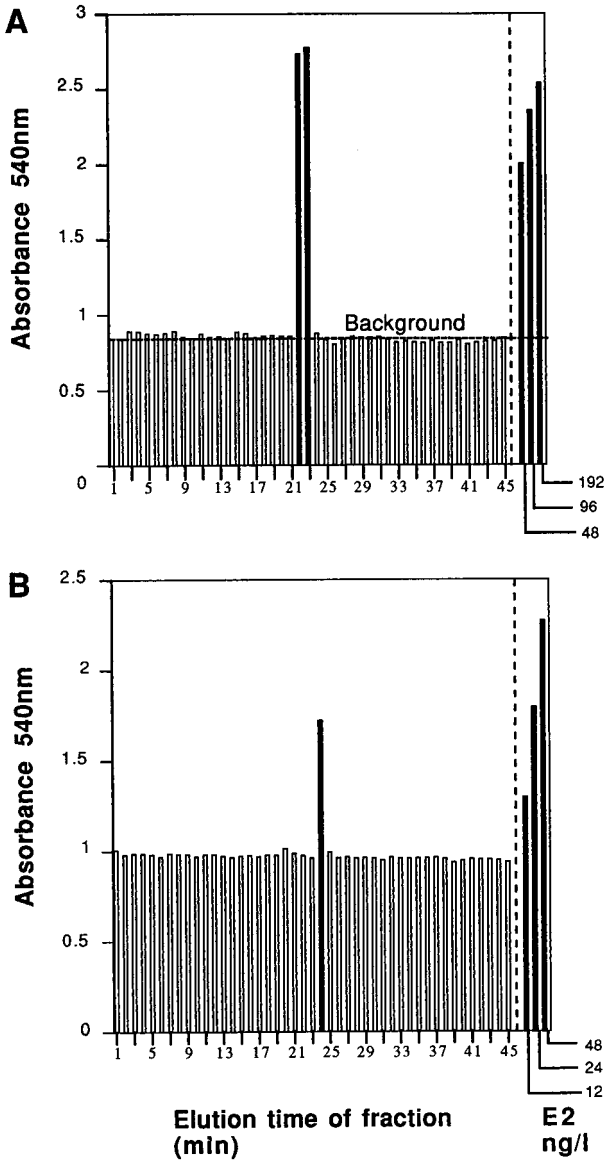


**FIGURE 4. Estrogenic activity profiles produced from domestic effluents following fine fractionation using reverse-phase HPLC. These figures depict the color change of the medium following incubation with a 10-$\mu$L aliquot of each fraction in the yeast screen: (A) Naburn STW effluent and (B) Harpenden STW effluent. In each case, the activity occurred over 1−2 fractions, which were eluted between 22 and 24 min following injection of the sample. See legend for Figure 2 for a fuller explanation of the results.**

unlikely to be volatile (results not shown).

Passing the filtered effluent through a SPE cartridge caused a >80% reduction in estrogenic activity (results not shown). Since most of the activity was retained on the cartridge, it appeared that the active compounds were not ionic substances, as these would have passed through the C18 column and produced a positive estrogenic response in the cartridge eluent. These results indicated that most of the estrogenic activity contained in the effluent was due to organic substances that bound to the C18 phase of the SPE cartridge. The details of these developmental stages, which determined the full direction of the fractionation procedures used subsequently on 20-L effluent samples, are published in a Government report (*15*).

**Results of C18 Fractionation.** Figure 2 illustrates a typical C18 elution profile produced when the compounds retained on the column were sequentially eluted using solvents of

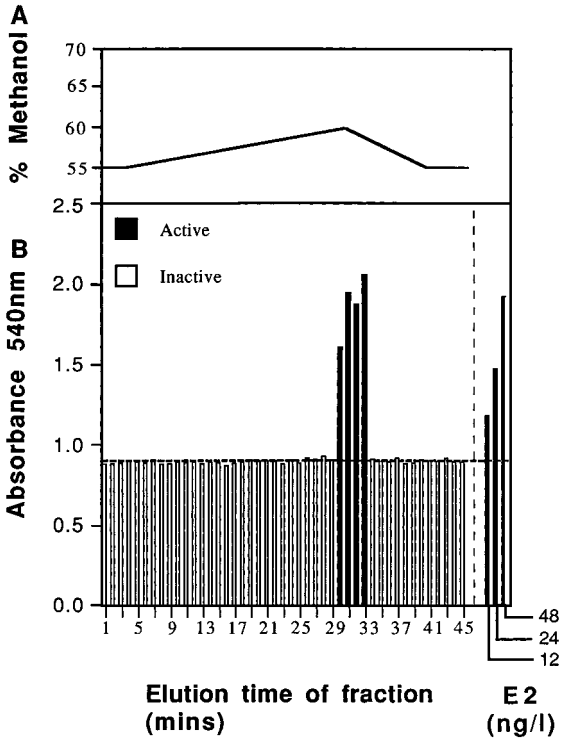


**FIGURE 5. Further separation of the active fractions produced previously during the fine fractionation of Southend STW effluent using reverse-phase HPLC: (A) methanol gradient and (B) estrogenic activity in the yeast screen following incubation of a 10-$\mu$L aliquot of each HPLC fraction. The figure shows that the estrogenic activity, previously in a single fraction, was resolved into at least two peaks of activity occurring over four fractions eluting between 30 and 33 min after injection of the sample. See legend for Figure 2 for a fuller explanation of the results.**

decreasing polarity. The figure shows that the majority of the estrogenic activity was eluted from the column between 50% and 85% methanol.

**Estrogenic Activity of HPLC Fractions from Southend STW Effluent.** Figure 3 depicts the results of the reversed-phase HPLC fractionation of Southend STW effluent. Figure 3C illustrates that the estrogenic activity occurred as a single peak (which eluted between 24.0 and 25.5 min) and was equivalent in activity to approximately 90 ng of E2/L. This suggested that the activity was due to a single compound or a set of closely related compounds that were eluted concurrently using the HPLC conditions employed. The UV profile of the chromatographic run (Figure 3B) indicated that the sample loaded remained a highly complex mixture and reverse-phase HPLC, at this stage, was unable to resolve the mixture into fractions composed of a few identifiable compounds. It was therefore impossible to determine whether the activity within the fraction was due to a highly abundant component or whether it was due to minor components concealed within the UV profile.

The estrogenic components present in the HPLC fraction were found to be liquid/liquid extractable into dichloromethane (result not shown), enabling their identification by GC−MS. Therefore dichloromethane (DCM) extracts of the active fraction as well as the bracketing fractions were prepared and analyzed separately using the ion-trap GC system. The major components of the extracts were found to be isomers of α-terpineol, a terpenoid alcohol widely used in detergents and cosmetics, which has been identified previously in sewage effluent (*16*). There were also around 20 unidentified minor components that occurred in all three extracts, but the highest concentration of α-terpineols

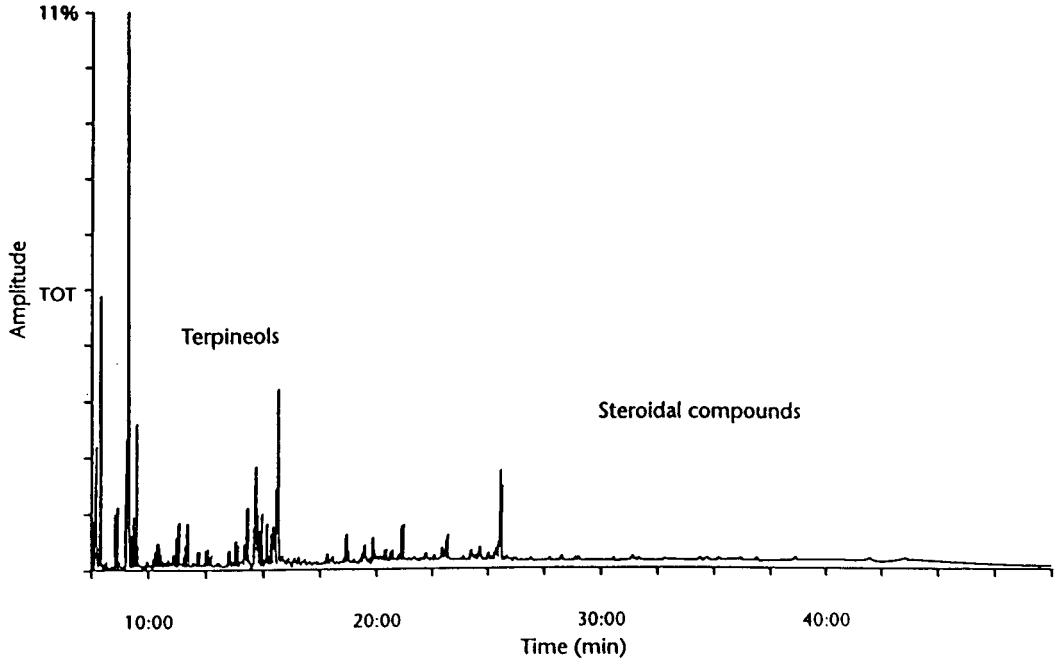


**FIGURE 6. Total ion chromatogram of the estrogenic fraction from Southend STW. The profile shows that the fraction contained mainly terpineols, with minor components tentatively identified as steroids.**

occurred in the active fraction. Standards of the identified terpineol, of both pure and technical (an isomeric mixture) grade, were purchased (Aldrich, Sigma, Fluka), and solutions spanning the concentration range present in the fraction were used to confirm the identifications by GC−MS and to test for estrogenic activity in the yeast screen. None of the terpineols tested were found to be active in the yeast screen. An additional fractionation step was therefore considered necessary to reduce the sample complexity further.

**Estrogenic Activity of HPLC Fractions from Other Domestic Effluent Samples.** Figure 4 depicts the reverse-phase HPLC profiles of two of the other effluents investigated using the fractionation conditions described previously. In contrast to Southend STW effluent, samples were collected at 1-min intervals to produce 45 5-mL fractions. In all cases, the HPLC profiles were similar, and the findings were consistent with the Southend STW effluent result. That is, the estrogenic activity appeared as a single fraction that eluted between 22 and 24 min after the sample injection. Small shifts in the retention time of the active fraction may be explained by differences in the composition (in terms of both the chemicals present and their quantity) of the extracts, which altered the chromatographic resolution of the column.

**Further HPLC Separation of Southend STW Effluent Using a Shallow Gradient.** Following the elimination of the terpineols as the estrogenic components in the active fraction, it was necessary to focus on the large number of potentially active trace compounds. An additional fractionation step was developed in order to reduce the sample complexity further.

Using the shallow elution gradient, the active fraction from the first HPLC run was resolved into two separate peaks of estrogenic activity, eluting between 29 and 31 min and between 31 and 33 min, respectively (Figure 5). A 50-$\mu$L aliquot of each active fraction was tested, producing an average response equivalent to between 25 and 50 ng of 17$\beta$-estradiol/L in the yeast screen (Figure 5). At this stage, it was also noted that the active fraction behaved in the yeast screen in a manner similar to steroid standards, in which the color development occurred rapidly as compared to the many xenoestrogens we have tested. On the basis of this observation, it was postulated that the estrogenic component could be a steroidal compound. As steroidal estrogens are very potent compounds, it was possible that the effects observed were caused by extremely low levels of these compounds in the effluent that were difficult to detect. Thus, it was at this stage in the developmental process that the methodology was up-scaled to handle larger volumes (20 L) of effluent in order to ascertain whether steroidal estrogens were present.

**Behavior of Steroids in the Fine Fractionation Procedure.** The behavior of steroids in the fractionation system was evaluated using the synthetic estrogen 17α-ethynylestradiol (EE2). If steroids (or steroid-like compounds) were responsible for the activity in the effluent, we expected their chromatographic retention time to coincide with that of the active fraction. Therefore, in separate chromatographic runs, 20 ng of EE2, procedural blanks, and the active effluent fraction were injected onto the HPLC column, and fractions were collected at 1-min intervals using both gradients (40−100% methanol and 55−60% methanol). In the 40−100% gradient run, the EE2 standard coeluted with the estrogenic activity of the effluent sample. In the 55−60% gradient run, the EE2 standard coincided with the second peak of estrogenic activity in the effluent sample. These results indicated that estrogenic steroids may be responsible for the estrogenic activity in domestic STW effluent. In both cases, the blanks were inactive, and a range of alkylphenolic standards did not coelute with the active fraction (result not shown).

**Identification of Estrogenic Chemicals in Effluent by GC−MS.** Figure 6 illustrates the mass chromatogram of the active fraction from Southend STW effluent. The standard GC−MS library searching routine identified potent natural and synthetic steroidal estrogens in the extract, namely, estrone (E1), 17$\beta$-estradiol (E2), and 17α-ethynylestradiol (EE2). This result was confirmed using a standard solution containing E1, E2 and EE2, which was analyzed under the same GC−MS conditions. The elution times of the standard compounds coincided with retention time of the peaks identified in the effluent extract, as is shown in Figure 7A,B.

**Extraction Efficiency and Detection Limits.** The extraction efficiencies for estrone, 17$\beta$-estradiol, $d_2$-17$\beta$-estradiol, and 17α-ethynylestradiol following liquid/liquid extraction

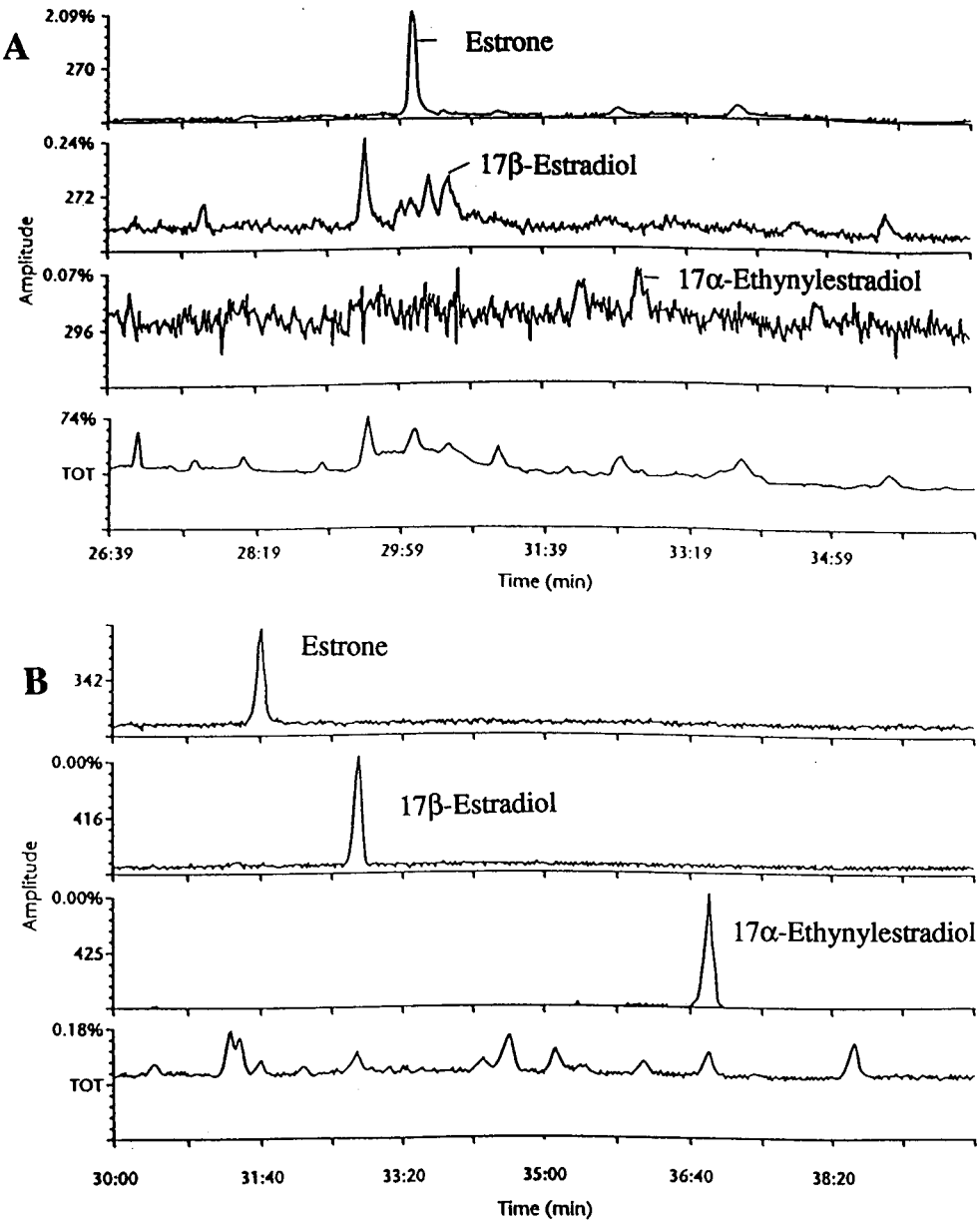


**FIGURE 7. Gas chromatograms comparing the retention times of the minor components (present in the 30−40 min range of Figure 6) of the estrogenic fraction from two STW effluents (A and B) with those of standards of natural and synthetic steroidal estrogens. The traces show that in both cases the retention times of the minor components (bottom traces), tentatively identified as steroids by GC−MS, correspond chromatographically to estrone, 17$\beta$-estradiol, and 17$\alpha$-ethynylestradiol standards (top three traces) under both derivatized and underivatized conditions and were unambiguously identified by MS library searching routines: (A) underivatized extract from Naburn STW collected in December 1995 with low 17$\alpha$-ethynylestradiol concentrations (B) derivatized sample from Southend STW collected in May 1995 with higher 17$\alpha$-ethynylestradiol concentrations.**

into dichloromethane were 85.4%, 83.3%, 88.6%, and 78.8%, respectively. Detection limits varied, depending on the performance of the GC−MS and the quality of the sample, but for a 20-L sample were generally around 0.2 ng/L in the original effluent sample. Potential improvements to the GC−MS method, by creating silyl derivatives of the sterols (less polar compounds) which were more amenable to gas chromatography, resulted in an improved peak shape for the steroids. However, this advantage was largely offset by a loss of sample during the additional manipulation.

**Concentration of 17$\beta$-Estradiol, Estrone, and 17$\alpha$-Ethynylestradiol in Domestic Effluent Samples.** The results from the effluent fractionations are listed in Table 2. The results indicate that estrone and 17$\beta$-estradiol were present in the effluents at concentrations ranging from 1 ng/L up to 80 and 50 ng/L, respectively. 17$\alpha$-Ethynylestradiol was not detected in two-thirds of the samples collected, but was detected in effluent collected from Southend STW (up to 7 ng of EE2/L) and in other effluents, for example, from Naburn STW (up to 4.3 ng of EE2/L), that contained a higher steroid load than some of the other effluents.

## Discussion

In this project the TIE approach, modified for our specific needs, was used to identify the estrogenic components in domestic STW effluent. The scale of the problem of isolating causal compounds can be gauged from reported estimates that 63 000 chemicals are in common use worldwide, of which 3000 account for 90% of the total global production. In addition, anywhere between 200 and 1000 new synthetic

**TABLE 2. Concentrations (ng/L) of Natural and Synthetic Steroidal Estrogens Detected in Domestic STW Effluents Discharging into British Waters[a]**

| site | date | estrone | 17$\beta$-estradiol | 17$\alpha$-ethynylestradiol |
|---|---|---|---|---|
| Southend STW | 15/5/95 | 48.0 ± 1.3 | 48.0 ± 6.0 | 7.0 ± 3.7 |
| | 17/5/95 | 45.0 ± 2.5 | 42.0 ± 1.4 | nd |
| | 22/5/95 | 32.0 ± 1.2 | 29.0 ± 1.2 | nd |
| Harpenden STW | 17/7/95 | 5.2 ± 0.6 | 3.7 ± 0.6 | nd |
| | 24/7/95 | 8.5 ± 0.6 | 7.1 ± 1.0 | nd |
| | 1/8/95 | 8.9 ± 0.8 | 4.4 ± 0.5 | nd |
| Rye Meads STW | 17/7/95 | 3.6 ± 0.4 | 2.7 ± 0.1 | nd |
| | 24/7/95 | 1.8 ± 0.5 | 5.5 ± 0.5 | nd |
| | 1/8/95 | 2.1 ± 0.4 | 6.3 ± 0.2 | nd |
| Deephams STW | 17/7/95 | 13.0 ± 4.6 | 12.0 ± 2.6 | nd |
| | 24/7/95 | 2.0 ± 0.05 | 4.9 ± 0.4 | nd |
| | 1/8/95 | 9.4 ± 0.9 | 4.3 ± 0.5 | nd |
| Naburn STW | 28/11/95 | 76.0 ± 10.3 | 10.0 ± 1.6 | 4.3 ± 0.5 |
| | 4/12/95 | 15.0 ± 1.0 | 6.5 ± 1.2 | 0.6 ± 0.2 |
| | 16/1/96 | 48.0 ± 2.9 | 9.8 ± 1.0 | 1.9 ± 0.2 |
| Horsham STW | 30/11/95 | 6.1 ± 0.6 | 4.9 ± 0.4 | 0.2 ± 0.1 |
| | 4/12/95 | 10.0 ± 0.3 | 5.7 ± 0.7 | 0.6 ± 0.1 |
| | 15/1/96 | 12.0 ± 0.5 | 4.0 + 0.4 | 0.8 ± 0.1 |
| Billing STW | 29/11/95 | 6.4 ± 0.5 | 6.1 ± 0.04 | nd |
| | 7/12/95 | 1.4 ± 0.15 | 7.4 ± 0.6 | nd |
| | 11/1/96 | 9.9 ± 1.2 | 6.9 ± 0.9 | nd |

[a] nd, not detected. Quantitation was based on *m/z* 213 270 (estrone), 213 272 (estradiol), 215 274 ($d_2$-17$\beta$-estradiol internal standard) and 213 296 (17$\alpha$-ethynylestradiol). Each value represents the mean and standard deviation of three replicate injections.

chemicals enter the market each year (*17*). Despite the complex composition of the effluents tested, only natural and synthetic steroidal estrogens were identified as candidate compounds responsible for the estrogenic activity observed in STW effluents with principally domestic inputs. This conjecture was supported by the very low concentrations of alkylphenolic compounds (NP and OP) measured in the effluents studied (*15*), which were generally less than 1 $\mu$g/L.

As the effluents tested contained little or no agricultural input (they came from STWs located in urban areas), we assume that the natural and synthetic steroidal estrogens detected were human in origin. Hepatic metabolism of natural steroidal estrogens occurs by 2-hydroxylation and 16$\alpha$-hydroxylation pathways (*18*). For example, 16$\alpha$-hydroxylation of 17$\beta$-estradiol (the main natural estrogen) eventually leads to the production of estriol, which was reported to be over 300-fold less active than E2 in vitro (*19*). Studies on the estrogen profile of urine samples indicate that women can excrete around 7 $\mu$g of estrone, 2.4 $\mu$g of 17$\beta$-estradiol, and 4.6 $\mu$g of estriol per day (*20*). Moreover, approximately 0.5 $\mu$g of estrone, 0.4 $\mu$g of 17$\beta$-estradiol and 1.25 $\mu$g of estriol is eliminated in the feces per day (*18*). Natural steroids are eliminated in the urine as inactive glucuronide or sulfated conjugates. In contrast, fecal elimination of steroids is reported to occur mainly as unconjugated forms (*21*), because the gut contains high levels of the bacteria *Escherichia coli*, which are able to deconjugate steroid metabolites due to their capacity to synthesize large quantities of the enzyme $\beta$-glucuronidase (*22*). As feces contain high levels of *E. coli*, it was postulated that STWs must also contain a large population of the bacteria, actively producing $\beta$-glucuronidase. Therefore it is possible that conjugated natural and synthetic steroidal estrogens eliminated from the body are deconjugated during the sewage-treatment process into the parent compounds, which we detected in the effluent, due to their restored biological activity. It is also interesting to note that the ratio of the levels of estrone to estradiol reported in urine (3.5 parts E1:1 part E2) is fairly similar to the ratio observed in the effluent (1.5 parts E1:1 part E2). Estriol (E3) was not detected among the minor components present in the active fraction, despite the fact that it was reported to be excreted in about the same quantity as 17$\beta$-estradiol. However, based on its potency in vitro, a concentration of 11 ng of E3/L (the mean concentration of E2 found in the effluents) would be equivalent in biological activity to approximately 0.03 ng of E2/L. On the basis of this calculation, if E3 was present, we would not have detected it, and it was unlikely to have contributed significantly to the estrogenic activity of the effluents. Moreover, E3 may not have been present in the same HPLC fraction as the other active steroidal compounds.

17$\alpha$-Ethynylestradiol (EE2), the main estrogenic component of the combined oral contraceptive pill, was also detected in some of the effluent samples. The oral contraceptive pill contains between 30 and 50 $\mu$g of EE2 per pill (*23*). In excretion studies, EE2 was identified as the principal component of the glucuronide and arylsulfate fraction of bile and urine (*24*). As a large proportion of the EE2 ingested was excreted as unmetabolized glucuronide conjugates (*24*), exposure to $\beta$-glucuronidase activity during the STW process may also release the biologically active form of EE2 into the aquatic environment. Reports from laboratory biodegradation studies indicated that EE2 was highly stable and persistent in activated sludge, with no detectable degradation occurring after 120 h of treatment as compared with gestagens, which were completely metabolized within 48 h of treatment (*23*). The relationship between sewage-treatment process and steroid concentration could not be accurately determined from this study as a real assessment would require measurements of concentrations in the influent and the effluent. However, there were indications that the better effluent treatments resulted in lower steroid concentrations in the effluent. The solubility of EE2 in pure water and sewage-treatment water was reported to be 4.2 and 4.7 mg/L, respectively, which was 3-fold less soluble than natural steroidal estrogens (*23, 25*). This fact is believed to contribute to the increased resistance of EE2 to biodegradation as compared with natural steroidal estrogens. Based on the limited data set in Table 2 it appears that, when EE2 was detectable, the ratio of E2 to EE2 was 9:1. Therefore, the mean concentration of EE2 was probably 0.6 ng/L, with a median concentration around half this value. These values correspond well with measurements taken in The Netherlands, which reported concentrations in river water of 0.3 ng of EE2/L, with concentrations 5-fold lower in drinking water (*26*). These concentrations of EE2 were at or just below the

best detection limits observed during the effluent fractionation method, indicating that, although EE2 was not always detected, it may still be present in the other effluent samples.

In the last two decades, a number of studies have reported the presence of natural and/or synthetic steroidal estrogens in sewage-treatment effluent, river water, or drinking water samples. In these studies, the objective of the research was to determine whether natural and synthetic steroids were present in the aquatic environment. In the present study, the objective was to identify the estrogenic components in effluent, without any preconceived ideas of their identity; they were found to be natural and synthetic steroidal estrogens. In 1970, Tabak and associates reported concentrations of 2000 ng of EE2/L, 25 ng of E1/L, 60 ng of E3/L, and 10 ng of E2/L (parent compounds plus conjugates) in STW effluents from the United States. Low levels (between 0.1 and 3.0 ng/L) of natural and synthetic estrogens were also reported in drinking water in southern Germany (*27*). In a British study, using an immunoassay procedure, EE2 was tentatively detected in STW effluent, river water, and potable water at concentrations in the low nanogram per liter range and below (*28*). Natural steroidal estrogens were detected in raw sewage from the Tel Aviv area (Israel) using a radioimmunoassay procedure at concentrations from 48 to 141 ng/L (depending on drought conditions) and in storage water (used for drinking) taken from Lake Kinneret (Northern Israel) at concentrations between 14 and 22 ng/L. Moreover, estrogen concentrations in treated sewage water (used for irrigation) discharged from small farm-based sewage-treatment units and municipal STWs in Israel were reported to be between 153 and 39 ng/L, respectively, with levels 2−3-fold higher in the summer months (*29*). In a more recent German study (employing sophisticated modern techniques), concentrations of up to 20 ng of E2/L and up to 62 ng of EE2/L were reported in effluents from STW plants, and EE2 was also occasionally detected at levels below 5 ng/L in river water (*30*). However, unlike the present study, the steroidal extraction procedures employed by Tabak (1970), Rurainski (1977), Shore (1993), and Stumpf (1996) included a pH adjustment step that may deconjugate the steroids present in the water samples (*25, 27, 29, 30*). Consequently, the figures derived from these studies may depict the total combined concentration of free and conjugated hormones and not necessarily the environmental concentration of unconjugated (active) steroids present in the samples.

In summary, in this study, the TIE approach was used to fractionate domestic effluent into samples of decreasing complexity, which were subsequently assessed for estrogenic activity in vitro. In all the effluents tested, the most active fraction (>80% total activity in domestic effluent) was found to contain low levels of natural and synthetic steroidal estrogens. This supports the earlier suggestions by Purdom et al. that the estrogenic activity was likely to be due to a common contaminant(s) and source (*1*). The results presented in Table 2 indicate that the concentrations of EE2 detected in the samples were generally too low to fully account for the magnitude of the vitellogenin response observed when male fish were exposed to the effluent (*31*). For example, in laboratory tank trials, exposure of male rainbow trout to 10 ng of EE2/L was required to produce a response of a similar magnitude to those observed following exposure to STW effluents (*1*). However, very little data address the issue of sensitivity of fish exposed to natural estrogens via the water. Hence, it was not possible to conclude whether the concentrations of E2 and E1 reported in this study would or would not be estrogenic to fish. Moreover, little, if any, information is available on whether different species, sexes, or life stages of fish differ in their sensitivity to estrogens. To address some of these issues and thus put the results obtained from the fractionation studies into an environmental context, in vivo laboratory tank trials were conducted in which rainbow trout and roach were exposed to low concentrations of estrogenic chemicals via the water. Therefore, as the second part to this project, in vivo tank trials were conducted in which trout (males) and roach (males and females) were exposed to environmentally relevant concentrations of E2 and E1 (*32*) to determine if the concentrations reported in this study were able to induce vitellogenin synthesis.

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