Transport and Transformation of Antibiotics and Hormones in the Aquatic Environment

Final Report to:
National Water Research Institute (NWRI)
Project: HRA 699-518-97

Principal Investigator:
David L. Sedlak
Department of Civil and Environmental Engineering
University of California, Berkeley
Berkeley, CA 94720

CONTENTS page

TABLES page

FIGURES

ACKNOWLEDGMENTS

We thank the University of California Toxic Substances Research and Teaching Program for providing financial support for this project. We also thank David Cwiertny, Dr. Ching-Hua Huang, Anna Schmid and James Gray for their assistance in conducting the research described in this report.

I. INTRODUCTION

Each year, approximately 50,000 kg of pharmaceutically active compounds (PhACs) are used in the United States (Glaser, 1996). Major classes of pharmaceutical compounds include antibiotics, analgesics and hormones. Many PhACs are not metabolized to a significant degree prior to excretion. Therefore, a significant fraction of the PhACs manufactured worldwide enter wastewater treatment systems. The concentration of PhACs discharged by wastewater treatment plants depends upon the treatability of the compound and the configuration of the wastewater treatment plant.

As a result of by the recent discovery of a variety of PhACs in wastewater, surface water and drinking water, scientists have raised concerns about the potential ecological and human health effects associated with wastewater effluent (Halling-Sørensen *et al.,* 1998; Ternes, 1998). Several recent studies have documented the presence of PhACs, such as antibiotics and cholesterol-lowering drugs, in wastewater effluents at concentrations ranging from 0.01 to 1 $\mu g\ L^{-1}$ (Herberer and Stan, 1996; Buser *et al.,* 1998; Ternes, 1998; Hartmann *et al.,* 1998). For example, clofibric acid, a cholesterol-lowering drug, has been detected in groundwater and tapwater in Berlin at concentrations as high as 0.17 $\mu g\ L^{-1}$ (Herberer and Stan, 1996). The fluoroquinolone antibiotic ciprofloxacin has been detected in wastewater effluents at concentrations of approximately 1 $\mu g\ L^{-1}$ (Hartmann *et al.,* 1998).

Although the presence of any xenobiotic compound in the environment is a concern, the threats posed by PhACs in wastewater effluent are difficult to assess because concentrations are typically very low (*i.e.,* generally less than 1 µg/L). At

these concentrations, consumption of several liters of water would result in a dose that is orders of magnitude less than the typical therapeutic dose (Richardson and Bowron, 1985). The absence of any adverse human health effects associated with exposure to these levels of PhACs has led many scientists and regulators to conclude that the human health risks associated with exposure to PhACs in the environment is minimal. However, some researchers have expressed concerns about ecological effects, such as the development of antibiotic-resistant bacteria, associated with the release of wastes containing low concentrations of antibiotics (Levy, 1993). At present, no evidence of antibiotic resistance resulting from the release of antibiotics in wastewater effluent has been documented.

In addition to antibiotics and other PhACs, it is estimated that wastewater effluents could contain several estrogenic hormones at concentrations ranging from approximately 1 to 20 ng/L (Table 1). While there is no reason to believe that such low concentrations of hormones pose a threat to human health, recent evidence indicates that they could be responsible for ecological problems. For example, Routledge *et al.* (1998) demonstrated that male rainbow trout (*Oncorhynchus mykiss*) cultured in water containing concentrations of 17β-estradiol as low as 10 ng/L had elevated concentrations of vitellogen, relative to control populations. The induction of vitellogen production is used by aquatic toxicologists as an indication that the endocrine system of the fish has been disrupted by an exogenous toxicant. Elevated levels of vitellogen and intersex characteristics also have been observed in wild fish and in fish caged in rivers that receive significant inputs of wastewater effluent (Purdom *et al.*, 1994; Harries *et al.*, 1996; Harries *et al.*, 1997; Jobling *et al.*, 1998).

Although estimates based on hormone use or hormone concentrations in urine (Table 1) indicate that untreated wastewater could contain hormone concentrations high enough to explain endocrine disruption observed in wild or caged fish, additional research is needed to assess the hypothesis that hormones are responsible for the observed effects. One important issue that must be resolved is the extent of hormone removal during wastewater treatment. Previous laboratory studies, performed at elevated concentrations of hormones, suggest that a significant fraction of the hormones discharged to wastewater treatment plants should be removed during treatment (Stumm-Zollinger and Fair, 1965; Tabak and Bunch, 1970; Norpoth et al., 1973; Rathner and Sonneborn, 1977). However, these experiments were performed at concentrations that were several orders of magnitude higher than those expected in wastewater, and removal could be much less significant at the relatively low concentrations expected in wastewater.

Table 1: Comparison of concentrations of hormones predicted in wastewater influent with concentrations reported in wastewater effluent.

Compound	Predicted Wastewater Influent Concentration* (ng/L)	Reported Concentration (ng/L)	Reference
17β-estradiol	3.5	3.7-48	Desbrow *et al.*, 1998
		0-20	Tabak *et al.*, 1981
ethinyl estradiol	2.2	0.8-7.0	Desbrow *et al.*, 1998
		250-1,800	Tabak *et al.*, 1981
estrone	20	1.4-76	Desbrow *et al.*, 1998
		0-70	Tabak *et al.*, 1981
equilin	9.8	NA	
dihydroequilin	5.9	NA	

notes:
*Predicted concentrations of ethinyl estradiol, equilin, dihydroequilin in wastewater influent are based upon manufacturers data (Arcand-Hoy *et al.*, in press). Predicted concentrations of 17β-estradiol and estrone are based on estimates derived from excretion data reported by Adlercreutz *et al.*, 1994.

To assess the role of estrogenic hormones as endocrine disrupters in wastewater effluent, several research groups have attempted to quantify the compounds using conventional analytical techniques (*e.g.*, gas chromatography/mass spectrometry). The reliability of these results is questionable because conventional analytical techniques are notoriously problematic when used to quantify part-per-trillion levels of organic compounds in complicated matrices. Early attempts to quantify hormones in wastewater effluents using thin layer chromatography as a cleanup technique and gas chromatography for quantification (Tabak *et al.*, 1981) yielded concentrations that are approximately an order of magnitude higher than the estimated concentrations in wastewater (Table 1). A more recent attempt to quantify hormones in wastewater effluents produced results that are in better agreement with predictions in Table 1; however, these results still require confirmation.

In their recent study, Desbrow *et al.* (1998) used gas chromatography/mass spectrometry to quantify 17β-estradiol and ethinyl estradiol in wastewater effluents from seven wastewater treatment plants in the United Kingdom. After concentrating 20 L of wastewater by solid phase extraction, fractionating the samples by liquid chromatography and concentrating the extracts to 0.15 mL, hormones were quantified by single ion monitoring with a mass spectrometer. Chromatograms presented by Desbrow *et al.* (1998) indicated the presence of numerous unidentified peaks of similar or greater size near the retention times of the hormones. In several cases, these other peaks were not resolved from the putative hormones. The presence of these unidentified peaks adds considerable uncertainty to the results and any

quantitative information from these studies must be confirmed with other analytical techniques.

To test the hypothesis that endocrine disruption in fish living near wastewater treatment plants is attributable to hormones in wastewater effluent, and to develop tools for studying the fate of hormones in engineered and natural systems, better analytical techniques are needed. The purpose of this project is to develop two independent analytical techniques for quantifying estrogenic hormones in the complicated matrices encountered in wastewater treatment plants and in the aquatic environment. The two analytical techniques developed as part of this project are simple, relatively inexpensive and sensitive enough to study the removal of hormones by wastewater treatment plants as well as the transformation processes that occur in the aquatic environment.

II. MATERIALS AND METHODS

Unless otherwise specified, all chemicals were obtained from Fisher Scientific at the highest possible purity. Aqueous solutions were prepared in deionized water produced by a Nanopure system (Barnsted Corporation).

Wastewater samples were collected after disinfection at three municipal wastewater treatment plants (Table 2). Wastewater treatment plants (WWTPs) 1 and 2 use activated sludge for secondary treatment and chlorination for disinfection. WWTP 2 also subjects wastewater to biological nutrient removal and effluent filtration. WWTP 3 is a 0.5 MGD advanced wastewater treatment plant. It uses a water hyacinth pond for primary and secondary treatment followed by lime precipitation, sand filtration, membrane filtration, reverse osmosis, ozonation and UV disinfection.

Table 2: Characteristics of wastewater treatment plants sampled in this study.

WWTP	Design Flow MGD (m^3/s)	Primary Treatment	Secondary Treatment	Tertiary Treatment	Advanced Treatment	Disinfection
1	12 (0.50)	X	X			HOCl
2	170 (7.3)	X	X	X		HOCl
3	0.50 (0.022)	X	X	X	X	O_3 & UV

Samples were collected from WWTPs 1 and 2 using a peristaltic pump equipped with Teflon tubing and a 0.22 μm polypropylene in-line filter cartridge (MSI, Inc.). Samples from WWTP 3 were collected by plant personnel from sampling valves in the treatment plant and were not filtered prior to analysis. Samples were stored in PFE-lined bottles at 5°C until extraction and analysis. Most samples were extracted and analyzed within 5 hours of collection.

The process used to quantify estrogenic hormones involved a series of steps to concentrate the hormones, separate them from interfering compounds and, for GC

analysis, to convert them into derivatives that were more easily detected. A schematic representation of the analytical technique is provided in Figure 1.

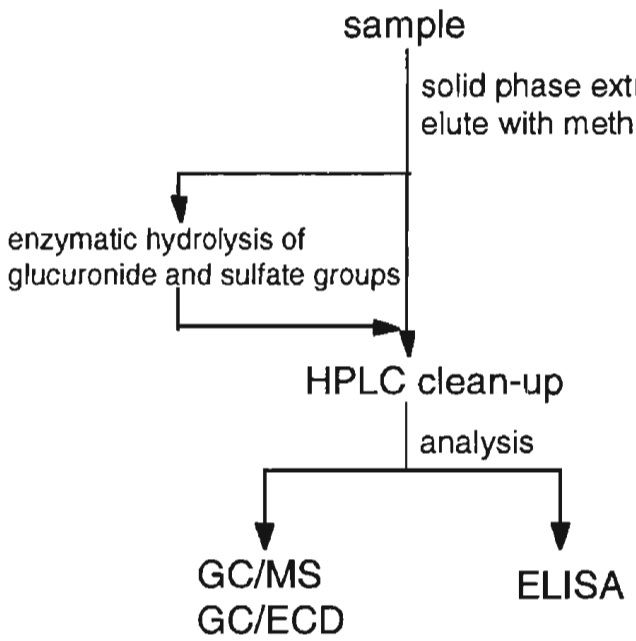

Figure 1: Schematic representation of analytical methods used to quantify hormones.

Estrogenic hormones were extracted from filtered wastewater samples using C-18 solid phase extraction discs (Empore, 3M Corporation). The extraction discs were conditioned prior to use by washing with three times with 10 mL-aliquots of methanol followed by three washes with 10 mL-aliquots of water. Between 300 and 1,250 mL of wastewater effluent sample was extracted at a flow rate of 0.5 to 5 mL/min.

After extraction, hormones were eluted by washing the extraction disc with 13 mL of methanol. The methanolic extract was transferred from a Pyrex collection tube

into 25-mL Pyrex volumetric flasks prior to drying the sample under a gentle stream of nitrogen. The dried extracts were resuspended in 600 µL of water.

Some samples were subjected to enzymatic hydrolysis to convert glucuronide and sulfate conjugates back into active hormones. Hydrolysis was performed using glucuronidase enzyme (Sigma Scientific, Type H-1 isolated from *Helix pomatia*), which was prepared at a concentration of 1.9 g/L in 0.1 M acetic acid buffer adjusted to pH 5.0 with 0.1 N NaOH. The final concentration of enzyme in corresponded to 800 units/mL. The enzyme was stored at -5°C and a stock solution was prepared immediately before each experiment. The hydrolysis was allowed to proceed for 16-20 hours at 35°C. The activity of the enzyme was confirmed in every experiment using a stock solution of 3,000 ng/L 17β-estradiol glucuronide (Sigma Scientific), which was analyzed by ELISA.

Hydrolyzed and unhydrolyzed samples were subjected to high pressure liquid chromatography (HPLC) to remove interfering organic compounds. The HPLC conditions are summarized in Table 3. The hormones typically eluted between 16 and 18 minutes. In some samples, 1 minute eluent samples were collected in conical glass vials between approximately 2 and 22 minutes to characterize interference in the ELISA technique. In addition, data on sample absorbance between 200 and 320 nm was collected using a diode array detector.

Table 3: HPLC conditions used for sample cleanup.

Stationary phase	Alltech Econosphere C-18
Column length	250 mm
Mobile phase	Methanol/water (63/37)
Flow rate	1 mL/min
Injection volume	90-200 µL

To evaluate the retention time of the estrogenic hormones, standards of 17β-estradiol and ethinyl estradiol (concentration 1 mg/L) were injected before and after wastewater effluent samples were separated. Retention times were determined by UV/visible detection. To prevent the possibility of sample contamination from the high concentration standard, the autosampler needle was washed with water several times after the high concentration standards were analyzed. ELISA analysis of deionized water samples fractionated immediately after the concentrated standard was injected indicated that carryover of the hormones did not occur.

HPLC fractions were dried at room temperature under a gentle stream of nitrogen prior to analysis by enzyme-linked immunosorbent assay (ELISA) or gas chromatography (GC). For ELISA analyses, samples were suspended in 200 μL of water and analyzed using commercially available ELISA kits (Neogen or Biotek). After adding the enzyme conjugates and chromophores supplied by the manufacturer, absorbance was measured at 450 nm using an automated microplate reader (Biotek).

Analysis of extracts by gas chromatography required several additional steps. First, dried HPLC fractions were resuspended in acetonitrile. The extracts were then derivatized with heptafluorobutyric anhydride at 50°C for one hour. The derivatized samples were cooled to room temperature and dried under a gentle stream of nitrogen. In some cases, samples were derivatized a second time using bis(trimethylsilyl)trifuoroacetamide/trimethylchlorosilane (10/1 v/v) in pyridine. The derivatized samples were dried, resuspended in *n*-hexane and analyzed by gas chromatography with an electron capture detector using the conditions listed in Table

4. 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE) was added as an internal standard before or after the samples were resuspended. Some samples also were analyzed by GC/MS.

Table 4: GC conditions used for sample analysis.

Stationary phase	Alltech AT-5, 0.25μm thickness		
Column length	60 meters		
Injection	2 μL, splitless, 250°C		
Flow rate	20 psi, N_2		
Oven conditions	Temp. (°C)	Rate (°C)	Hold (min)
	50		2
	185	40	25
	230	3	25
	280	40	10

To evaluate the accuracy and precision of the method, duplicate and spike recovery samples were analyzed with each batch of samples. Recoveries were measured in two ways: (1) 3-10 ng/L of 17β-estradiol, 17β-estradiol glucuronide and ethinyl estradiol were added to three different wastewater effluent samples prior to extraction; (2) 3-10 ng/L of 17β-estradiol, 17β-estradiol glucuronide and ethinyl estradiol were added to three different aliquots of deionized water prior to extraction. Duplicate analyses (*i.e.*, completely separate extractions and analyses of the same sample) were performed on approximately 60% of the samples.

III. RESULTS

A. Evaluation of the Analytical Method

The first step in the evaluation of the analytical method involved recovery studies in distilled water and in wastewater effluent samples amended with hormones. To calculate recoveries in wastewater effluents, concentrations of hormones and their conjugates measured in the same samples were subtracted from concentrations measured in the recovery studies. For 17β-estradiol sulfate, it was also necessary account for losses during enzymatic hydrolysis because only 30% of the compound is converted into 17β-estradiol by the enzyme (*vide infra*).

To assess the potential for adsorption of dissolved hormones onto filters, we added 0.1 to 1.0 mg/L of 17β-estradiol to distilled water, buffered at pH 7.0 with phosphate. Analysis of the samples collected after filtration indicated that significant concentrations of hormones were adsorbed onto the filters during the initial stages of filtration (Figure 2). After approximately 100 mL of sample was filtered, sorption losses became insignificant. Therefore, filters were equilibrated by passing at least 100 mL of sample through the cartridge prior to collection of samples. To evaluate potential losses of hormones during filtration of wastewater, 17β-estradiol was added to unfiltered wastewater effluent at a concentration of 10 ng/L. After filtration, pre-concentration and cleanup, a recovery of 99% was obtained (Table 5). Because sorption of compounds onto the filter was negligible in wastewater effluent samples, further studies were performed using pre-filtered samples.

Results of recovery experiments, in which 3-10 ng/L of 17β-estradiol, ethinyl estradiol, 17β-estradiol glucuronide and 17β-estradiol sulfate were added to filtered

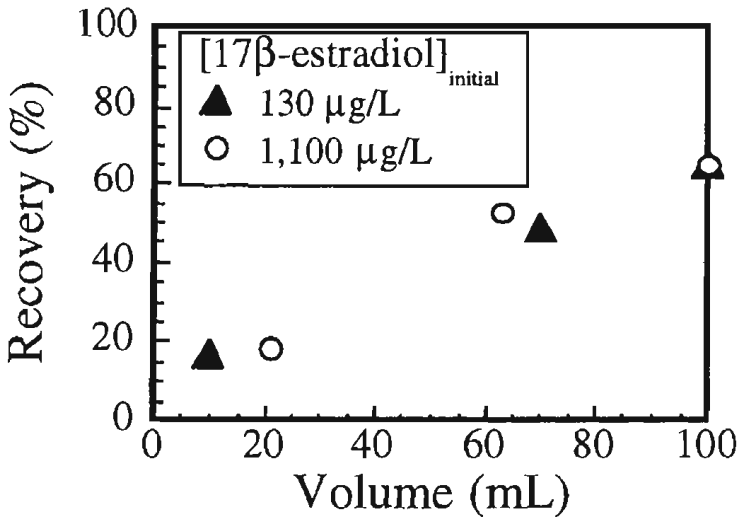

Figure 2: Results of recovery experiments for estrogenic hormones added to distilled water prior to filtration with a 0.22 µm cartridge filter.

samples yielded recoveries ranged from 48 to 112% (Table 5). The lowest recoveries were observed for 17β-estradiol sulfate (mean recovery = 60%). Recoveries did not depend upon the nature of the treatment processes or the organic carbon content of the samples.

To evaluate the efficacy of the enzymatic hydrolysis process, kinetic experiments were conducted in distilled water amended with 800 units/mL of Type H-1 and Type L-2 glucuronidase enzyme (Sigma). Results indicated that the enzymes quantitatively convert 17β-estradiol glucuronide into 17β-estradiol (Figure 3). Both enzymes only convert approximately 30% of the 17β-estradiol sulfate into 17β-estradiol. This is consistent with previous studies in which incomplete conversion of sulfate conjugates into active hormones has been reported (Tang and Crone, 1989).

Table 5: Recovery of hormones added to wastewater effluent samples. Concentrations and recoveries are indicated with ± standard error. NA: No recovery data available.

Date	Conc. Added (ng/L)	Conc. In Unspiked (ng/L)	Conc. Recovered (ng/L)	Recovery (%)	Conc. Estimated (ng/L)	Average	WWTP
17β-estradiol:							
12/9/97	10.0	3.00 ± 0.28	9.34	72	4.18		WWTP 1
1/27/98	8.0	2.53	8.19	78	3.26	***4.00 ± 0.67***	WWTP 1
3/11/98	8.0	3.70 ± 0.21	9.47	81	4.57		WWTP 1
3/31/98	3.0	1.43	2.25	51	2.82		WWTP 3 – MF
3/31/98	3.0	0.07	1.94	63	0.11		WWTP 3 – RO
4/6/98	3.0	0.03	1.82	61			DI-H_2O
6/4/98	3.0	0.01	2.05	68			DI-H_2O
6/19/98	8.0	0.60 ± 0.17	4.17	49	1.23		WWTP 2
7/23/98	3.0	0.55 ± 0.20	3.98	112	0.49	***0.95 ± 0.40***	WWTP 2
9/28/98	3.0	0.69 ± 0.29	2.28	62	1.13		WWTP 2
7/23/98	10.0	0.692	10.64	99			WWTP 2 - unfiltered
			ave. recov.=	***72 ± 19***			
17β-estradiol glucuronide:							
12/9/97	2.4	0.00	4.10	76	0.00		WWTP 1
1/27/98	8.0	0.28	6.81	63	0.28	***0.09 ± 0.16***	WWTP 1
3/11/98	8.0	0.00	4.81	48	0.00		WWTP 1
3/31/98	NA	1.66	NA	NA	1.66		WWTP 3 – MF
3/31/98	NA	0.17	NA	NA	0.17		WWTP 3 – RO
6/19/98	8.00	NA	NA	NA	NA		WWTP 2
7/23/98	3.0	0.00	3.01	85	0.00	***0.00 ± 0.00***	WWTP 2
9/28/98	3.0	0.00	3.79	106	0.00		WWTP 2
			ave. recov.=	***68 ± 16***			
17β-estradiol sulfate:							
12/9/97	2.4	0.00	2.72	73			WWTP 1
1/27/98	8.0	0.28		65			WWTP 1
3/11/98	8.0	0.00	2.65	51			WWTP 1
3/31/98	NA	1.66	NA	NA			WWTP 3 – MF
3/31/98	NA	0.17	NA	NA			WWTP 3 – RO
6/19/98	8.00	NA	1.40	59			WWTP 2
7/23/98	NA	NA	NA	NA			
9/28/98	6.0	0.00	1.21	53			WWTP 2
			ave. recov.=	***60 ± 9***			
ethinyl estradiol:							
12/9/97	NA	NA	NA	NA	NA		
1/27/98	NA	NA	NA	NA	NA		
3/11/98	8.0	1.68 ± 0.15	7.15	74	2.28		WWTP 1
3/31/98	3.0	0.32	2.52	76	0.43		WWTP 3 – MF
3/31/98	3.0	0.11	1.58	51	0.21		WWTP 3 – RO
6/19/98	8.0	0.30 ± 0.03	5.86	71	0.42		WWTP 2
7/23/98	3.0	0.34 ± 0.19	3.69	110	0.31	***0.43 ± 0.12***	WWTP 2
9/28/98	3.0	0.41 ± 0.15	2.54	75	0.55		WWTP 2
			ave. recov.=	***76 ± 19***			

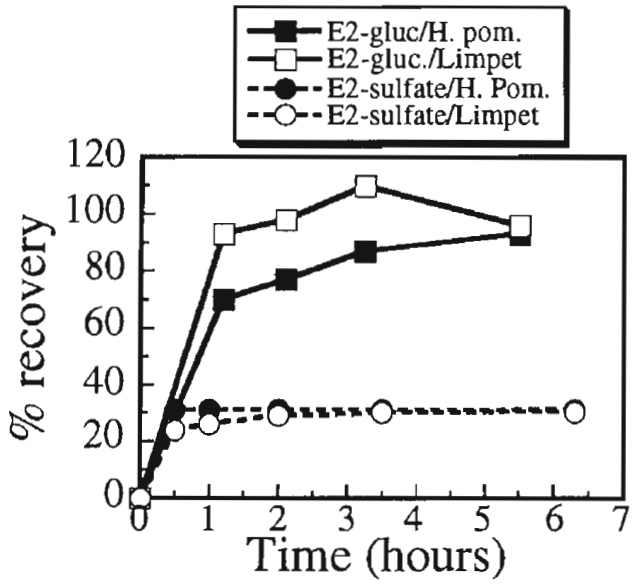

Figure 3: Enzymatic hydrolysis of conjugated hormones 17β-estradiol-glucuronide (E2-gluc.) and 17β-estradiol-sulfate E2-sulfate with glucuronidase enzymes (H. Pom. = H-1 and Limpet = L-2).

Preliminary analysis of wastewater effluent samples by ELISA indicated the presence of hormone concentrations that were considerably higher than the predicted concentrations listed in Table 1. To determine if these results were attributable to interfering compounds, sample fractionation was performed using HPLC. Eluent from the HPLC was collected in 1-minute fractions, which were evaporated under nitrogen, resuspended in water and analyzed by ELISA. Results indicated the presence of one or two regions of the chromatograms where a signal corresponding to approximately 3 to 10 ng/L of 17β-estradiol eluted prior to the estradiol (Figure 4). UV-visible chromatograms (Figure 5) indicated the presence of significant concentrations of UV-absorbing compounds during the period when the early-eluting

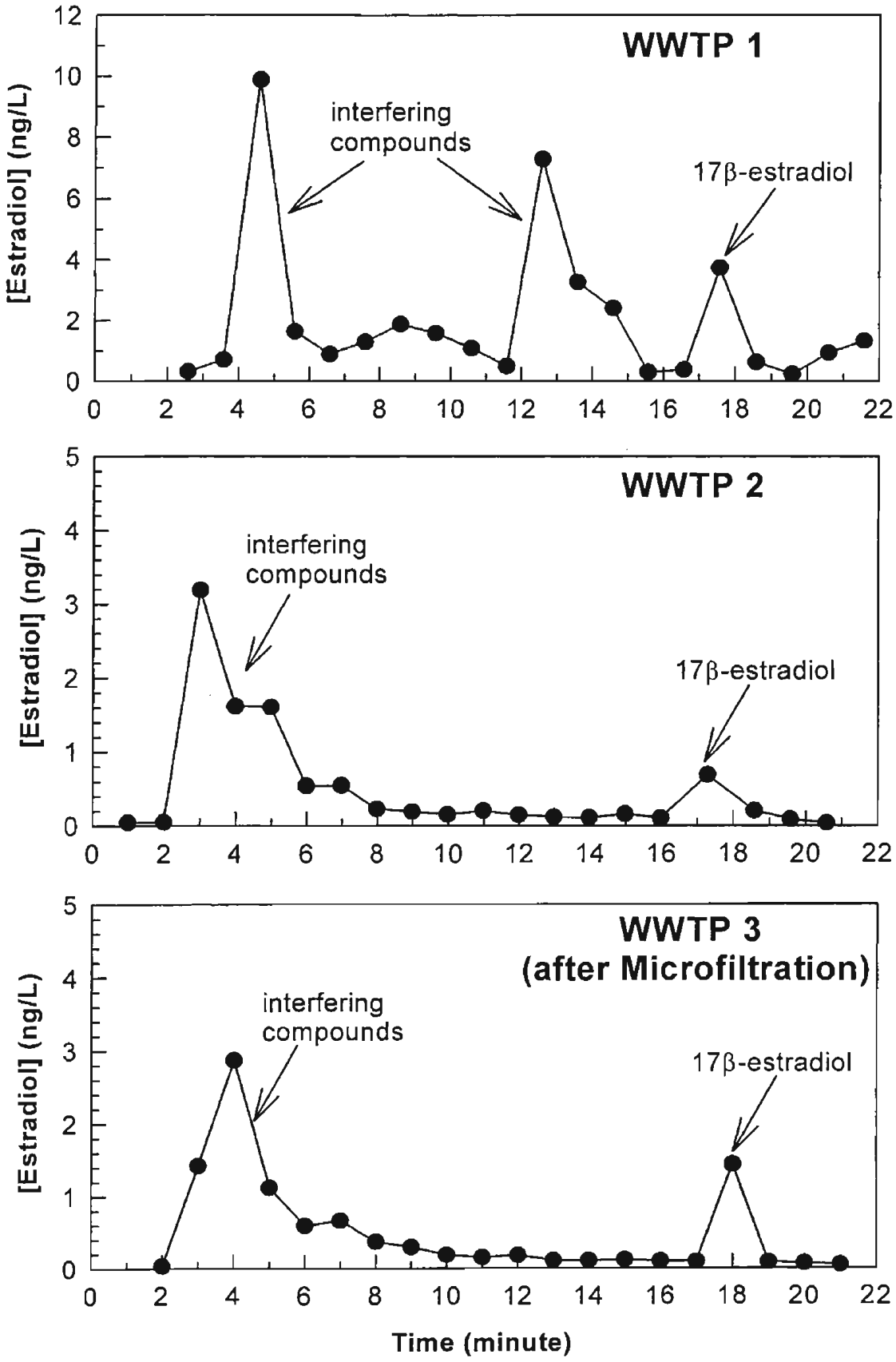

Figure 4: ELISA signal measured in HPLC fractions collected from wastewater treatment plants 1-3. Sample collection dates: WWTP 1: 3/12/98; WWTP 2: 9/28/98; WWTP 3: 4/3/98.

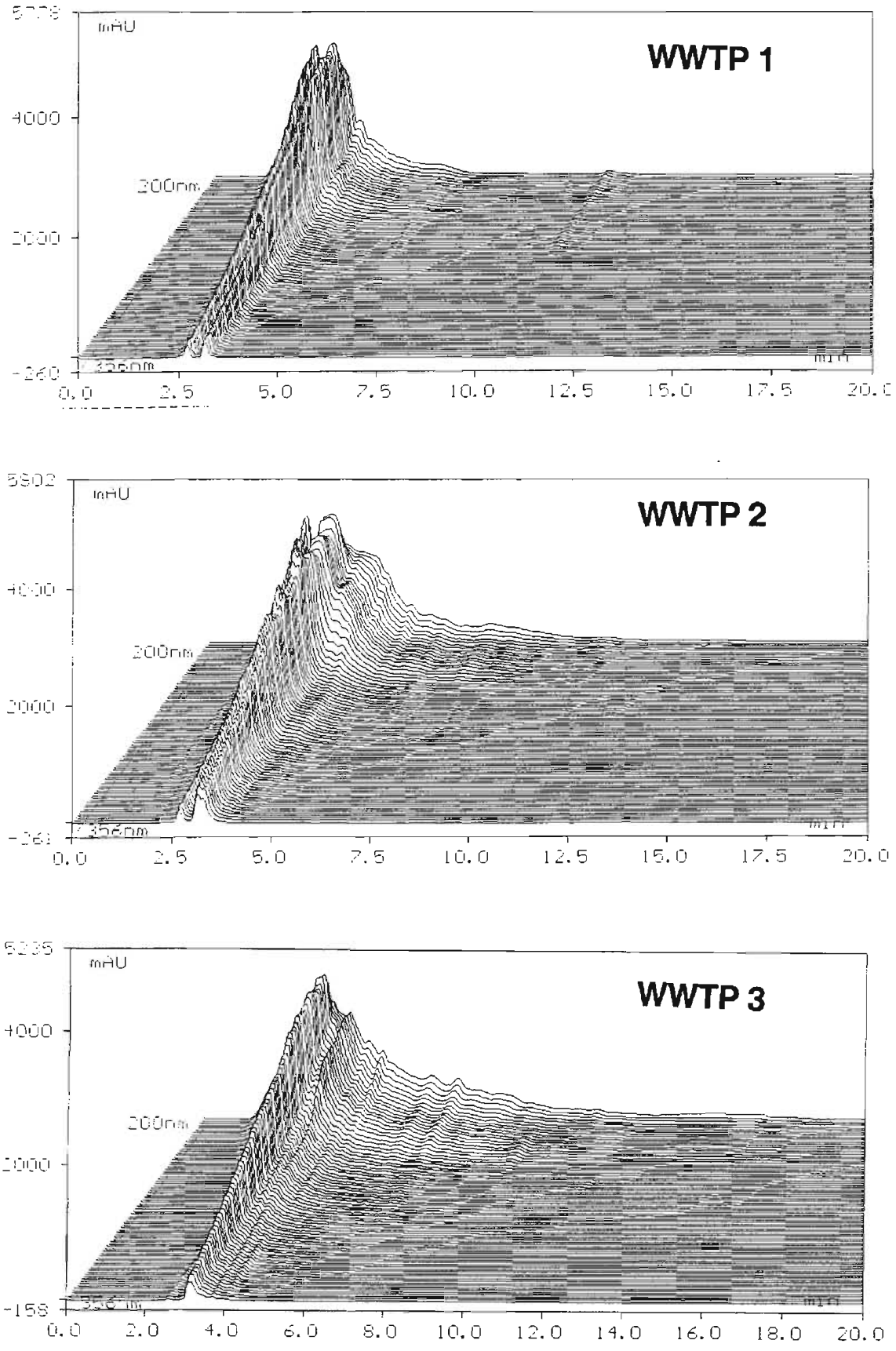

Figure 5: UV/Visible absorption measured during HPLC cleanup of samples collected from WWTP 1-3. For sample collection dates see Figure 4.

ELISA-active signal was observed. Little UV-visible signal was detected during the retention time when the hormones eluted. The results also indicate that 17β-estradiol can be separated from the interfering compounds if the appropriate HPLC conditions are used.

Attempts to analyze the samples using gas chromatography/mass spectrometry yielded broad peaks with low responses. The poor chromatography of the compounds was attributable to the reactivity of the alcohol and phenolic functional groups on the compounds (Figure 6). To improve the chromatography of 17β-estradiol, these reactive functional groups were derivatized. The initial step in the derivatization yielded 3,17-heptafluorobutyrate derivatives (*i.e.*, E_2-2,17-$(HFB)_2$) that gave good responses and linear calibration curves when analyzed using GC/ECD (Figure 7). Under these conditions, a detection limit of approximately 1,000 ng/L (*i.e.*, 1 ppb) was obtained. This corresponds to an overall detection limit of 0.06 ng/L for the extraction and processing of a 1.7 L sample.

Preliminary experiments indicated that the 3,17-heptafluorobutyrate derivative of estradiol was unstable when stored for longer than approximately 4 weeks. We hypothesized that the instability of the derivative was attributable to the hydrolysis of the phenolic heptafluorobutyrate ester (position 3). In an attempt to produce a more stable derivative, we used the protocol recommended by Dehennin (1989) to convert the 3,17-heptafluorobutyrate derivative into a mixed derivative (*i.e.*, estradiol-3-trimethylsilyl ether-17-heptafluorobutyrate) by reacting the compound with bis(trimethylsilyl)trifuoroacetamide/trimethylchlorosilane (Figure 6). The mixed derivative was more stable; however, it was less sensitive than the

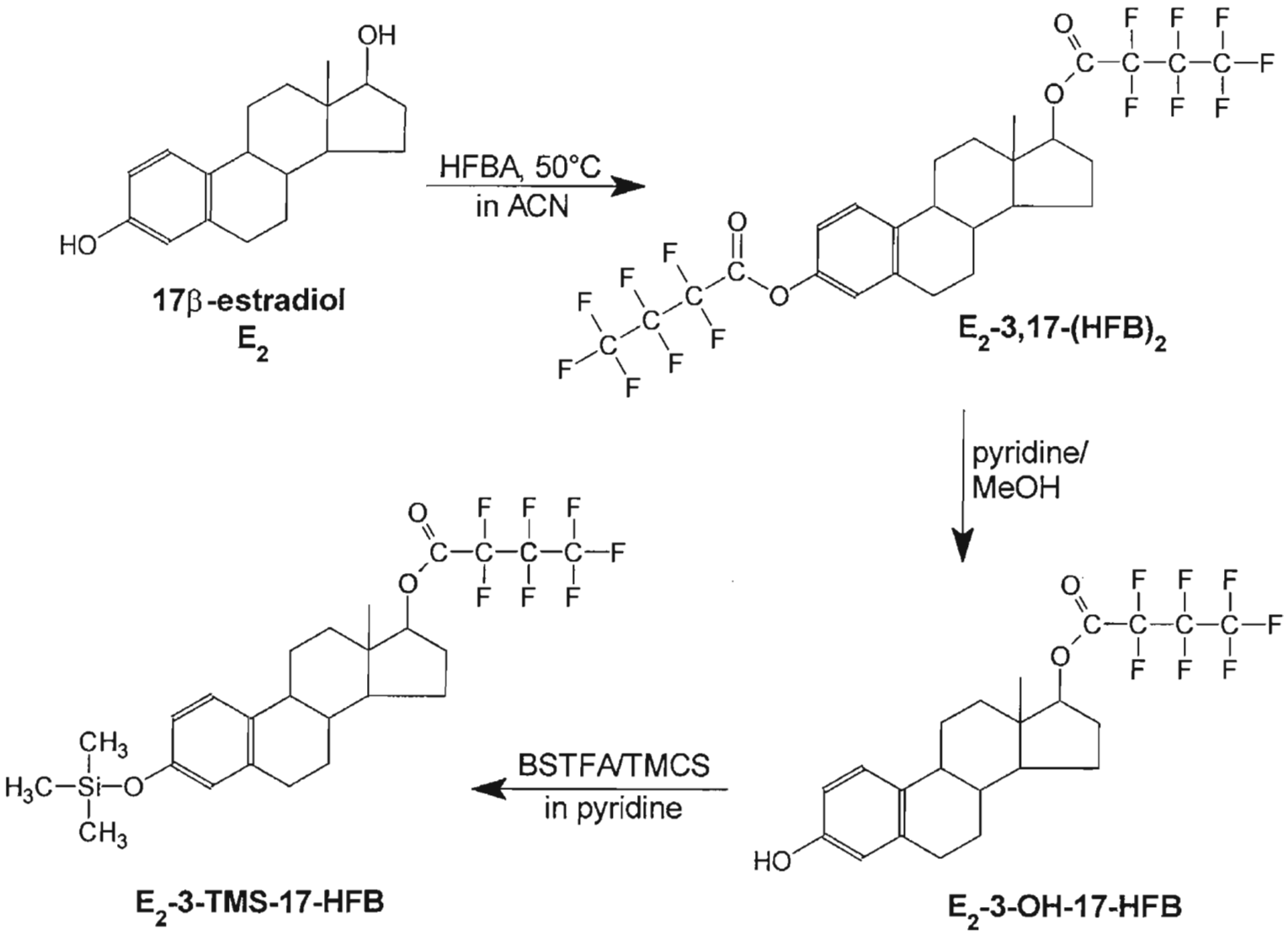

Figure 6: Analytical scheme used to derivatize 17β-estradiol.

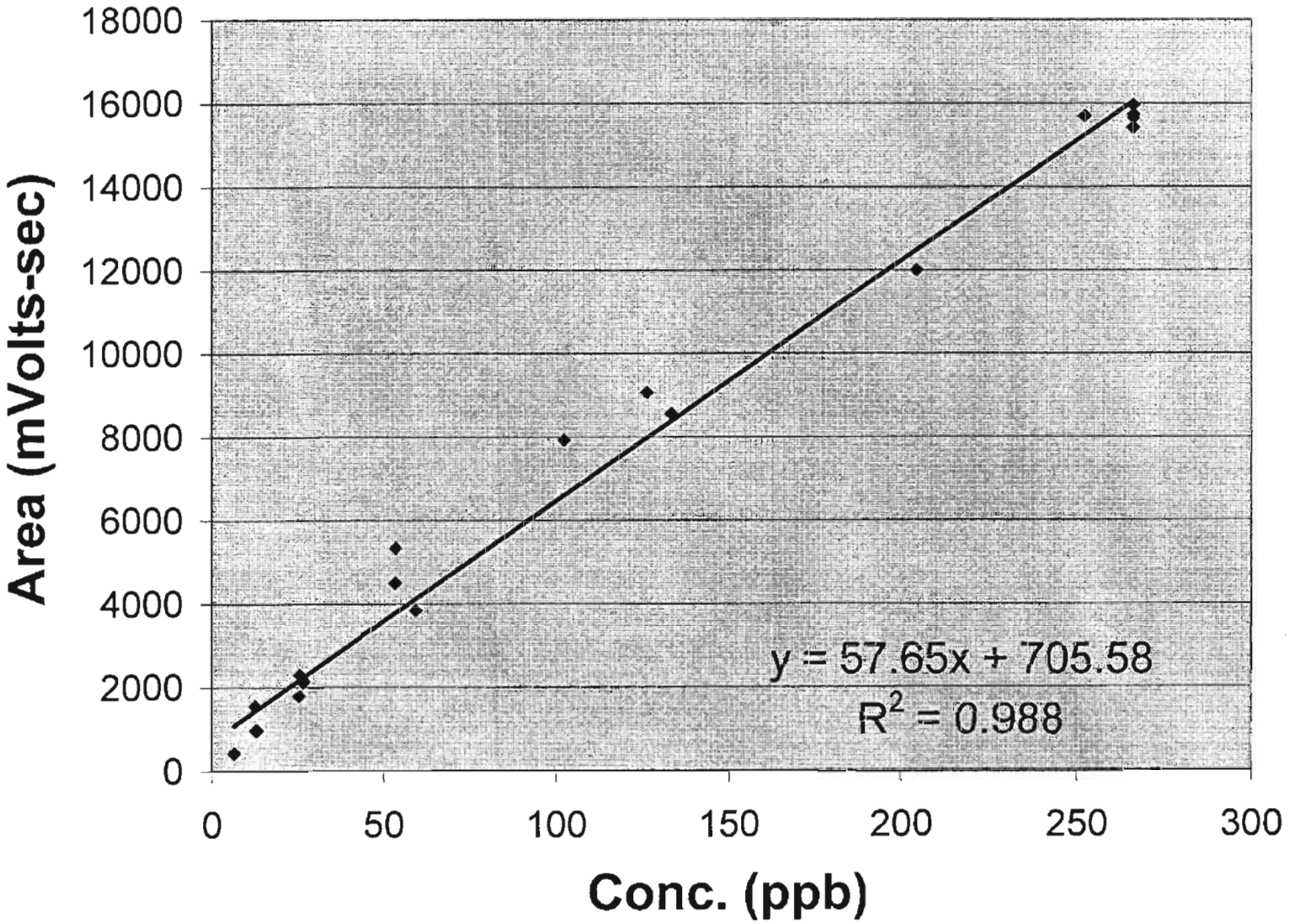

Figure 7: Calibration curve for 3,17-heptafluorobutyrate derivatives of 17β-estradiol (*i.e.*, E_2-2,17-$(HFB)_2$)

heptafluorobutyrate derivative when analyzed by GC/ECD. Furthermore, conversion of the 3,17-heptafluorobutyrate derivative into the mixed derivative was not reproducible, presumably because the alcoholic heptafluoroacyl ester also reacted with trimethylsilane under certain conditions. Because the 3,17-heptafluorobutyrate derivatives were more sensitive, easier to produce and stable for up to 4 weeks, further GC analyses were conducted using the 3,17-heptafluorobutyrate derivatives, which were analyzed within one day of derivatization.

Attempts to detect the derivative in wastewater effluent were unsuccessful due to the large number of coeluting peaks (Figure 8). Further experiments indicated that these peaks were attributable to organic compounds present in the wastewater effluent that were eluted from the HPLC along with the hormones. Attempts to use different chromatographic conditions to reduce the interference were unsuccessful.

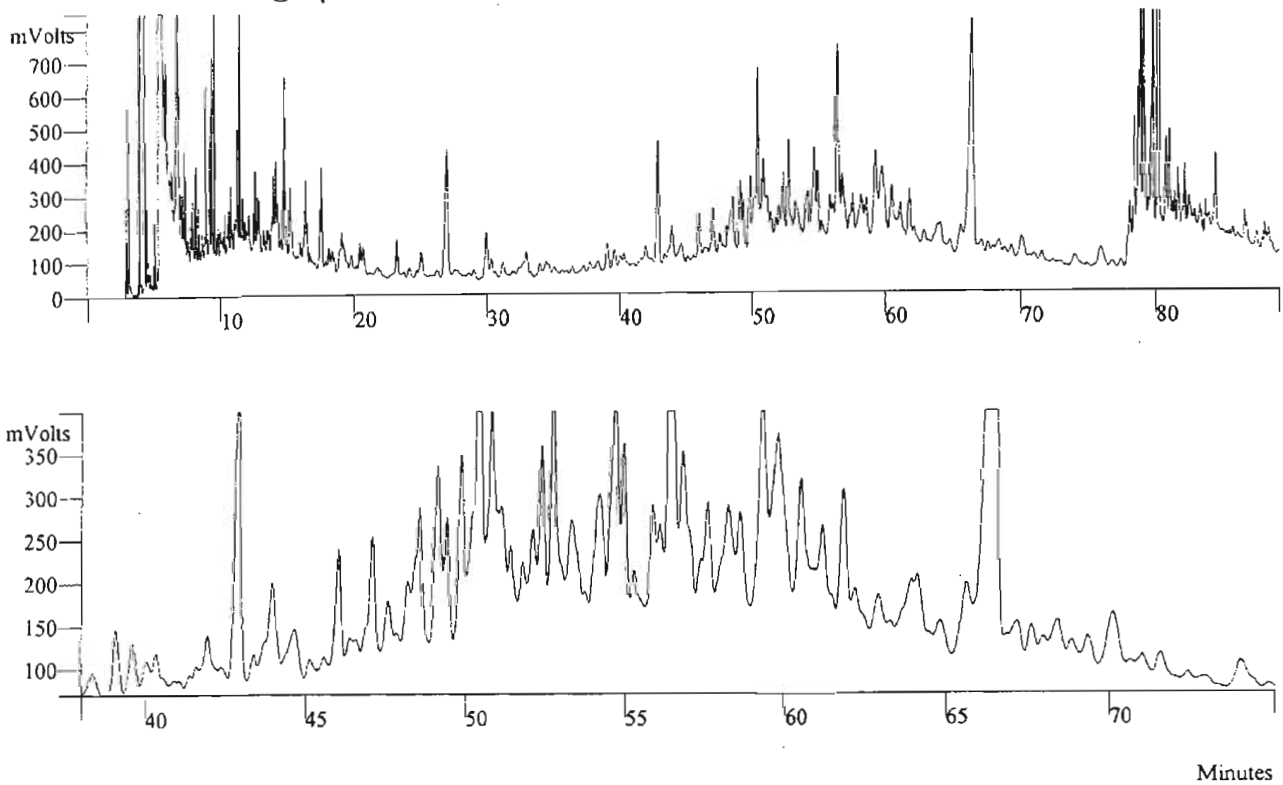

Figure 8: GC chromatogram from analysis of wastewater extract after derivatization with heptafluorobutyric anhydride. 17β-estradiol elutes at approximately 59.4 minutes.

B. Analysis of Samples from Wastewater Treatment Plants

Results from ELISA analyses conducted at WWTPs 1-3 are summarized in Table 6 and Figure 9. In general, the concentrations of hormones were related to the sophistication of the treatment system. Concentrations of hormones were generally highest in the effluent of WWTP 1, the plant that employs only primary and secondary treatment. Lowest concentrations of hormones were observed in the effluent of WWTP 3, the advanced wastewater treatment plant.

At WWTP 1, the concentration of 17β-estradiol was consistent in samples collected on three different days *(i.e.,* 3.3 to 4.6 ng/L). Glucuronide and sulfate conjugates of 17β-estradiol accounted for 0 to 7% of the estradiol released. The concentration of ethinyl estradiol, which was only measured on one date, was 2.3 ng/L.

At WWTP 2, the treatment plant equipped with biological nutrient removal and effluent filtration, the concentrations of 17β-estradiol and ethinyl estradiol were approximately half of the concentrations measured at WWTP 1 (*i.e.,* mean values of 0.95 and 0.43 ng/L, respectively). Concentrations of conjugated hormones, which are only available for one date, also indicate the presence of low concentrations of conjugated hormones (Table 6).

At WWTP 3, the advanced wastewater treatment plant, samples collected after microfiltration yielded concentrations of 17β-estradiol and ethinyl estradiol that were similar to those detected in the effluent of WWTP 2. Samples collected from the effluent of the reverse osmosis system of WWTP 3 yielded concentrations of all three types of hormones that were near method detection limits (*i.e.,* 0.1 ng/L).

Table 6: Concentrations of hormones measured in WWTPs 1-3. Concentrations and recoveries are indicated with ± standard error. NA: No recovery data available.

WWTP	Date	17β-Estradiol (ng/L)	Ethinyl Estradiol (ng/L)	17β-Estradiol Conjugates (ng/L)
WWTP 1	12/9/97	4.18 ± 0.39	NA	<0.1
WWTP 1	1/27/98	3.26	NA	<0.3
WWTP 1	3/11/98	4.57 ± 0.26	2.28 ± 0.34	<0.1
WWTP 2	6/19/98	1.23 ± 0.34	0.42 ± 0.05	<0.1
WWTP 2	7/23/98	0.49 ± 0.18	0.31 ± 0.17	<0.1
WWTP 2	9/28/98	1.13 ± 0.47	0.55 ± 0.19	<0.1
WWTP 3 – MF	3/31/98	1.43	0.32	<1.7
WWTP 3 – RO	3/31/98	<0.1	<0.1	<0.2

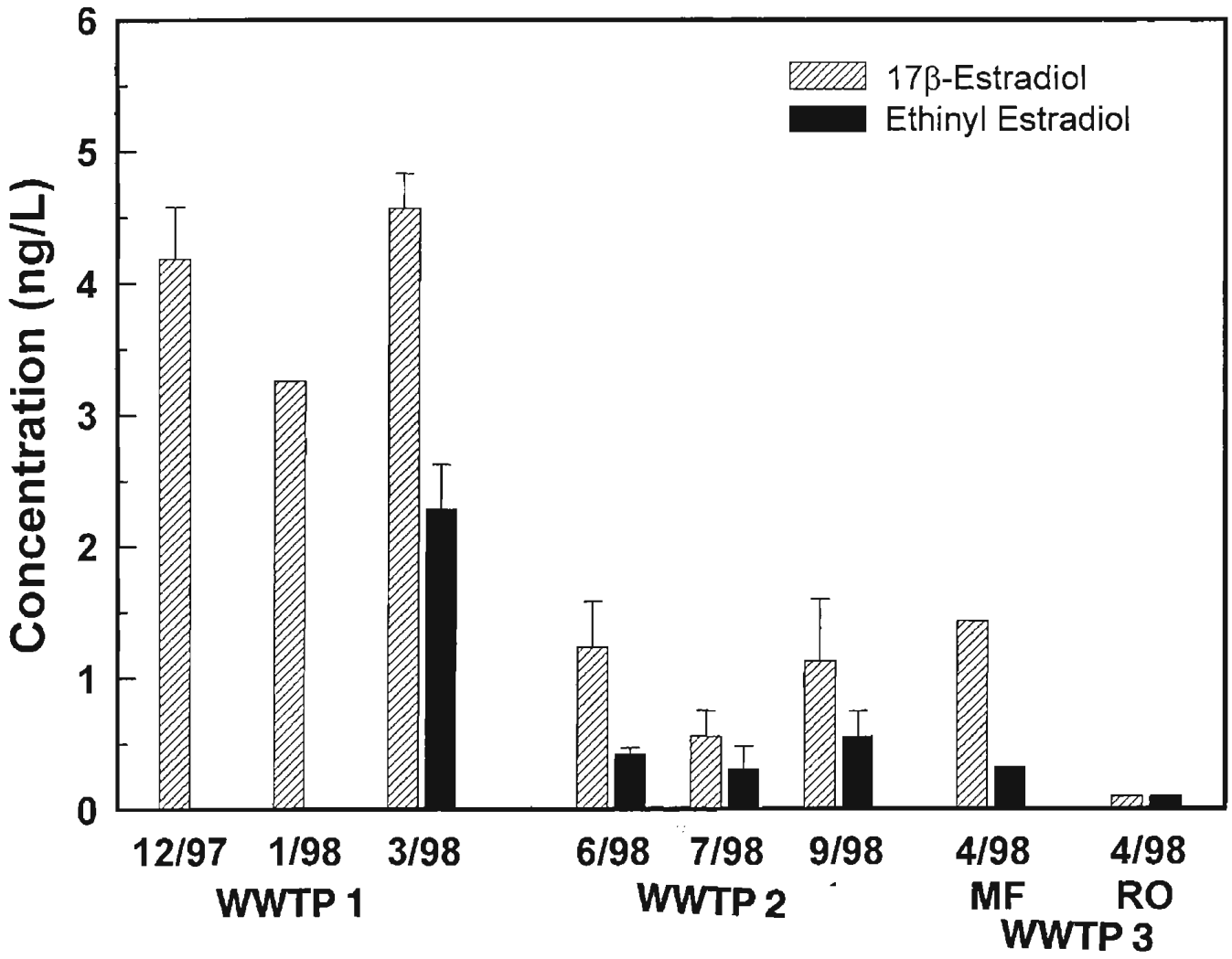

Figure 9: Concentrations of hormones measured in WWTPs 1-3.

IV. DISCUSSION

Results from the method development studies and analysis of samples from three WWTPs indicate that the ELISA techniques is a sensitive, robust and accurate method of measuring the concentrations of 17β-estradiol, 17β-estradiol glucuronide and ethinyl estradiol in wastewater effluents. Under a wide variety of conditions, the technique yields reproducible recoveries. Concentrations of hormones measured in all samples were above method detection limits by a factor of 3 to 40. When low concentrations of hormones are expected in a sample, the detection limit can be increased further by passing a larger volume of sample through the solid phase extraction disk. The volume of sample that can be passed through the disc in a reasonable period is related to dissolved organic carbon (DOC) content of the sample. Therefore, samples with low DOC, such as the effluent from reverse osmosis systems, can be concentrated more than samples from treatment plants that use only primary or secondary treatment.

HPLC cleanup was required to remove interference prior to employing ELISA. When samples were fractionated by HPLC, interfering compounds always eluted prior to the hormones (Figure 4). A significant fraction of the interfering compounds eluted at the same time that strong UV absorbance was observed in the chromatograms (Figure 5). Given the low cross-reactivity of the ELISA antibodies and the high concentrations of surface-active organic compounds in these fractions, we hypothesize that the interference was attributable to non-specific binding of humic substances to the microplate well. Similar types of interference have been described in immunoaffinity chromatography systems.

In contrast to the ELISA technique, attempts to use GC/ECD on wastewater effluent samples were impeded by organic matter that could not be separated from the hormones during HPLC cleanup. The small amount of organic matter that coeluted with the hormones reacted with the derivatizing agent to produce many interfering peaks on the GC/ECD. As a result of the large pre-concentration factor and the high sensitivity of the GC/ECD for fluorinated compounds, these interfering compounds could still cause interference at extremely low concentrations of reactive organic matter. Because initial dissolved organic carbon concentrations in wastewater are typically as high as several milligrams per liter, it is extremely difficult, if not impossible, to completely separate the estrogenic hormones from organic matter using reverse-phase chromatography. The organic compounds detected in this study are probably the same types of compounds that produced coeluting peaks in the method described by Desbrow *et al.* (1998). Preliminary attempts to separate the coeluting peaks from the hormones by solid-phase immunoaffinity extraction have been successful. Further efforts to use immunoaffinity chromatography prior to GC/ECD and GC/MS are beyond the scope of this project and will be reported elsewhere.

Our measurements of 17β-estradiol in a secondary wastewater treatment plant (mean concentration of 4.0 ng/L) is comparable to results reported by Desbrow *et al.* (1998), who reported a mean concentration of 6.2 ± 0.6 ng/L, excluding data from a WWTP that employs only primary treatment. Our data also are consistent with results recently obtained by researchers at Michigan State University (Snyder, 1998). All of these concentrations are consistent with estimates of the concentration of

17β-estradiol in wastewater influent (*i.e.*, 3.5 ng/L). The poor removal of 17β-estradiol during secondary wastewater treatment is not surprising, given the incomplete removal of similar compounds during secondary wastewater treatment. For example, Elhmmali *et al.* (1981) reported the removal of approximately 50% of the dissolved deoxycholic acid, a bile acid, during secondary treatment. The biological transformation of hormones may not be important in wastewater treatment plants because transformation rates decrease at low concentrations. Therefore, the main removal mechanism for hormones is likely to be adsorption onto particles. On the basis of hydrophobicity (*i.e.*, the octanol/water partition coefficient for 17β-estradiol is approximately 15,000) it would be expected that estrogenic hormones would have some affinity for particles in wastewater; however, our data suggest little adsorption occurs.

One possible explanation for the poor removal of these dissolved hydrophobic compounds during secondary treatment is partitioning into organic colloids (*i.e.*, macromolecular humic substances). The association of hydrophobic organic compounds with organic colloids would lower their affinity for particles and slow their rate of biotransformation. Evidence for the colloid-facilitated transport of hydrophobic organic compounds was first reported twenty years ago (Hassett and Anderson 1979; Carter and Suffet, 1982). For example, data from Hassett and Anderson (1979) indicate that as much as 60% of the coprostanol added to surface waters binds to colloidal humic substances. The extent of colloid association of the less hydrophobic steroid hormones is unknown and merits further research.

The low concentrations of conjugated hormones detected in our samples is consistent with enzymatic hydrolysis of conjugates in the wastewater collection and treatment system. Previous studies of conjugated compounds suggests that glururonidase and sulfatase enzymes in manure rapidly hydrolyze conjugated PhACs (Halling-Sørensen *et al.*, 1998) into active forms. Although estrogenic hormones are excreted mainly in conjugated forms, they are converted into active hormones prior to effluent discharge.

Concentrations of ethinyl estradiol detected in this study also are comparable to concentrations reported by Desbrow *et al.* (1998), who reported concentrations as high as 4.3 ng/L in secondary wastewater effluent. Although the concentrations of ethinyl estradiol were below detection limits in most of their samples, Desbrow *et al.* (1998) estimated that mean ethinyl estradiol concentrations should be approximately 0.3 ng/L in secondary wastewater based on the ratio of ethinyl estradiol to 17β-estradiol detected In their samples. This compares favorably with the concentrations we detected (*i.e.*, 2.3 ng/L at WWTP 1 and 0.4 ng/L in WWTP2 and after microfiltration at WWTP3).

Assuming that concentrations of estrogenic hormones are similar in wastewater influent received by all three WWTPs, we can assess the ability of different treatment processes to remove these compounds. In general, the more efficient the wastewater treatment plant is at removing dissolved organic carbon, the lower the concentrations of estrogenic hormones in the effluent (Figure 10). Assuming the predicted influent concentrations (Table 1) are correct, we can estimate the removal efficiency of the three WWTPs (Table 7). Results of these calculations indicate that

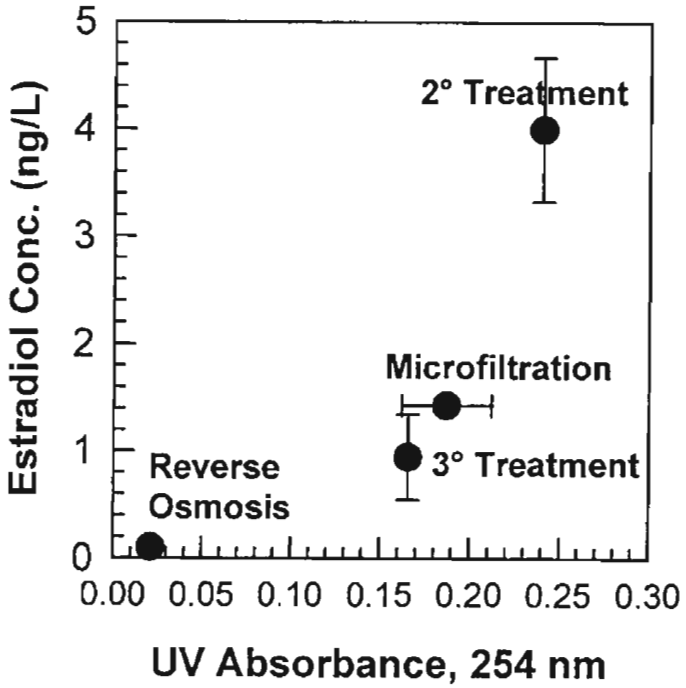

Figure 10: Relationship between concentrations of 17β-estradiol detected in wastewater effluents and UV absorbance at 254 nm.

Table 7: Estimated removal efficiencies of WWTPs 1-3.

Treatment Plant or process	Predicted Wastewater Influent Concentration* (ng/L)	Mean Effluent Concentration (ng/L)	% removal
17β-estradiol			
WWTP 1	3.5	4.0	0
WWTP 2	3.5	1.0	70
WWTP 3/microfiltration	3.5	1.0	70
WWTP 3/reverse osmosis	3.5	0.2	95
ethinyl estradiol			
WWTP 1	2.2	2.3	0
WWTP 2	2.2	0.4	80
WWTP 3/microfiltration	2.2	0.4	80
WWTP 3/reverse osmosis	2.2	0.1	95

notes:
* see Table 1.

the removal of both 17β-estradiol and ethinyl estradiol are similar within each of the treatment plants. The results also suggest that activated sludge secondary treatment

does not remove any of the hormones, whereas the plants that use effluent filtration or microfiltration remove approximately 80% of the hormones. Reverse osmosis removes most, but not all of the hormones.

Results of this study indicate that the concentrations of estrogenic hormones 17β-estradiol and ethinyl estradiol discharged by wastewater treatment plants are high enough to explain vitellogenesis observed in fish exposed to wastewater effluent. However, additional research is needed to ecological effects of these compounds and engineering controls that can be used to remove these compounds. Important areas of future research include:

1. Development of better gas chromatography methods for confirming hormone measurements made by ELISA techniques.
2. Quantification of the fate of hormones after their discharge to surface waters.
3. Detailed assessment of the efficacy of different wastewater treatment processes.
4. Analysis of other PhACs that could cause adverse ecological or human health effects.

V. REFERENCES

Adlercreutz H., Gorbach S.L., Goldin B.R., Woods M.N., Dwyer J.T. and Hamalainen E. (1994) Estrogen metabolism and excretion in oriental and caucasian women. *J. Nat. Cancer Inst.*, 86, 1076-1082.

Arcand-Hoy L.D., Nimrod A.C. and Benson W.H. (1998) Endocrine modulating substances in the environment: estrogenic effects of pharmaceutical products. *Internat. J. Toxicol.* In press.

Buser H.R., Müller M.D. and Theobald N. (1998) Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea. *Environ. Sci. Technol.*, 32, 188-192

Carter C.W. and Suffet I.H. (1982) Binding of DDT to dissolved humic materials. *Environ. Sci. Technol.*, 16, 735-740.

Dehennin L. (1989) Estradiol-17β determined in plasma by gas chromatography-mass spectrometry with selected ion monitoring of mixed silyl ether-perfluoroacyl ester derivatives and use of various stable-isotope-labeled internal standards. *Clin. Chem.*, 3514, 532-536.

Desbrow C. Routledge E.J., Brighty G.C., Sumpter J.P. and Waldock M. (1998) Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in Vitro biological screening. *Environ. Sci. Technol.* 32, 1549-1558.

Elhmmali M.M., Roberts D.J. and Evershed R.P. (1997) Bile acids as a new class of sewage pollution indicator. *Environ. Sci. Technol.* 31, 3663-3668.

Glaser M. (1996) Annual prescription survey. *Drug Topics*. 97-104.

Halling-Sørensen B., Nielsen S.N., Lanzky P.F. Ingerslev F. Lützhøft H.C. and Jørgensen S.E. (1998) Occurrence, fate and effects of pharmaceutical substances in the environment-A review. *Chemosphere* 36, 357-394.

Hartmann A., Alder A.C., Koller T. and Widmer R.M. (1998) Identification of fluoroquinolone antibiotics as the main source of *umuC* genotoxicity in native hospital wastewater. *Environ. Toxicol. Chem.*, 17, 377-382.

Harries J.E., Sheahan D.A., Jobling S., Mattiessen P., Neall P., Routledge E.J., Rycroft R., Sumpter J.P. and Tylor T. (1996) A survey of estrogenic activity in United Kingdom inland waters. *Environ. Toxicol. Chem.* 15, 1993-2002.

Harries J.E., Sheahan D.A., Jobling S., Mattiessen P., Neall P., Routledge E.J., Rycroft R., Sumpter J.P. and Tylor T. (1997) Estrogenic activity in five United Kingdom

rivers detected by measurement of vitellogenesis in caged male trout. *Environ. Toxicol. Chem.* 16, 534-542.

Hassett J.P. and Anderson M.A. (1979) Association of hydrophobic organic compounds with dissolved organic matter in aquatic systems. *Environ. Sci. Technol.* 13, 1526-1529.

Herberer T and Stan H.J. (1996) Vorkommen von polaren organischen Kontaminanten im Berliner Trinkwasser. *vom Wasser.* 86, 19-31.

Jobling S., Nolan M., Tyler C.R., Brighty G. and Sumpter J.P. (1998) Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* 32, 2498-2506.

Levy S. (1993) Confronting multidrug resistance: a role for each of us. *JAMA*, 269, 1840-1842.

Norpoth K., Nehrkorn A., Kirchner M., Holsen H. and Teipel H. (1973) Untersuchungen zur Frage der Loeslichkeit und Staebilitat ovulationschemmender Steroide in Wasser, Abwaesser und Blelbschlamm. *Zbl. Bakt. Hyg., I. Abt. Orig. B,* 156, 500-511.

Purdom C.E., Hardiman P.A., Bye V.J., Eno N.C., Tyler C.R. and Sumpter J.P. (1994) Estrogenic effects from sewage treatment works. *Chemistry and Ecology*, 8, 275-285.

Rathner M. and Sonneborn M. (1977) Biologisch Wirksame Oestrogene in Trink- und Abwasser. *Forum Staedte-Hygeine.* 30, 45-49.

Richardson M.L. and Bowron J.M. (1985) The fate of pharmaceutical chemicals in the aquatic environment. *J. Pharm. Pharmacol.,* 37, 1-12.

Routledge E.J., Sheahan D., Desbrow C., Brighty G.C., Waldock M. and Sumpter J.P. (1998) Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. *Environ. Sci. Technol.* 32, 1559-1565.

Snyder S.A. (1998) personal communication with S. Snyder, Institute for Environmental Toxicology, Michigan State University, East Lansing, MI.

Stumm-Zollinger E. and Fair G.M. (1965) Biodegradation of steroid hormones. *JWPCF*, 37, 1506-1510.

Tabak H.H. and Bunch R.L. (1970) Steroid hormones as water pollutants. In: Developments in Industrial Microbiology. Volume 11, pp. 367-376. American Institute of Biological Sciences, Washington, D.C.

Tabak H.H., Bloomhuff R.N. and Bunch R.L. (1981) Steroid hormonmes as water pollutants II. Studies on the persistence and stability of natural urinary and synthetic ovulation-inhibiting hormones in treated and untreated wastewaters. *Developmental and Industrial Microbiology*, 22, 457-519.

Tang P.W. and Crone D.L. (1989) A new method for hydrolyzing sulfate and glucuronyl conjugates of steroids. *Anal. Biochem.*, 182, 289-294.

Ternes T.A. (1998) Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.*, 32, 3245-3260.