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Analytical Methods for Detection of Selected Estrogenic Compounds in Aqueous Mixtures

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Both natural estrogens and synthetic compounds that mimic estrogen can reach the aquatic environment through wastewater discharges. Because nonylphenol (NP), octylphenol (OP), nonylphenol polyethoxylates (NPE), 17β-estradiol (E2), and ethynylestradiol (EE2) have previously been found to be estrogenic and to occur in wastewater effluents, they were the primary analytes for which the method was developed. Water samples were extracted in situ using solid-phase extraction disks. Analytes were separated by high-pressure liquid chromatography and detected by fluorescence or competitive radioimmunoassay (RIA). Method detection limits (MDLs) using HPLC with fluorescence detection were 11, 2, and 52 ng/L of water for NP, OP, and NPE, respectively. The RIA MDLs for E2 and EE2 were 107 and 53 pg/L, respectively. Samples were collected from four municipal wastewater treatment plants in south central Michigan, eight locations on the Trenton Channel of the Detroit River, MI, and five locations in Lake Mead, NV. Concentrations of NP and OP ranged from less than the MDL to 37 and 0.7 μg/L, respectively. NPE concentrations ranged from less than the MDL to 332 μg/L. Concentrations of E2 and EE2 ranged from less than the MDLs to 3.7 and 0.8 ng/L, respectively.

Introduction

The potential effects of chemicals that alter the normal endocrine function and physiological status of animals have been an increasing concern in recent years (*1*). Reports of apparent increases in hormone-dependent cancers and decreases in sperm quantity and quality in humans have raised questions about the role of natural and synthetic chemicals in these trends (*2*−*7*). A number of pollutants including pesticides, certain polychlorinated biphenyls (PCBs), dioxins, furans, alkylphenols, synthetic steroids, and natural products such as phytoestrogens have been reported to disrupt normal hormonal pathways in animals and collectively have been referred to as endocrine disrupters or endocrine disrupting chemicals (EDCs) (*2, 8*). While EDCs can operate through a number of both direct and indirect mechanisms of action, of particular concern are those compounds that mimic endogenous estrogens. The Safe Drinking Water Act Amendments of 1995 (Bill No. S.1316) and the Food Quality Protection Act of 1996 (Bill No. P.L. 104-170), which mandate comprehensive screening for estrogenic and anti-estrogenic chemicals, are examples of the increasing public concern regarding endocrine disruption. While it is known that many natural and synthetic chemicals are estrogenic, it is unclear whether the concentrations of estrogenic agents present in the environment are sufficient to cause adverse physiological effects. One aspect of conducting human or wildlife risk assessments is an exposure assessment. This suggests the need for assays and techniques to monitor the quantity and effects of endocrine disrupters.

Wastewater treatment plants (WWTPs) receive natural and synthetic EDCs from urban and industrial dischargers. WWTPs use a variety of treatment processes of varying efficiency such that in some cases compounds are not completely removed by the treatment processes and are ultimately discharged into surface waters. Fish caged below WWTP outfalls have been reported to have abnormal ratios of sex steroid hormones as well as to exhibit histological changes in their reproductive organs (*9*−*11*). Wild fish living in waters influenced by WWTP effluents have been observed to express similar effects attributed to unknown or unspecified EDCs (*12*−*14*). Extracts of some WWTP effluents in the United Kingdom have been shown by use of in vitro assays to be estrogenic due to the presence of alkylphenols and natural and synthetic estrogens (*15*).

Alkylphenol polyethoxylates (APEs) are nonionic surfactants widely used in various industrial (55% of total demand), institutional (30% of total demand), and household applications (15% of total demand) (*16*). APEs are among the most widely used nonionic surfactants, with 1998 U.S. use of approximately 204 million kg/year (*16*). Most of the APEs used are of the nonylphenol polyethoxylate (NPE) types that contain a 9-carbon branched isomeric alkyl group (*16, 17*). NPEs and the less widely used octylphenol polyethoxylates (OPEs) degrade during wastewater treatment or in the environment to alkylphenol ethoxycarboxylates (APECs), lower oligomer APEs such as NPE_{1-3}, and alkylphenols such as octylphenol (OP) and nonylphenol (NP) (Figure 1) (*17*−*20*). NP and OP have been shown to be estrogenic in a variety of both in vitro (*9, 15, 21*−*23*) and in vivo bioassays (*24*−*26*). Some oligomers of NPE have also been found to be estrogenic in vitro (*9, 23, 24, 27*) and in vivo (*24*). Reports of APEs and their corresponding degradation products from the United States are scarce. The most comprehensive survey reports concentrations from 30 U.S. rivers that are influenced by municipal or industrial wastewater effluents (*17*). That study found that 60−75% of samples had no detectable levels of NP, NPE_1, and NPE_2 (*17*).

The endogenous hormone 17β-estradiol (E2) and the synthetic hormone 17α-ethynylestradiol (EE2) have been detected in several WWTP effluents (Figure 1) (*15, 28*−*31*). Synthetic hormones are generally more stable in water than are natural hormones and have greater potency (*31, 32*). EE2 was found to be unchanged after 120 h in activated sludge treatment, indicating possible release into surface waters after wastewater treatment (*29*). EE2 is a widely used pharmaceutical with daily oral doses ranging from 30 to 50 μg (*33*). Estrogens are excreted by humans in concentrations as great as 2.7 mg/L on a daily basis (*34*). Conjugated estrogens used in the treatment of cancer, hormonal imbalance,

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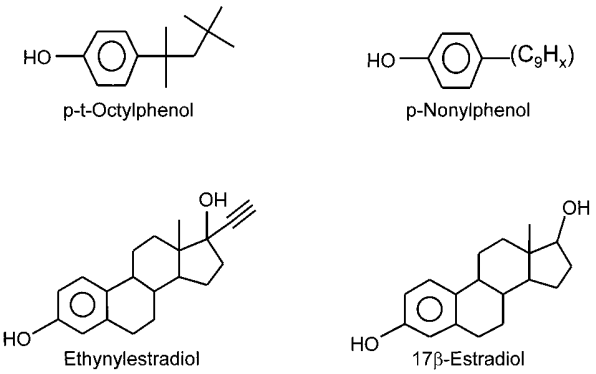

FIGURE 1. Structures of target compounds.

osteoporosis, and other ailments are the second most prescribed drug in the United States and are administered orally in doses ranging from 0.3 to 2.5 mg (*33*). Estrogens are excreted by mammals as water-soluble conjugates in urine or as "free" estrogens in feces (*30, 35, 36*). Conjugated forms of these estrogens can be deconjugated during wastewater treatment and in the environment to generate the more potent free estrogen (*15, 31*). Environmental concentrations of E2 and EE2 range from less than detection to greater than 140 ng/L and from less than detection to 15 ng/L, respectively (*15, 28, 29, 32*). Fish exposed to E2 and EE2 exhibit changes in biomarkers for estrogenicity at concentrations as low as 0.5 ng/L (*10, 15, 37, 38*).

In this paper, we describe a rapid and sensitive method for detecting and quantifying EDCs in surface waters. The method was designed to isolate and concentrate a broad range of organic compounds from surface waters so that it could be used as an analytical tool for toxicity identification and evaluation (TIE). An in situ extraction technique using 90-mm styrenedivinylbenzene (SDB) Empore solid-phase extraction (SPE) disks was developed. This technique permits the extraction of greater volumes of water more rapidly and efficiently than the typical vacuum filtration methods (*39*). This method also provides detection limits less than the reported effects concentrations for these compounds in the aquatic environment. The SDB matrix is effective for extracting polar organics, which is necessary as the compounds of interest all have polar hydroxyl functional groups. Previously, Empore disks have been used for the extraction of natural water sucessfully (*18, 39*−*41*). However, these compounds of interest were not investigated. APs and certain steroids are extracted by this method without requiring the transport of large volumes of water, thus reducing losses due to adsorption to transport containers and due to microbial degradation (Figure 2). To demonstrate the utility of this method, samples of effluents and surface waters were collected from several locations in the Trenton Channel of the Detroit River, MI; below the outfalls of municipal WWTPs in south central Michigan; and in the Las Vegas (LV) Wash and in Lake Mead, NV.

Experimental Section

Standards and Reagents. All standards and reagents used were of the highest purity commercially available. High purity standards (98% pure) of *p*-nonylphenol (NP), *p-tert*-butylphenol (BP), and *p-tert*-octylphenol (OP) were obtained from Schenectady International (Freeport, TX). Standards of nonylphenol monoethoxylate (NPE1) and nonylphenol polyethoxylates (Surfonic N-95) were obtained from Huntsman Corporation (Austin, TX). Standards of E2 and EE2 of 98% purity were obtained from Sigma Chemical Company (St. Louis, MO). High-purity pesticide residue grade acetonitrile (ACN), methanol (MeOH), hexane, dichloromethane ($MeCl_2$),

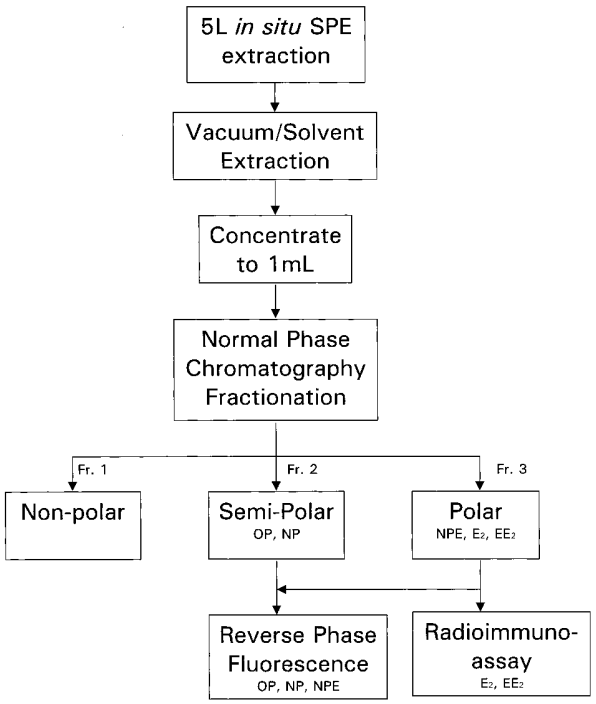

FIGURE 2. Flow diagram of analytical method. SPE, solid-phase extraction.

isooctane, 2-propanol, and acetone were obtained from Burdick and Jackson (Muskegon, MI) or Sigma-Aldrich (Milwaukee, WI). Solvents were also tested for interferences by examining 150-fold concentrated solvents using high-pressure liquid chromatography (HPLC) with fluorescence detection and gas chromatography/mass spectrometery (GC/MS). Reagent water was first purified by reverse osmosis (RO) followed by Nanopure (Barnstead, Dubuque, IA) treatment. Glass fiber filters, fine grade (GF/F) of 0.7 μm nominal pore size and coarse grade (GF/C) of 1.2 μm nominal pore size (Whatman, Maidstone, England), were heated at 450 °C for 4 h in aluminum foil prior to use. Anhydrous granular sodium sulfate (EM Science, Gibbstown, NJ) was heated at 550 °C overnight and then stored at 125 °C until the day of use.

Sample Collection and Preservation. Water samples were collected from eight sites in the Trenton Channel of the Detroit River, MI; four WWTPs in south central Michigan, and five sites in Lake Mead, NV. To minimize effects due to dilution, effluent samples were collected as close to the source as possible. Samples from the Trenton Channel and Lake Mead were collected at 50% of the maximum depth or 3 m, whichever was less. Samples were collected between April and October 1997.

All samples were filtered and extracted at the sample location (Figure 2). Water samples were pumped through fluoropolymer (PFA) tubing (3/8 in. o.d.) by a Fluid Metering (Oyster Bay, NY) DC pump (model QB) fitted with a ceramic-lined stainless steel pump head (model Q1-CSC) to a 90-mm stainless steel pressure filtration holder (Millipore, Bedford, MA). Flow rate was maintained at 100 mL/min until 5 L of total volume had been extracted or until the pressure exceeded 100 psi. Stainless steel screen (250 μm mesh, Aquatic Ecosystems, Apopka, FL) was used to prevent large particles from entering the inlet tubing. A glass fiber filter was placed on each side of a 90-mm poly(styrenedivinylbenzene) (SDB-XC) Empore disk (3M Corporation, St. Paul, MN). The bottom filter (90 mm GF/C filter) was used to improve flow characteristics, and the top filter (90 mm GF/F) was used to filter particulates from the sample. Fifteen milliliters each of

acetone, $MeCl_2$, and MeOH and 20 mL of reagent water were injected sequentially through a custom Luer lock fitting on the relief valve of the filtration holder with a 2-min residence period to condition the Empore disk. A McMillan (Georgetown, TX) electronic flow meter (model 102-6TP), calibrated to ±1% accuracy with National Institute of Standards and Technology (NIST) traceable standards, was installed downstream of the filtration holder. This digital rate meter and totalizer was used to monitor flow rate and to record the total volume. Once the desired sample volume had been reached, a volume of 20 mL of air was pushed through the filter holder to move headspace water through the holder. The filter holder then was disassembled, and the GF/F and Empore disk were stored separately in clean aluminum foil and kept on ice for transport to the laboratory. The GF/C filter was discarded.

At each sampling site, prior to collecting the samples, the apparatus was purged with sample for 10 min by bypassing the filter holder. At sites with concentration gradients, samples were taken sequentially from the least to greatest predicted concentrations. Flow meter calibration was verified prior to each sampling trip by comparing the reported totalizer output to the measured volume of water exiting the meter. The sampling apparatus was cleaned with water and solvents before and after each sample.

Field blanks were taken daily by passing 5 L of Nanopure water through the system as previously described. Laboratory blanks were performed on each day of extractions, and instrument blanks were conducted daily. None of the compounds of interest were detected in the blanks. Breakthrough tests were performed by placing two Empore disks in series and analyzing each disk separately. Breakthrough was detected only at the greatest concentrations spiked, and the breakthrough was determined to be less than 11%.

Extraction. Empore disks were eluted on a 90-mm vacuum manifold (3M Corporation, St. Paul, MN). Fifteen milliliters of acetone, 25 mL of $MeCl_2$, and 10 mL of hexane were added sequentially to the reservoir and collected in a 50-mL centrifuge tube. Slight vacuum was applied to initiate solvent transfer, and the remainder was permitted to flow without vacuum. Vacuum was applied once all solvents had passed through the disk to remove residual solvents from the disk. Moisture was removed from the solvent extract by passing it through 30 g of anhydrous sodium sulfate placed in a custom glass funnel containing a stopcock. The eluate was collected in a flat-bottom flask and rotary evaporated at 30 °C to approximately 5 mL. The sample was concentrated and solvent-exchanged to 1 mL of isooctane under a gentle stream of nitrogen at 30 °C. One milliliter of 40% hexane in $MeCl_2$ was added to the test tube and vortexed for 5 s. The sample was collected in a 2.5-mL Hamilton syringe (Reno, NV) and injected into a normal-phase HPLC system for fractionation.

Normal-Phase HPLC Fractionation. The HPLC fractionation system consisted of a Rheodyne 7725i injector (Cotati, CA) with a 5-mL sample loop, a quaternary pump (Perkin-Elmer, Series 410, Norwalk, CT), and an electronic fraction collector (ISCO Foxy 200, Lincoln, NE). A Phenomenex Luna 5-μm silica column (250 mm × 4.6 mm, Torrance, CA) was used for normal-phase separations with 3-step isocratic elution. The mobile-phase solvent profile was 30% $MeCl_2$ in hexane for 15 min, $MeCl_2$ for 20 min, and MeOH for 20 min, each at 1 mL/min with no gradient curves. The column was returned to initial conditions by passing $MeCl_2$ for 10 min followed by 30% $MeCl_2$ in hexane for 35 min at 1 mL/min. Fractions were collected from 0 to 20 min (F1), 20−45 min (F2), and 45−70 min (F3) with no delay. F1 contained the most nonpolar compounds such as PAHs, PCBs, organochlorine (OC) pesticides, and fluorescent compounds leached from the Empore SDB-XC disk. F2 contained BP, NP, and OP. F3 contained the most polar compounds, including E2, EE2, and NPEs. Each fraction was concentrated by rotary evaporation at 30 °C to approximately 5 mL. The sample was evaporated and solvent-exhanged to 1 mL of ACN under a gentle stream of nitrogen. No internal or surrogate standards were added to the samples since these could interfere with the in vitro bioassays used to screen for total estrogenic and dioxin-like activity.

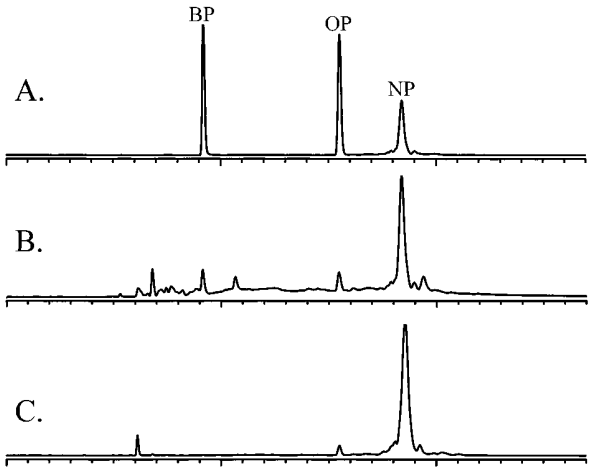

FIGURE 3. Reverse-phase HPLC of fraction 2: (A) spiked standards; (B) Trenton Channel−Black Lagoon 8/30/97; (C) WWTP−BV effluent 10/8/97.

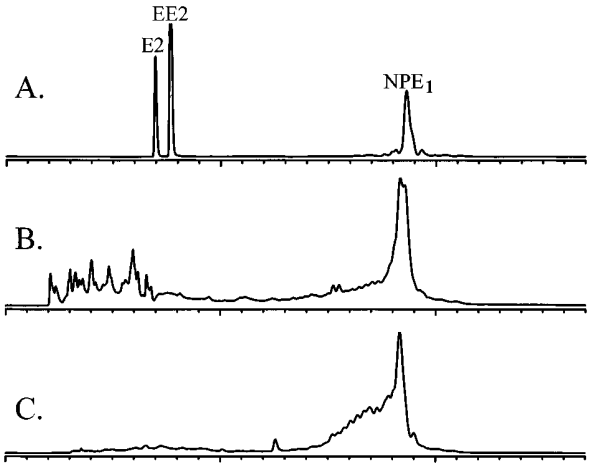

FIGURE 4. Reverse-phase HPLC of fraction 3: (A) spiked standards; (B) Trenton Channel−Black Lagoon 8/30/97; (C) WWTP−BV effluent 10/8/97.

Quantitation of Compounds. F2 and F3 were analyzed by reverse-phase HPLC (Figures 3 and 4). The reverse-phase HPLC system included a Perkin-Elmer (PE) (Norwalk, CT) series 200 autosampler, a PE series 200 binary pump, a Hewlett-Packard (HP) 1046A flourescence detector, and a PE TurboChrome 4.0 data software package. The column used was a Phenomenex Prodigy 5-μm octadecylsilica (ODS) 100 Å (250 mm × 4.6 mm, Torrance, CA) preceded by a 30 mm × 4.6 mm guard column of the same packing material. For all compounds of interest, the fluorescence detector settings were as follows: 229 nm excitation, 310 nm emission, PMT gain of 12, lamp time of −1, response time of 2 s, stop time at 27 min, gate and delay at zero.

Elution solvents for gradient elution were reagent water and ACN delivered at a constant flow rate of 1 mL/min. The elution profile was a 20-min gradient (curve = −2) from 50% water and 50% ACN to 2% water and 98% ACN followed by a 10-min isocratic ACN purge. Each sequence began with

one blank and one standard. The injection volume of standards and samples was 10 μL. Peak areas were determined by electronic integration, and sample concentrations were determined using Microsoft Excel version 7.0. Chromatography and retention times for the compounds of interest are shown in Figures 3 and 4. All calibration curves were linear ($r^2 > 0.99$) across the entire calibration range. An HP 5890 series II plus GC with a 30-m DB5-MS capillary column (J&W Scientific, Folsom, CA) and a HP 5972 series mass selective detector (MSD) were used for confirmation of peak assignments.

Radioimmunoassay (RIA). Competitive radioimmunoassay (RIA) was used for detection of E2 and EE2 following a procedure previously described for measurement of these compounds in plasma and adapted for environmental samples (*14*). The most polar fraction (F3) from each of the HPLC fractionation procedures was analyzed in duplicate for both E2 and EE2. A 20-μL aliquot of sample was evaporated to dryness in a 5-mL glass test tube by use of a vortex evaporator (Labconco, Kansas City, MO) and nitrogen gas. Each sample was reconstituted in 0.5 M sodium phosphate buffered saline buffer (PBSGA). Standard curves were prepared in PBSGA.

Accuracy and precision of the assay were determined in several ways. Cross-reactivities of the E2 antiserum with other steroids are 11.2% for estrone; 1.7% for estriol; <1.0% for 17α-estradiol and androstenedione; and <0.1% for EE2, DES, and all other steroids examined. Cross-reactivities of the EE2 antiserum with other steroids were 0.3% for E2; <0.1% for norethindrone, estrone, 17α-estradiol, diethylstilbestrol, hexoestrol, and dienoestrol; and <0.01% for all other steroids and compounds tested. To determine recovery during reverse-phase HPLC fractionation, known amounts (1, 2, 5, 10, 25, 50, 100, 250, and 500 pg) of E2 or EE2 were fractionated by HPLC, and the masses of the recovered materials were determined. No significant losses occurred during fractionation. Parallelism between the dose−response relationships for standards and samples were evaluated by assaying dilutions of HPLC fractions (2.5, 5, 10, 15, and 20 μL HPLC fraction volumes). The standard curves were determined to be parallel to the dilution curves for HPLC fractions.

Recovery and Precision. Spike/recovery experiments were performed using reagent water or untreated groundwater to determine the accuracy and precision of the method. Nineteen liter glass carboys were spiked with NP, BP, OP, and EE2 and mixed for at least 1 h. Three 4-L samples were sequentially extracted from the 19-L mixture using the procedure described as above. Three spikes were also performed to determine the effect of larger volumes by spiking 19 L and extracting 17 L. Flow rate was optimized by spiking 4 L of water with the compounds of interest and loading the Empore disks at different rates.

For NPE, quantitation was based on a sum of all oligomers and reported as total NPE (NPE). The instrument was calibrated using Surfonic N-95. Therefore, all results relative to NPE concentrations are semiquantitative in relation to the defined conditions. To ensure NPE oligomer distribution integrity, oligomer distribution of extracted NPE was compared to that of an NPE (as Surfonic N-95) standard. The NPE oligomer distribution was determined by normal-phase HPLC using a Phenomenex Phenosphere 5 μm cyanopropyl column (250 mm × 4.6 mm). The PE binary HPLC instrument and fluorescence detector have been described previously. The mobile phase solvents consisted of 35% 2-propanol in MeOH (A) and hexane (B). The elution profile was 3% A and 97% B for 7 min at a flow rate of 0.5 mL/min isocratic followed by a 20-min gradient to 95% A and 5% B at a curve of −5 and flow rate of 1 mL/min. The column was purged a 1 mL/min with 3% A and 97% B to return the column to the initial conditions. It was determined that the oligomer distribution remained unchanged throughout the procedure (Figure 5). Oligomer-specific distributions were not determined for environmental samples.

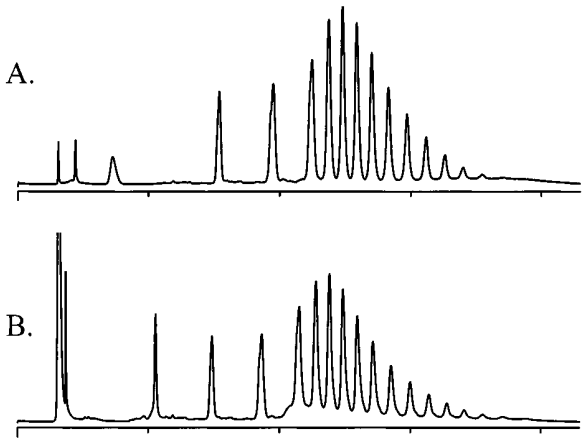

FIGURE 5. Normal-phase HPLC of NPE oligomer distribution: (A) NPE standard; (B) extracted NPE.

TABLE 1. Recoveries through the Analytical Procedure (n = 3) (% Recovered ± SD)

spike concn (ng/L)	vol	EE2	BP	OP	NP
240	5	78 ± 6	65 ± 7	76 ± 4	77 ± 7
1128	5	78 ± 4	62 ± 3	72 ± 3	74 ± 5
2374	5	76 ± 5	61 ± 8	74 ± 4	76 ± 5
5934	5	74 ± 3	65 ± 6	73 ± 6	72 ± 8
23737	5	72 ± 7	46 ± 4	68 ± 9	69 ± 4
10761	17	63 ± 7	48 ± 5	67 ± 10	67 ± 9

To determine losses of EE2, OP, NP, and BP during the evaporation procedure, these compounds were spiked into 250-mL flat-bottom flasks containing the correct amounts and ratios of solvents used for the Empore extraction. Solvents were rotary evaporated, and the volume was adjusted as shown earlier.

Detection Limits. The instrumental detection limits (IDLs) and limits of quantitation (LOQs) for NP, OP, BP, NPE, and EE2 by reverse-phase HPLC with fluorescence detection were determined by injecting 10 μL of a 50 ng/mL mixture of each of these compounds 5 times. The IDLs and LOQs are defined as 3 and 10 times the standard deviation (SD) of the quantified peak for each compound in the mixture, respectively. The method detection limits (MDLs) using reverse-phase HPLC with fluorescence detection (10 μL injections) were estimated from the mass of analyte at the LOQ divided by 5-L sample size plus 25% to account for a 75% average recovery. The IDLs for E2 and EE2 using RIA were determined using ImmunoFit EIA/RIA Data Analysis Software (Beckman, Fullerton, CA). The minimum concentration distinguishable from zero for E2 and EE2 was 427 and 211 pg/mL extract, respectively. The MDLs for E2 and EE2 using RIA detection were calculated in the same manner as for HPLC with fluorescence detection.

Results

Recoveries for these compounds at various concentrations are shown (Table 1). It was determined that recovery did not vary significantly between untreated groundwater and reagent water. The compounds of interested added to a natural water sample from Lake Mead were recovered within the range determined by laboratory spike/recovery experiments. Flow rates greater than 500 mL/min resulted in poor recoveries (<50%) possibly due to breakthrough. The greatest average recovery of >80% for all analytes was achieved using

TABLE 2. Dection Limits for Selected Xenoestrogens[a]

method		E2	EE2	BP	OP	NP	NPE
HPLC/fluorescence	IDL (ng/mL)	nm	5	4	3	13	62
HPLC/fluorescence	LOQ (ng/mL)	nm	16	13	9	42	208
HPLC/fluorescence	MDL (ng/L)	nm	4	3	2	11	52
RIA	IDL (pg/mL)	427	211	na	na	na	na
RIA	LOQ (pg/mL)	na	na	na	na	na	na
RIA	MDL (pg/L)	107	53	na	na	na	na

[a] nm, not measured; na, not applicable.

a flow rate of 50 mL/min; however, this resulted in extraction times >1.5 h. A flow rate of 100 mL/min resulted in consistent recoveries of the compounds of interest with an acceptable average recovery >70% (Table 1). BP was the only compound with poor recoveries (average 60%). BP was tested for use as a possible surrogate standard in future studies because it has been used previously as an internal standard (*42*). BP has a greater volatility than the other compounds of interest, which is the likely cause for its lesser recoveries (Table 1). If rotary and nitrogen evaporation temperatures and final volumes were not carefully monitored, losses of up to 50% were observed. By maintaining the evaporation temperatures at 30 °C and avoiding overconcentration, evaporation losses could be kept consistent and less than 10%.

It was determined that EE2 could function as a surrogate for E2. Recoveries for E2 and EE2 were virtually the same for each experiment performed. Additionally, E2 required fresh standards weekly, while EE2 was stable and remained in calibration for at least 6 months. NPE was also spiked and recovered 4 times with an average recovery of 71% ± 12. In environmental samples, oligomers greater than NPE_4 rarely were observed (qualitatively) by GC/MS.

The IDLs, LOQs, and MDLs for the compounds of interest for each method of detection are presented in Table 2. The coefficient of variation for duplicate RIA analyses was less than 12% in each case.

The compounds of interest were detected in some samples (Table 3). NP was detected in 17 of the 23 samples collected (not including replicate samples) with concentrations ranging from less than detection to 37 μg/L. OP was detected in 13 of the 23 samples analyzed (not including replicate samples) with concentrations ranging from less than the MDL to 0.67 μg/L. NPE was detected in 16 of the 23 samples analyzed (not including replicate samples) with concentrations ranging from less than the MDL to 332 μg/L. E2 was detected in 16 of the 22 samples analyzed (not including replicate samples) with concentrations ranging from less than the MDL to 3.7 ng/L. EE2 was detected in 8 of the 22 samples analyzed (not including replicate samples) with concentrations ranging from less than the MDL to 0.76 ng/L.

Discussion

The Trenton Channel of the Detroit River, MI, is in an industrialized and urbanized area with point sources of industrial and municipal wastewater effluents (*20, 43, 44*). Concentrations of NP and OP at Grosse Ile, an island that forms the Trenton Channel, have been reported to be 0.12 and 0.045 μg/L, respectively, in June 1994 (*20*). These concentrations are similar to those observed in our studies. The Trenton Channel is influenced by these compounds not only in the headwaters but also by point sources entering the Channel. Monguagan Creek appears to provide the greatest loading of NP, OP, and NPE to the Trenton Channel (Table 3). This Creek was previously reported to be a source for unique *tert*-alkylphenols such as 4-*tert*-pentylphenol (*45*). Many of these unique alkylphenols were also detected during GC/MS screening in this study. However, no concentrations are reported here. No trend was observed from the concentrations of E2 and EE2 detected in the Trenton Channel. The greatest concentration of E2 and the only detectable concentration of EE2 were at the Black Lagoon site.

Concentrations of NP, OP, NPE, E2, and EE2 were determined in four WWTP effluents in south central Michigan. Samples were also analyzed upstream from three of the

TABLE 3. Concentrations of Selected Xenoestrogens in Water Samples (Corrected for Recovery)

location	date	NP (ng/L)	OP (ng/L)	NPE (ng/L)	E2 (pg/L)	EE2 (pg/L)
			Lake Mead			
LV Wash[a]	4/30/97	1140 ± 28	43 ± 9	8990 ± 230	2670 ± 152	480 ± 68
LV Bay	4/30/97[a]	750 ± 34	27 ± 7	4850 ± 122	2210 ± 175	520 ± 117
	9/5/97	160	nd[c]	3180	188	253
LV Marina	9/5/97	nd	nd	nd	270	nd
Saddle Island[a]	4/30/97	nd	nd	nd	nd	nd
Callville Bay	9/5/97	nd	nd	nd	nd	nd
			Trenton Channel			
lower transect	8/30/97	993	26	6970	nd	nd
Trenton WWTP	8/30/97	479	5	5390	1070	nd
chemical plant	8/30/97	862	15	7310	911	nd
power plant	8/30/97	721	17	8600	nd	nd
Elizabeth Park	8/30/97	269	nd	1910	435	nd
Black Lagoon	8/30/97	936	66	8680	1290	359
Monguagan Creek[b]	8/30/97	1190 ± 91	81 ± 8	17800 ± 2390	1060 ± 202	nd
upper transect	8/30/97	665	23	8330	1280	nd
			WWTPs			
ER effluent[b]	5/16/97	806 ± 83	nd	9020 ± 712	nm	nm
EL effluent	5/16/97	1020	72	9310	656	248
BV effluent	5/16/97	22800	249	21800	3230	242
BV upstream	10/8/97	nd	nd	nd	711	nd
BV effluent	10/8/97	37000	673	332000	3660	759
MA upstream	10/8/97	nd	nd	nd	nd	nd
MA effluent	10/8/97	590	16	4850	905	357
ER upstream	10/8/97	ND	nd	nd	nd	nd
ER effluent	10/8/97	171	nd	nd	477	nd

[a] Duplicate samples (reported as mean ± relative deviation). [b] Triplicate samples (reported as mean ± standard deviation). [c] nd, not detectable; nm, not measured.

WWTP discharges. Concentrations of these compounds varied greatly among locations (Table 3). NP and E2 were detected in each effluent sample analyzed. No upstream site contained detectable concentrations of NP, OP, NPE, or EE2. The only upstream location with a detectable concentration of E2 was the BV site. This site is located in an agricultural area with many livestock farms, which may have contributed to the detection of E2, but we do not have enough data to draw conclusions on the reason for this occurrence. It should be noted, however, that the BV site uses a lagoon system for wastewater treatment. Operators of that plant during the October sampling event indicated that only primary treatment was in operation at that time. This effect can be seen in the F3 chromatogram (diluted 50×) of the sample collected during that time period (Figure 4). The NPE oligomer distribution is different from that of the other sites (Figure 4). This is an indication of inefficient treatment. Removal rates for NP during wastewater treatment have been shown to be 95−99% (*46*). Concentrations of NP in municipal wastewater effluents in the United States have been previously demonstrated to range from less than detection to 4.9 μg/L (*46*). These concentrations are similar to the concentrations observed in our studies. In U.S. rivers, NP has been reported to be undetectable (MDL = 0.11 μg/L) in most samples collected and averaged 0.12 μg/L in samples where it was detected (*17*).

Concentrations of analytes varied greatly among locations within Lake Mead. The LV Wash site was located at the confluence of WWTP effluents from the city of Las Vegas and the Clark County Santitation District, NV. This combined effluent stream represents an average of approximately 460 million L/day of tertiary-treated wastewater. The LV Bay site is located approximately 8 km downstream of the LV Wash site and is located in Lake Mead near the entry point of the LV Wash. The LV Bay site would be expected to have some mixing of the effluent with Lake Mead water and some dilution from nonpoint source groundwater entry. Concentrations of NP, OP, and NPE decreased by nearly 50% from LV Wash to LV Bay, while E2 and EE2 concentrations decreased and increased, respectively. The increase in EE2 could be due to the deconjugation of EE2 conjugates (*15, 31*). The LV Marina site lies approximately 2.3 km downstream of the LV Bay site. While only E2 was detected at this site, it should be noted that previous research by the U.S. Bureau of Reclamation has shown an interflow of the dense wastewater extending from the Las Vegas Wash to the Hoover Dam (*47*). This interflow lies on top of the hypolimnion, and its depth changes seasonally. It is likely that our samples collected at 3 m of depth do not represent the influence of the wastewater effluent at the LV Marina and Saddle Island sites, thus offering a possible explanation of the nondetectable concentrations of the compounds of interest. Both of these sites lie in the path of the wastewater interflow as it moves toward the Hoover Dam. Samples collected in September followed a rain event that resulted in a 3-fold increase of the LV Wash average daily flow (personal communication from Clark County Sanitation District). The addition of large amounts of stormwater in the LV Wash could explain the lesser concentrations of the compounds of interest from this sampling event. To our knowledge, this paper offers the first reported data for these compounds in the waters of Lake Mead, NV.

This method permits the sensitive detection of NP, OP, NPE, E2, and EE2 without the transportation of large volumes of water. This equates to savings in time and costs while achieving biologically relevant detection limits. While HPLC fluorescence provides sensitive detection of E2 and EE2, polar interferences were encountered in all environmental samples. RIA allowed a very sensitive detection of E2 and EE2 without a rigorous cleanup. However, RIA is also sensitive to compounds that are structurally similar to the analyte. Future efforts will include greater sample volumes and derivatizations to achieve structural confirmations of E2 and EE2 by GC/MS.

Although the sites studied represent only one point in time and were chosen to demonstrate the utility of the method rather than the dynamics of the target compounds, it can be concluded that estrogenic compounds are widespread contaminants of WWTP effluents. A monitoring program would be required to better describe the flux and fate of these compounds in the environment. While the concentrations of these compounds determined in this study are in the range of those predicted to cause physiological responses in certain fish (*10, 15, 37, 38*), no direct link currently exists between the sites evaluated and a direct cause−effect relationship.

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