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IN VIVO BIOASSAY-GUIDED FRACTIONATION OF MARINE SEDIMENT EXTRACTS FROM THE SOUTHERN CALIFORNIA BIGHT, USA, FOR ESTROGENIC ACTIVITY

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Abstract—The exposure and uptake of environmental estrogenic compounds have been reported in previous studies of demersal flatfish species in the central Southern California Bight (SCB), USA. The objective of this study was to evaluate the estrogenic or feminizing activity of marine sediments from the SCB by using in vivo vitellogenin (VTG) assays in male or juvenile fish. In 2003, sediments were collected near wastewater outfalls serving the counties of Los Angeles (LACSD) and Orange (OCSD), and the city of San Diego (SD), California, USA. Cultured male California halibut (CH; *Paralichthys californicus*) were either directly exposed to sediments for 7 d or treated with two intraperitoneal injections of sediment extract over 7 d. The 17β-estradiol (E2) equivalent values ranged from 1 to 90 μg/kg with LACSD > SD > OCSD. Measurable concentrations of E2 were observed in all sediment extracts and ranged from 0.16 to 0.45 ng/g. Estrone (E1) was only observed in sediments near the LACSD outfall (0.6 ng/g). Alkylphenols and alkylphenol ethoxylates were observed in all sediment samples, but were highest near the OCSD outfall, where concentrations of nonylphenol were 3,200 ng/g. Fractionation studies of the LACSD sediment extract collected in 2004 failed to demonstrate relationships between VTG expression and 62 analytes, including E2, which was observed in the whole extract (2.9 ng/g). Oxybenzone (1.6 ng/g) was identified in bioactive fractions as well as unknown compounds of relatively high polarity. These results indicate that estrogen receptor-based assays may underestimate environmental estrogenic activity and estrogenic compounds other than classic natural and xenoestrogens may contribute to estrogenic activity of sediments from the SCB.

Keywords—17β-Estradiol Vitellogenin Sediments Pharmaceuticals Oxybenzone

INTRODUCTION

Hormonally active steroids with feminizing/estrogenic activity have been measured in surface waters receiving wastewater throughout North America and Europe [1–3]. Through the use of in vitro estrogen receptor (ER) assays, the natural estrogens, 17 β-estradiol (E2), estrone (E1), and the synthetic estrogen (17α-ethinylestradiol) have been identified as being the causative agents for estrogenic activity in wastewater effluents and streams receiving wastewater [1]. Other studies have reported alkylphenol and other industrial chemicals may also contribute to feminization [4]. Recent in vivo-guided fractionation studies of New York City (NY, USA) wastewater have indicated the presences of nonylphenol isomers, as well as a host of other compounds causing vitellogenin expression in male fish [5,6]. Noticeably absent, however, were the high affinity ER ligands such as the natural and synthetic steroids.

Previous studies in 2000 reported the occurrence of the egg-yolk protein, vitellogenin, in male flatfish collected from the central Southern California Bight, USA [7]. The causative agent(s) for the in vivo estrogenic activity in California waters have yet to be identified. As flatfish reside within sediments for much of their life history, sediment and extracts of sediment receiving input from three of the four largest wastewater treatment facilities in the western United States were evaluated with an in vivo flatfish assay and compared with sediment residues for two steroids and several aklylphenol/alkylphenol ethoxylate agents in 2003. Further studies were carried out in 2004 to identify causative agents using in vivo-guided fractionation and analytical chemistry targeting eight steroids, as well as 62 legacy and pharmaceutically active agents previously observed in wastewater [8]. Although sediments contained concentrations of various known xenoestrogens, biologically active fractions failed to correlate with sediment concentrations of measured xenoestrogens, including sex steroids. The results indicated the occurrence of novel compounds of relatively high polarity in biologically active fractions of sediments demonstrating feminizing activities within fish.

MATERIALS AND METHODS

Chemicals

Ninety-six-well plates were purchased from Fisher Scientific (Los Angeles, CA, USA). The 17β-estradiol was purchased from Merck KGaA (Darmstadt, Germany). All other consumable reagents were purchased from Sigma Chemical (St. Louis, MO, USA). Anti-turbot and anti-trout vitellogenin antibodies were purchased from Biosense (Bergen, Norway).

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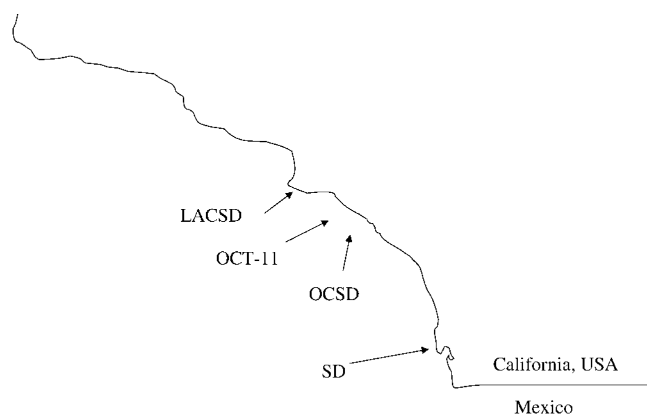

Fig. 1. Map of the Southern California Bight, USA, and sampling locations (see Table 1 for description of sites). LACSD = outfall for the Los Angeles County Sanitation District; OCT-11 = reference location; OCSD = outfall for the Orange County Sanitation District; SD = outfall for the City of San Diego.

Sediment collection and extract preparation

Sites for sediment collection were selected based on monitoring (quarterly, biannual, annual) requirements set by the regional U.S. Environmental Protection Agency office. Site OC-T11 was a far-field reference area located approximately 8 km north of the Orange County Sanitation District (OCSD) outfall. Establishment of this site as a reference was based on more than 30 years of monitoring data carried out by OCSD. Sediments were collected with a paired 0.1-m^2 Van Veen grab sampler (Kahlsico International Corporation, El Cajon, CA, USA) at sites adjacent to the wastewater outfalls of the Joint Water Pollution Control Plant operated by the Los Angeles County Sanitation Districts (LACSD), the Orange County Sanitation District (OCSD), and the Point Loma Wastewater Treatment Facility operated by the City of San Diego (SD), California, USA (Fig. 1). Treatment levels, output, total organic carbon, particle type/size, and population base varied between the three facilities (Table 1). Samples were stored on ice during transport and at 4°C until analyzed. Sediment samples were first passed through Buchner filters (Fisher Scientific, Pittsburgh, PA, USA) to separate pore water and freeze dried (−20°C). Five grams of freeze-dried sediment were extracted with a methanol-acetone mixture (1:1) three times for 5 min each using an ultrasonic disruptor (550 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA), as described by Lopez de Alda and Barcelo [9], with slight modifications. The extracts were centrifuged for 5 min at 4,000 rpm, with the resulting combined supernatants reduced in volume to dryness and resuspended in 1 ml of ethanol to which 4 ml of water was added and used for initial in vivo analyses.

Fish

California Halibut (CH) (*Paralichthys californicus*) were obtained from the Hubbs SeaWorld Research Institute hatchery in SD and were maintained in a 10,000-L circular tank at the SEA Lab, in Redondo Beach (CA, USA). Approximately 50 three-year-old adult male halibut (655 ± 50 g and 41 ± 14 cm) were kept in flowing sand-filtered ambient seawater and fed every other day until used for sediment exposures or intraperitoneal injections.

Sediment-only exposures

Individual halibut were kept in 40-L glass aquaria receiving sand-filtered flow-through seawater at ambient temperature (18°C) under natural light:dark conditions during the winter and spring of 2003. Approximately 2 L of sediment were applied to the bottom of the aquaria and allowed to settle before the addition of fish. The fish were fed every other day and removed on day 7 for serum vitellogenin analyses. Due to limited sediment availability, sediment-only exposures were only conducted on sediments collected near the OCSD outfall and OCT-11. Sand was used as a negative control.

Intraperitoneal injection

Intraperitoneal injections were performed on days 1 and 3, with subsequent bleeding of the fish on day 7. These particular dosage concentrations and exposure durations were determined in range-finding studies. To determine estradiol equivalents, an E2 dose–response curve was calculated using the two-injection design. Final doses were 0, 0.1, 1, 10, and 100 μg/kg body weight, and two animals at each dose were used. Following injections, animals were maintained in 40-L aquaria having 2 L of sand with free-flowing seawater, as above. There was no loss of sediment following exposure. Sediment extracts reconstituted in ethanol were administered by injecting 0.05 ml per 100 g fish (which was the same volume administered for E2). On day 7, the plasma samples were collected and the vitellogenin levels were determined. Seven to eight animals in each group (vehicle control and sediment extract) were used to evaluate the estrogenic activity of the sediments.

Blood samples were collected from the dorsal aorta using a latex-free 1-ml syringe and a 26-gauge needle (Becton Dick-

Table 1. Characteristics of sediment at associated wastewater treatment plant outfalls and wastewater treatment plants that discharge into the Southern California Bight, USA. Sampling was conducted in March of 2003, and Los Angeles County Sanitation District (LACSD) began 100% secondary discharge in January of 2003[a]

	Daily discharge (million gallons/day)	Population (millions)	Treatment regime	% TOC	Sand	Silt	Clay	Medium grain size (phi)
LACSD	330	3.5	40% primary, 60% secondary	2.5	18.3	62.8	18.7	5.1
SD	270	2.0	100% primary	0.43	65.9	31.0	3.2	4.0
OCSD	240	2.3	50% primary, 50% secondary	0.48	83.7	12.1	4.1	3.6
OCT-11 reference	—	—	—	0.42	73.8	21.3	4.9	3.7

[a] LACSD = Los Angeles County Sanitation District; SD = city of San Diego; OCSD = Orange County Sanitation District; TOC = total organic carbon.

inson Labware, Franklin Lakes, NJ, USA). Animals were anesthesized with 1 g/L Tricaine methane sulfonate (MS-222) (Sigma) and blood removed at the caudal peduncle. The samples were immediately centrifuged at 5,000 *g* for 5 min and the supernatants immediately frozen on dry ice, where they remained until transported to the laboratory. Previous studies with other flatfish indicated no significant degradation of protein with a single thaw [7]. Total protein concentrations of the serum were determined using the Coumassie Blue protein-staining reagent from the Pierce Chemical Company (Rockford, IL, USA). Relative concentrations of serum vitellogenin expressed in male halibut were determined using an enzyme-linked immunosorbent assay developed for measuring CH vitellogenin. Immunological detection was obtained using commercial anti-turbot vitellogenin (BioSense) and purified CH vitellogenin as a standard [10].

Estrogen, alkylphenol ethoxylate, and akylphenol measurements in sediments

Deuterated standards of d_4 estradiol and d_4 estrone (5 ng of each) were spiked into each of the extracts, and the samples were dried under a gentle stream of nitrogen. The dried extracts were reconstituted in 25 ml of 5% methanol:water and purified over an immunoaffinity column, as described previously [11]. The eluates from the affinity column were dried and reconstituted in 190 μl of 25% acetonitrile/water with addition of 5 ng of d_4 equiline (10 μl) as internal standard, and analyzed by immunoaffinity extraction coupled with liquid chromatography/mass spectroscopy (LC/MS) [12]. Method detection limits for estrone were 0.03 ng/g and 0.04 ng/g for estradiol. Standards and surrogates were analyzed in the range from 0.25 ng/ml to 70 ng/ml using d_4 equiline as internal standard (5 ng/ml). The Micromass Liquid Chromatography Technologies instrument (Waters, Milford, MA, USA) used for analysis was optimized to obtain the best possible signal for the steroids, with the electrospray ionization (ESI) capillary voltage set at 2,000 V, cone at −60 V, source temperature at 100°C, desolvation gas flow rate at 433 ml/h, and the gas at a temperature of 200°C. The limit of detection of the Liquid Chromatography Technologies instrument was 0.25 ng/ml for each of the estrogens analyzed at a microchannel plate voltage of 2,900. Standard curves constructed for E1 and E2 were linear in the range of calibration. Deuterated standards of the estrogens also showed linear calibration curves. The internal standard d_4 equiline showed the same response in the extracts and the standards, suggesting that there was no matrix-related ion suppression in the extracts after the immunoaffinity clean up. Surrogate standards were spiked into the extracts before immunoaffinity cleanup to determine the recovery of the steroids after purification. The recovery of d_4 E1 was 130%; however, the recovery of d_4 E2 was greater than 200%.

For analysis of alkylphenol ethoxylates (APEs) and their metabolites, filtered extracts were purified by nonaqueous reverse phase high performance liquid chromatography (HPLC) on two 250 × 4.6 mm Beckman Ultrasphere columns (Fullerton, CA, USA) connected in series, with a methanol mobile phase flow of 2 ml/min as described previously [11]. Collected fractions from sediment and blank extracts were again evaporated to dryness and reconstituted with 1 ml of methanol:water (50:50). Internal standards consisting of 50 ng each of *n*-nonylphenol (NP) and *n*-nonylphenol-3-ethoxylate (NPE3O) were added to the reconstituted extracts before HPLC-MS analyses [11]. The MDLs ranged from 2.9 (NPE3O) to 21.5 (NP) ng/g, and average recoveries were 123 ± 13% (NPE30) to 95 ± 14% (NP).

Fractionation studies

To determine specific causative agents responsible for the feminizing effects observed in CH exposed to sediments from the Southern California Bight (SCB), bioassay-guided fractionation was performed on another batch of sediments collected in the summer of 2004, using the vitellogenin response in treated fish. To accommodate the larger number of samples and smaller volumes, fractions (0.1 ml) were injected into juvenile rainbow trout (*Oncorhynchus mykiss*) and serum vitellogenin measured using a commercial enzyme-linked immunosorbent assay kit (Biosense) after 7 d. Estradiol equivalents (EEQs) were calculated using an E2 dose–response curve as previously described [13]. Extract fractionation was carried out as previously described [5,6]. Initial fractions were obtained by subjecting the 5-ml ethanol/water extract (see above) to a Sep-Pac plus C18 cartridge (Waters) with sequential elutions of 5-ml volumes of 10, 25, 50, 75, and 100% ethanol/water solutions. Approximately 75% of E2, 95% of akylphenols (APs), and 95 to 123% of dichlorodiphenyldichloroethylene (DDE) was recovered after elution. Each fraction was evaluated by bioassay using three to four animals for each fraction.

Following evaluation by bioassay, fractions were reduced in volume to 400 μl, and 200 μl was injected into an HPLC system using a J'sphere™ ODS-L80 (YMC Europe, Schermbeck, Germany) column with a flowrate of 1 ml/min. The mobile phase was methanol:water with an elution program of 0 to 3 min 40% methanol:water, followed with a gradient of 3 to 30 min of 40 to 100% methanol. Column effluent was monitored at 254 nm and fractions were collected every 3 min. Fractions were evaluated for vitellogenin expression as described above, using three to four animals for each fraction.

Chemical evaluation of fractions

To evaluate sex steroid concentrations in specific fractions, the fraction was spiked with 100 ng/L of mesterolone as a surrogate standard, completely dried under vacuum, and derivatized with hepta-fluorobutyric anhydride (purity 98%) as previously described [14]. Steroid derivatives were analyzed by gas chromatography/tandem mass spectroscopy ([GC/MS/MS]; Thermoquest, San Jose, CA, USA). A 30-m, 0.25-mm (inner diameter), 0.25-mm (film thickness) MDN-5S column (Supelco, Bellefonte, PA, USA) was used for separation. Positive identification of steroids was based on retention time and MS/MS daughter-ion abundance ratios [14]. For analyte identification and quantification, retention times for the analytes had to match retention times of reference compounds within 0.1 min. Also, the abundance ratios of the MS/MS daughter ions had to match the abundance ratios of the reference compounds within 20%. The limit of quantification was based on the lowest calibration point of the calibration curve (i.e., 0.3 or 0.4 ng/L after accounting for sample preconcentration). Quantification was accomplished using the summed areas of the MS/MS base peak ion and any confirmatory qualifier ions. Peak areas were normalized to the surrogate standard (mesterolone) area count to correct for variations in derivatization efficiency, analyte recovery, and GC/MS/MS performance. The data also were normalized using the internal standard, hexachlorobenzene. Recovery was found to be 74.1 ± 27.4% and was correlated with the recovery of mesterolone. Little

variation in recovery was observed among the five analytes, indicating similar loss mechanisms through the analytical method for these steroids. Some positive interference, as evidenced by an increased response relative to that of the standards, was observed in certain environmental samples. This interference, presumably a result of organic matter matrix effects, was accounted for by the surrogate standard.

Unknown evaluations of fractions

Thirty-two compounds were targeted for analyses using liquid chromatography with tandem mass spectroscopy (LC-MS/MS), as previously described [8]. An additional 30 contaminants were evaluated by GC-MS/MS, which included several organochlorine pesticides and polycyclic aromatic hydrocarbons. Analytes (with MS source) included the following: (ESI-positive, MDL < 1.0 μg/L)—hydrocodone, trimethoprim, acetaminophen, caffeine, erythromycin-H_2O, sulfamethoxazole, fluoxetine, pentoxifylline, meprobamate, dilantin, Tris(2-carboxyethyl)phosphine, carbamazepine, *N*,*N*-diethyl-meta-toluamide, atrazine, diazepam, oxybenzone, progesterone; (atmospheric-pressure chemical-ionization-positive, <1.0 μg/L)—estriol, ethynylestradiol, estrone, estradiol, testosterone, androstenedione; (ESI-negative)—iopromide, naproxen, ibuprofen, diclofenac, triclosan, gemfibrozil. Legacy analytes (<10 ng/L) included naphthalene, acenapththylene, acenaphthene, fluorine, α-hexachlorocyclohexane (α-BHC, β-BHC, γ-BHC), phenanthrene, anthracene, δ-BHC, galaxolide, heptachlor, metolachlor, musk ketone, aldrin, heptachlor epoxide, pyrene, γ-chlordane, fluoranthene, α-chlordane, DDE, dieldrin, endrin, dichlorodiphenyldichloroethane, DDT, benz[*a*]anthracene, chrysene, methoxychlor, mirex, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene. Analyses were conducted using a triple-quadrupole mass spectrometer and ion-trap mass spectrometer for LC and GC methods, respectively. Quantitation was performed using external calibration. All curves had a linear fit with regression coefficients of 0.98 or better. Quality-control check standards were evaluated every six samples and passed within 100 ± 20% of the correct concentration, or the samples were reanalyzed. Additionally, extracts were screened for nontarget compounds using GC-MS, followed by database matching using the National Institute of Standards Mass Spectrometry database (1998; see http://www.nist.gov/srd/webguide/nist01/01-struc.htm).

Statistics

Bartlett's test of homogeniety was used before Student's *t* test comparing two treatments or analysis of variance for multiple treatments. Dunnett's multiple range was then used as a post hoc test for significance in multiple treatment comparisons. Linear regression analyses were used to extrapolate EEQs from a log-transformed E2 dose–response curve.

RESULTS

2003 sediment evaluations

Chemistry. The concentration of E2 determined in sediment extracts ranged from 0.16 ng/g to 0.45 ng/g sediment with extracts from sediments collected near the OCSD outfall having the highest concentration of E2 (Table 2). The identity of E2 in the extracts was confirmed by determining the accurate mass of E2 with respect to the surrogate standard d_4 E2. The accurate mass of E2 computed was 2.5 mDa higher than the expected mass. Nevertheless, the similar differences in accurate mass in the E2 standards with respect to d_4 estradiol were within the error of the instrument. Estrone was only observed in sediments collected near the LACSD outfall at 0.6 ng/g and was not detected (<0.3 ng/g) in any of the other sediments. The OCSD sediments also had the highest NP (3,200 ng/g) and nonylphenol ethoxylate concentrations (330–3,900 ng/g). The LACSD sediments possessed the next highest concentrations of NP (198 ng/g), followed by SD and the proposed reference sediments. Octylphenol was only observed in sediments collected near the LACSD outfall.

Table 2. 17β-Estradiol, estrone, alkyl phenol, and alkylphenol ethoxylate concentrations (ng/g dry wt) of sediments collected from the Southern California Bight, USA, in 2003 (<cal = detected by instrument, but below calibration curve; <DL = below detection limit[a]

	LACSD	SD	OCSD	OCT-11 (reference)
E1	0.6	<0.03	<0.03	<0.03
E2	0.3	0.3	0.45	0.16
NP	198	122	3200	130
NPCl	<DL	<DL	100	<DL
NPBr	<DL	<DL	<DL	<DL
NP 1EO	36	11.6	330	76
NP 2EO	19.2	3.2	600	92
NP 3EO	15.6	1.9	3900	92
OP	8.2	1.9	<DL	<DL
OP 1EO	<DL	<DL	<cal	21
OP 2EO	<DL	<DL	<cal	8
OP 3EO	<DL	<DL	42	58

[a] E2 = 17β-estradiol; E1 = estrone; NP = nonylphenol; NP (X)EO ethoxylate = nonylphenol (carbon chain) ethoxylate; OP (X)EO ethoxylate = octylphenol (carbon chain) ethoxylate; LACSD = Los Angeles County Sanitation District; SD = city of San Diego; OCSD = Orange County Sanitation District.

Bioassay evaluation. Following 7 d of exposure to sediments from near the OCSD outfall and the proposed reference site, measurable expression of vitellogenin (VTG) was observed in male CH compared with animals exposed to sand and OCT-11 (reference) (Table 3).

When the extract of the OCSD sediment was injected into CH twice over a 7-d period, approximately 1.3 ng/g body weight of EEQ was observed (Table 3). In vivo estrogenic/feminizing activity was highest in the extracts of LACSD sed-

Table 3. In vivo (California halibut [CH] vitellogenin [VTG]) estrogenic activity of sediment extracts collected near outfalls of three wastewater discharge outfalls and a reference site in the Southern California Bight, USA, in 2003. Each value represents the mean ± standard deviation of seven (CH vitellogenin) replicates. ND = not detected; NM = not measured[a]

	Extract		Sediment only
	CH VTG (μg/g) plasma protein	CH VTG EEQ (μg/kg wet wt) fish	CH VTG (μg/g) plasma protein
LACSD	17.7 ± 4.1	90.4 ± 48.3	NM
SD	6.8 ± 1.4	13.7 ± 2.9	NM
OCSD	2.8 ± 0.4	1.3 ± 0.12	2.0 ± 0.2
OCT-11 reference	ND	<1.0	ND
Sand	ND	<1.0	ND

[a] LACSD = Los Angeles County Sanitation District; SD = city of San Diego; OCSD = Orange County Sanitation District; EEQ = estradiol equivalents. Detection limits (1 μg/kg CH).

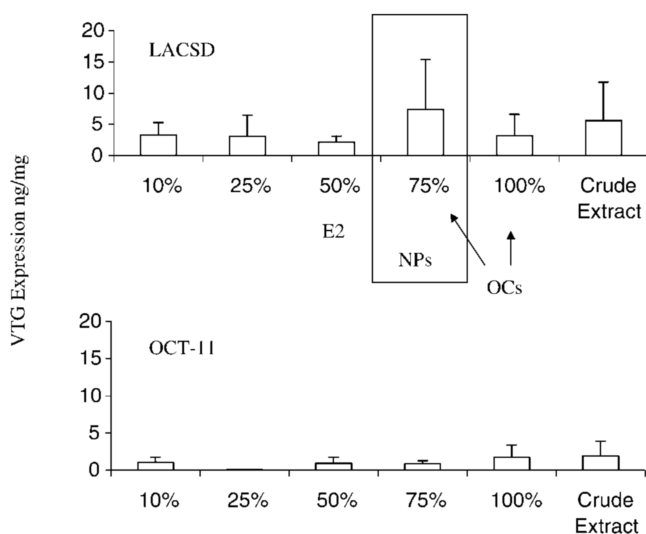

Fig. 2. Vitellogenin (VTG) expression in fish treated with solid-phase extraction fractions of Los Angeles County Sanitation District (LACSD) and reference (OCT-11) sediment extracts collected in 2004, California, USA. Each value represents the mean of three to four animals ± standard deviation. The 17β-estradiol (E2) concentrations were 2.9 ng/g in the 50% fraction and 0.4 ng/g in the 75% fraction. Testosterone was 0.1 ng/g in the 50% fraction, and progesterone was 1.8 ng/g in the 50% fraction. NP = nonylphenol; OCs = organochlorines.

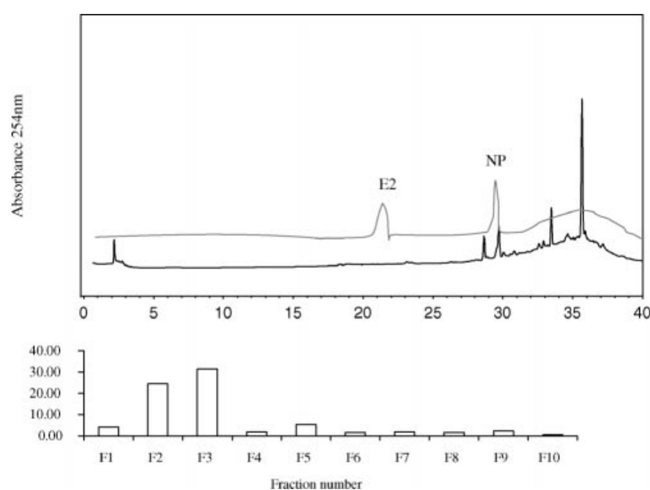

Fig. 3. Chromatogram and associated expression of vitellogenin of the 75% ethanol fraction of the Los Angeles County Sanitation District (LACSD) extract collected in 2004, California, USA. Fractions evaluated for vitellogenin were collected every 3 min. Each value represents the mean of three to four animals ± standard deviation. Retention times of 17β-estradiol (E2) and nonylphenol (NP) were 22.7 and 29.2 min, respectively.

iments compared with SD or OCSD sediments (Table 3). Activity of LACSD was roughly sixfold greater than SD and 90-fold higher than OCSD. The SD had nearly 10-fold higher in vivo activity than OCSD, and no detectable in vivo activity was observed in OCT-11 or sand extracts.

Fractionation analyses. The LACSD sediment extracts in 2004 had approximately 10-fold higher VTG expression in fish relative to OCT-11 sediments (Fig. 2), which served as a reference based on chemistry and bioassay results above. The 75% extract caused the highest nominal VTG expression in fish, even though E2 and other steroids were found to elute in the 50% fraction (Fig. 2). Consequently, the 75% fraction was subjected to subsequent HPLC fractionation. Fractions 1, 2, and 3, which corresponded to 1- to 3-, 4- to 6-, and 7- to 9-min retention times, respectively, demonstrated significant VTG expression in fish (Fig. 3) and were subsequently screened for 62 compounds, including several sex steroids and legacy contaminants. Only oxybenzone was positively identified in the three fractions at 0.36, 0.86, and 0.38 ng/g sediment concentrations. At least one unknown compound was identified in each of the three fractions having a 79 to 51 *m*/*z* fragmentation profile reminiscent of dieldrin. However, further analyses with dieldrin standards failed to confirm its identity. None of the fractions demonstrated absorbance at 254 nm, suggesting either absence of chromophore or concentrations that were below ultraviolet detection.

DISCUSSION

Previous studies carried out in the summer of 2000 indicated feminization of demersal flatfish species collected near the OCSD outfall [7]. As flatfish tend to contact sediment throughout much of their life history, sediments from the locations where flatfish were previously found were subsequently evaluated in order to ultimately determine the potential source of estrogenic/feminizing activity. To our knowledge, this is the first report of feminizing activity identified in sediments from the Southern California Bight. Although compounds possessing feminizing activity, such as various legacy organochlorine-containing contaminants, have been measured in sediments and biota of the SCB [15], the relationship between sediment exposure and biological response has not been documented.

Various natural and xenoestrogens were identified in sediment extracts from locations where feminized fish were previously collected. Contrasting other sediment evaluations of estrogen mimics [16–18], E1 was not detected in the OCSD outfall or proposed reference sediment. Estrone was observed in the LACSD outfall sediments (0.6 ng/g). 17β-Estradiol tends to rapidly degrade to E1 [19], which has been found previously in concentrations ranging from 1.2 to 388 ng/g [16–18,20]. Reasons why E1 was not uniformly present in all the extracts are unclear but may be due to differences in E2-degradation capacity in the sediments or wastewater-processing facilities.

Of the four sediments examined, E2 concentrations were highest in the OCSD outfall sediment extracts and lowest at the OCT-11 site. The lowest value, 0.16 ng/g, was higher than values observed in the United Kingdom (UK) and Spain, where bed sediment concentrations of E2 were below limits of detection (<0.1 ng/g) [17–18]. More recent evaluations of sediments from the UK observed concentrations of 0.006 to 0.014 ng/g for E2 and 0.025 to 0.052 ng/g for E1 [21]. However, in river sediments of wastewater-dominated streams from Germany, E1 and E2 were detected up to 2 ng/g and 1.5 ng/g, respectively [16].

With approximately the same average daily discharges from their oceanic outfalls and serving approximately the same number of residents, the in vivo feminizing activity of the sediments from SD was more than 10-fold higher than OCSD. The most significant difference between the SD and OCSD effluents is the level of treatment, with 100% of the SD discharge undergoing primary treatment, and the OCSD discharge existing as a blend of 50% primary and 50% secondary treatments. Earlier studies in the UK failed to demonstrate relationships between treatment process, population base, and estrogenic activity. However, it was suggested that treatment

facilities having more industrial effluents were likely to influence estrogenic activity through the input of alkylphenol ethoxylates and other industrial phenolic derivatives [4]. In the current study, OCSD had the highest AP and APE concentrations in the sediments, but in vivo activity was higher in the SD sediments. Derivatives of APEs, APs tend to be very persistent within anerobic sediments and have been found to occur in significant concentrations within embayments that are not well flushed [22]. In vitro fractionation studies evaluating estrogenic activity of river sediments in France indicated nonylphenols as likely candidates for the observed activity [23]. Evaluations of wastewater effluent in the UK have likewise identified APs or APEs as likely candidates for estrogenic activity observed in caged and wild fish populations residing within receiving streams [4]. In a recent fractionation study of New York City wastewater, fractions possessing the highest estrogenic/feminizing activity did not contain measurable amounts of steroids but did have several unidentified nonylphenol and bisphenol isomers [6]. Reasons for the differences between SD and OCSD may be related to differing sediment parameters (i.e., silt), which may affect bioavailability of the (xeno)estrogens from the sediments.

Although sediment concentrations of targeted estrogenic compounds were relatively high in the OCSD outfall sediment extracts, in vivo activity of the extract was slightly above detection (1.3 ng/g EEQ). The magnitude of response was consistent with sediment-only exposures, where vitellogenin expression was likewise just above detection. The lack of biological response relative to the chemical residues may be due to coextraction of antiestrogenic or androgenic compounds from the sediments, which may have antagonized the in vivo estrogenic assays. Androgenic compounds were shown to be particularly prevalent in sediments throughout the UK [24]. Antagonistic chemicals, such as polynuclear aromatic hydrocarbons and polychlorinated biphenyls, have been observed in these sediments previously [25]. A second potential mechanism for the differences may be stress resulting from handling and the injection process, which may reduce the signal-to-noise ratio. The stress hormone, cortisol, which is released during handling stress, has been shown to reduce VTG expression [26]. However, the relatively similar response in sediment-only exposures suggest handling stress was not likely a contributing factor.

Although OCSD sediment extracts possessed the highest E2, APE, and AP concentrations, sediments from near the LACSD outfall had the highest in vivo feminizing/estrogenic activity. Prior to the time of sampling (winter 2003), LACSD was discharging a primary (40%) and secondary (60%) blend of treated wastewater. The LACSD has subsequently begun to discharge wastewater undergoing 100% secondary treatment. The treatment facility of LACSD serves the largest population base in Southern California and has the largest daily output of the treatment facilities examined in the current study (Table 1). The LACSD system historically was a large source of chlorinated organics, particularly DDT, to the Palos Verdes shelf. Between 1950 and 1971, an estimated 1,800 metric tons of DDTs (primarily as DDE) from a chemical manufacturer tributary to the LACSD wastewater system was discharged to the local marine environment via the LACSD outfall [27,28]. The DDTs and other chlorinated organics have been observed in sediments and biota near the LACSD outfall [15], and contamination is widespread throughout the SCB, with 100% of sand-dab guild species in 1998 having concentrations of DDT or its metabolites exceeding predator threshold concentrations established for avian species [29].

Because LACSD sediment extracts caused consistently higher VTG expression in fish, extracts were fractionated using VTG expression in fish in an attempt to find causative agents. Of the 62 targeted analytes, half of which included legacy compounds such as DDT, only one compound was identified in each of the active fractions, the sunscreen additive, 2-hydroxy-4-methoxy benzophenone, also known as oxybenzone. Benzophenone, 2-hydroxy-4-methoxybenzophenone, and 2,2′-dihydroxy-4-methoxybenzophenone exhibited in vitro estrogenic activities after incubation with an S-9 mix [30]. The estrogenic metabolites of 2-hydroxy-4-methoxybenzophenone were fractionated by HPLC, and one was identified as 2,4-dihydroxybenzophenone. In vivo assays of the estrogenic activity of benzophenone derivatives carried out primarily in rodents demonstrated only slight estrogenic effects [31]. The effects of oxybenzone or benzophenone derivatives in fish are unknown, but studies are currently underway to determine threshold concentrations for VTG induction in fish.

In contrast with the plethora of studies based on in vitro ER-ligand activity, chemically screening bioactive fractions for known xenoestrogens, including organochlorine legacy contaminants and sex steroids, failed to demonstrate any associations in the current study. The E2 and 4-nonylphenol eluted at 23 and 29 min, respectively, using this chromatographic system and mobile phase. The E2 values were nearly an order of magnitude higher in 2004 (2.9 ng/g) compared with 2003 (0.3 ng/g). The VTG expression was not observed in fish treated with the fraction containing E2 or other steroids. This is not surprising, as concentrations of E2 administered via injection (0.00058 μg/kg) using this fraction were well below determined threshold values for VTG induction in fish (1.0 μg/kg fish wt). Only the more polar fractions caused VTG expression in fish. Peck et al. [21] demonstrated the occurrence of numerous unidentified estrogen receptor ligands in UK sediments. Polar ER-ligands were also previously observed to be abundant in sediment extracts for other wastewater-impacted areas in Europe [32]. Polar fractions with estrogenic/feminizing activity in fish were also observed in studies with New York City wastewater [5,6]. Although organochlorine contaminants, selected pharmaceuticals, and APs did not associate with bioactivity in the current study, it should not be assumed that they can be ruled out as contributing compounds. The current assay was developed using E2 as a standard. Consequently, agents with lower ER affinity or other indirect mechanisms of action, such as DDT or its metabolites, may require higher concentrations or longer durations of exposure before VTG may be induced. Further study is warranted, perhaps increasing duration and/or dose of fractions to find other compounds capable of causing feminization of fish.

In summary, in vivo feminizing/estrogenic activity was observed in sediments and extracts of sediments receiving wastewater effluent from three of the largest municipal wastewater treatment facilities that discharge into the SCB. All sediments possessed detectable concentrations of E2, AP, and several APE compounds. However, bioassays of extracts using VTG expression in male or juvenile fish failed to correspond to any of the sediment concentrations of classic (xeno)estrogens. Fractionation studies of subsequent sediment extracts failed to identify sex steroids as potential agents and demonstrated the occurrence of polar compounds within extracts that demonstrated VTG expression in fish. Whether these compounds are

bioavailable to demersal fish or whether a longer duration or dietary route of exposure would yield additional compounds warrants further study.

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