

## THE RELATIONSHIPS OF BIOCHEMICAL ENDPOINTS TO HISTOPATHOLOGY AND POPULATION METRICS IN FERAL FLATFISH SPECIES COLLECTED NEAR THE MUNICIPAL WASTEWATER OUTFALL OF ORANGE COUNTY, CALIFORNIA, USA

LUKE A. ROY,<sup>†</sup> JEFFREY L. ARMSTRONG,<sup>‡</sup> KEN SAKAMOTO,<sup>‡</sup> SCOTT STEINERT,<sup>§</sup> EDWIN PERKINS,<sup>||</sup>DANIEL P. LOMAX,<sup>#</sup> LYNDAL L. JOHNSON,<sup>#</sup> and DANIEL SCHLENK\*<sup>†</sup><sup>†</sup>Department of Environmental Sciences, University of California, Riverside, California 92521, USA<sup>‡</sup>Orange County Sanitation District, 10844 Ellis Avenue, Fountain Valley, California 92708-7018, USA<sup>§</sup>CSC Biomarker Laboratory, Computer Services Corporation, San Diego, California 92110, USA<sup>||</sup>Marine Environmental Biology Section, Department of Biological Sciences, University of Southern California, Los Angeles, California 90089, USA<sup>#</sup>Environmental Conservation Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Seattle, Washington 92112, USA

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**Abstract**—In July 2000, 330 individuals of three flatfish species were collected from reference locations and nine sites surrounding the outfall of the Orange County (CA, USA) Sanitation District (OCSD) municipal wastewater discharge. Species included hornyhead turbot (*Pleuronichthys verticalis*), English sole (*Pleuronectes vetulus*), and bigmouth sole (*Hippoglossina stomata*). Livers of sampled animals were examined for the expression of vitellogenin (in males), cytochrome P450 1A (CYP1A), and DNA damage (comet assay). Biliary fluorescent aromatic compounds (FACs) were also measured. Comparisons with tissue and sediment contaminant residues, liver histopathology, and population estimates were assessed to determine whether relationships exist between levels of biological organization. The CYP1A results indicated exposure to planar aromatic hydrocarbons at one nearshore site. Regression analysis of all English sole CYP1A to total sediment polychlorinated biphenyls (PCBs) ( $r^2 = 0.97$ ;  $p < 0.05$ ) indicated a significant correlation. Widespread exposure to estrogens was observed at all sites without correlation to the abundance of the three species examined. Fluorescent aromatic compounds demonstrated no correlation to CYP1A expression. Overall, histopathology and biochemical endpoints did not indicate significant adverse effects in fish at the OCSD outfall.

**Keywords**—Vitellogenin Cytochrome P450 DNA damage Fluorescent aromatic hydrocarbons Polycyclic aromatic hydrocarbons

## INTRODUCTION

Until 1976, when the manufacturing of PCBs was banned, an estimated 10 metric tons of PCBs were being discharged annually into the Southern California Bight through municipal wastewater outfalls [1], including the highly agricultural Orange County Sanitation District (OCSD) sewage outfall. These compounds accumulated in offshore sediments [2], and while their input is not as pronounced as in the 1970s, PCBs still present a problem for both benthic invertebrates and demersal fishes [3]. Since the 1970s, the concentrations of PCBs in the sediments and local fish have been steadily decreasing [4]. In addition, because of increasing evidence of the biological impacts associated with offshore oil drilling operations [5], effects of PAHs from local oil drilling platforms have been of increasing concern. The OCSD has been conducting extensive annual ocean monitoring surveys since 1986. During the 2000–2001 monitoring year, OCSD discharged an average of 240 million gallons per day (MGD) of treated wastewater into the Pacific Ocean. Sediment quality and fish population and health data are included in the annual marine monitoring surveys (<http://www.ocsd.com>). However, OCSD has just recently begun to employ the use of various biochemical endpoints into their arsenal of monitoring techniques.

Vitellogenin is a precursor egg yolk hormone found in female fish. The synthesis of vitellogenin in male fish indicates

exposure to estrogenic compounds and is commonly used as a biomarker for exposure to estrogens [6]. The presence of chemicals such as DDT in the sediments off the coast of California [3] and the discharge of domestically derived estrogens from OCSD sewage outfall raised the question as to whether the male fish were synthesizing vitellogenin and, if this were the case, whether compounds in the sediment could be correlated to vitellogenin induction in male animals.

Liver cytochrome P450 (CYP1A) expression has been correlated to exposure to polycyclic aromatic hydrocarbons (PAHs) and PCBs in fish [7]. Elevated liver CYP1A expression, due to PAH exposure, has been linked to increased levels of biliary FACs [8,9]. Strong evidence exists in the chemical etiology of sediment PAHs for liver lesions in benthic fish that have been used as indicators of environmental quality in both saltwater and freshwater ecosystems [10,11].

Modifications of DNA, including strand breaks, DNA base modifications, cross-linking, and depurination, have been observed to be associated with exposure to a number of contaminants, including PAHs, pesticides, furans, dioxins, PCBs, and trace metals, that can produce reactive oxygen radicals [12]. The Comet Assay is a genetic biomarker technique that measures the relative integrity of DNA following denaturation or unwinding under alkaline conditions in individual cells. Exposure to DNA damaging agents can result in an increased level of DNA damage repair along with increases in cellular turnover rates [13], thus increasing DNA strand breakage.

The objectives of this study were to assess the expression

\* To whom correspondence may be addressed ([daniel.schlenk@ucr.edu](mailto:daniel.schlenk@ucr.edu)).

Table 1. Trawl station locations off Orange County (CA, USA)

Station	Latitude	Longitude	Depth (m)
T11 (reference)	33°36.055'N	118°05.199'W	60
T1 (outfall)	33°34.641'N	118°00.567'W	55
T2	33°35.688'N	117°59.561'W	35
T3	33°34.856'N	117°57.345'W	55
T6	33°35.946'N	118°02.785'W	36
T10	33°33.771'N	118°00.250'W	137
T12	33°34.868'N	118°01.60'W	57
T13	33°35.535'N	118°03.637'W	60
T14	33°34.672'N	118°03.200'W	137
T0	33°37.672'N	117°59.250'W	18

of various hepatic biochemical endpoints of hornyhead turbot (*Pleuronichthys verticalis*), English sole (*Pleuronectes vetulus*), and bigmouth sole (*Hippoglossina stomata*) located at and around the California OCS D sewage outfall and to determine whether sediment or tissue concentrations of PCBs or PAHs were related to histopathological or biochemical effects on these fish species and their respective populations.

## MATERIALS AND METHODS

### Sampling locations

The OCS D outfall is located 7 km offshore within the Southern California Bight. Trawl stations varied in depth from 18 to 137 m (Table 1). In July 2000, sampling was conducted at nine stations. The prevailing surface current is upcoast. Five stations are located at the outfall depth along the 60-m contour (outfall station T1, upcoast intermediate stations T12 and T13, upcoast reference station T11, and downcoast reference station T3). In addition, one shallow (18 m) station (T0), two middepth (60 m) shelf stations (T2 and T6), and two deep (137 m) stations (T10 and T14) were also sampled. Station T0 was the previous OCS D municipal outfall site until 1971, when the outfall pipe at T1 was completed. Station locations (Table 1) and trawling paths were determined using differential global positioning system (dGPS) navigation to accurately locate the area sampled and to control the speed of the trawl (2–2.5 knots).

### Fish sampling

English sole (*P. vetulus*), bigmouth sole (*H. stomata*), and hornyhead turbot (*P. verticalis*) were collected in July 2000 utilizing a 7.6-m-wide semiballot otter trawl. Three daily replicate samples for population analysis were collected at stations T1, T11, T12, and T13, and two replicate samples were collected at the remaining five stations (T2, T3, T6, T10, and T14). Two additional trawls at T1, T11, T12, T13, and T0 were taken to collect samples for liver histopathology analyses. All specimens are taxonomically identified, counted, and individually weighed and measured. If significantly more than 30 specimens of any one species were collected in a single trawl, the first 30 specimens were individually weighed and measured, and the balance were tallied and size classed, and a batch weight was recorded. Blood samples were taken immediately prior to necropsy and dissection of the fish. Gross liver histopathology of each fish was examined and recorded prior to removal. Liver and bile samples were immediately stored in liquid nitrogen prior to transport to a  $-80^{\circ}\text{C}$  freezer. Blood (~0.5 ml) was collected via nonheparinized syringe from the dorsal aorta and immediately spun with a portable centrifuge unit for 2 min at 750 g. The supernatant was re-

moved and stored in liquid nitrogen until transport to a  $-80^{\circ}\text{C}$  freezer where it was stored until analysis.

### Sediment chemistry

Sediment samples were collected at 10 stations located along the 60-m contour. Three replicate samples were collected at each station. Bottom sediments were collected using a paired 0.1-m<sup>2</sup> Van Veen grab sampler. Only samples from grabs penetrating to depths exceeding 10 cm with a minimum sample volume of 4 L and an undisturbed surface were accepted for processing. Sediment samples were collected from the top 2 cm using a stainless-steel scoop. Approximately 300 and 50 g of sediment were removed from each grab for extractable organic compounds (PAHs, pesticides/PCBs) and metals analysis, respectively. Sediments were stored on ice until transport to the lab in precleaned glass jars with Teflon<sup>®</sup>-lined lids. The sediment samples were frozen in a  $-20^{\circ}\text{C}$  freezer until analysis. Field quality control samples, consisting of equipment rinses (cross-contamination blanks) and field blanks, were collected during each survey. These samples were analyzed for metals, PAHs, pesticides, and PCBs (PAHs and PCBs will only be discussed in the current paper). Sample storage, preservation, and holding times generally followed the 301(h) quality assurance/quality control guidance document recommendations and/or other guidance established for the National Status and Trends and EMAP programs [14]. All samples are transferred under chain-of-custody documentation to the chemistry lab for subsequent analyses.

Chlorinated pesticides, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons were analyzed according to established methods [14]. Predried sediments were extracted with a mixture of methylene chloride/hexane. Polycyclic aromatic hydrocarbons were analyzed by gas chromatography/mass spectrometry using selected-ion monitoring (GC/MS-SIM). A gas chromatograph with an electron capture detector (GC-ECD) was utilized to analyze for pesticides and polychlorinated biphenyls. Fish tissue residues of liver and muscle were also analyzed with GC/MS. Detection limits and recovery values for all compounds may be found at <http://www.ocsd.com/main.htm>.

### Biochemical endpoint measurements

**CYP1A.** Hepatic CYP1A levels were quantified using Western slot-blotting techniques followed by semiquantitative measurements of densitometry in optical density units (ODU). Liver samples were homogenized in a glass homogenizer at 4 times volume in 0.1 M Tris-HCl (pH 7.4 with HCl), 0.15 M KCl, 1 mM ethylenediaminetetraacetate acid (EDTA), and 0.1 mM phenylmethyl sulfonyl fluoride (added in a minimal volume of ethanol). Postmitochondrial supernatants (S12) were obtained using centrifugation at 10,000 g for 30 min. Supernatants were then recentrifuged at 100,000 g for 90 min to obtain microsomal pellets and cytosolic fractions (the latter used in vitellogenin analysis). The microsomal pellet was then resuspended in 0.1 M potassium phosphate (pH 7.4), 20% (v/v) glycerol, and 1 mM EDTA. Microsomal and cytosolic samples were stored at  $-80^{\circ}\text{C}$  until analyzed. All procedures were carried out at  $4^{\circ}\text{C}$ . The CYP1A protein was assayed utilizing monoclonal mouse antipeptide CYP1A IgG as the primary antibody [15]. Microsomal protein was measured using the Pierce kit (Pierce, Rockford, IL, USA) method with bovine serum albumin as the standard. Protein samples were normalized to contain 50  $\mu\text{g}$  of protein per slot. Initial Western

blot analyses indicated the presence of a single immunoreactive band of approximately 55 kD [15], allowing the use of slot-blot analyses.

**FACs.** Fluorescent biliary metabolites of benzo[*a*]pyrene (BaP), naphthalene (NAP), and phenanthrene (PHN) were analyzed in fish bile using a Shimadzu (Columbia, MD, USA) high-pressure liquid chromatography (HPLC) system with fluorescence detection. The assays were conducted according to previously reported methods [16]. Fluorescence was measured with a Shimadzu fluorescence detector (RF-10 AXL) at 380/430 nm, 256/380 nm, and 290/335 excitation/emission for BaP, PHN, and NAP, respectively. Gallbladders of English sole were thawed, and the bile was removed and diluted 1:500 in 99% methanol, which was then injected into the HPLC. The HPLC analysis was conducted with a 5- $\mu$ m (2.0  $\times$  150 mm) Spherisorb S5- $\mu$ m ODS2 reverse-phase column with a 2.0- $\mu$ m (23 mm) YMC ODS-A S5- $\mu$ m guard cartridge (Waters, Milford, MA, USA) using a Shimadzu LC-10ATvp solvent pump and a SCL-10Avp ultraviolet detector (Shimadzu). A 56:44 methanol/0.001% acetic acid (v/v) isocratic mobile phase was used at a flow rate of 0.25 ml/min. Concentrations were calculated from standard curves developed from BaP, NAP, and PHN standards.

**DNA damage.** Tissue samples were collected as fish were being examined and dissected for the histopathology component of the OCSO environmental monitoring. Blood was preserved by gently mixing and freezing a small volume (<100  $\mu$ l) in 1 ml of ice-cold cryopreservation solution, phosphate-buffered saline/10% dimethyl sulfoxide (DMSO). Small sections of liver and gonad (collected from male fish) were placed in 1 ml of ice-cold cryopreservation solution. Within 20 min, all samples were frozen in liquid nitrogen. Samples were transported to the Comet Analysis Laboratory and transferred to a -80°C freezer. To prepare samples for DNA damage analysis, cryopreserved samples were thawed on ice; 10 to 100  $\mu$ l of blood were added to 140  $\mu$ l ice-cold phosphate-buffered saline; liver was homogenized using dissection scissors, and 25  $\mu$ l of suspended cells were added to 100  $\mu$ l ice-cold phosphate-buffered saline (PBS); and 10  $\mu$ l cryopreservation solution from a gonad sample vial were added to 140  $\mu$ l ice-cold PBS in a clean 1.5-ml centrifuge tube, and the remaining sample was refrozen. Cells were pelleted at 600 g for 2 min, supernatant was discarded, and the pellet was resuspended in 50 to 600  $\mu$ l 0.65% low-melting-temperature agarose/low-melting DNA-grade agarose (Fisher Scientific, Pittsburgh, PA, USA) in PBS at 30°C (PBS low-melting agarose). Twenty-five microliters of the resuspended cells were then transferred onto slides of GelBond plastic support media (BioWhittaker Molecular Applications, Rockland, ME, USA), and the cell/agarose suspension was allowed to solidify on an ice-chilled stainless-steel tray covered with a topcoat of 25  $\mu$ l PBS/LMA. After solidifying, the slides were placed in 4°C lysing solution (LS), 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, 1% Triton X-100 (Sigma, St. Louis, MO, USA), and 10% DMSO, pH 10.0 (LS), in polycarbonate trays and incubated at 4°C for at least 1 h. Slides were then transferred from LS to trays filled with distilled water, and the water was replaced three times over a 10-min period and then placed in a submarine gel electrophoresis chamber filled with 300 mM NaOH and 1 mM EDTA, and the DNA was denatured under alkaline conditions for 15 min. After unwinding, electrophoresis was performed at 300 mA, 25 V, for 10 min. The slides were then neutralized with three 2-min rinses in 0.4 M Tris and removed, and excess solution

was blotted away and placed in ice-cold ethanol for 5 min. The fixed slides were dried in an oven at 37°C for 20 min and transferred to slide boxes. For analysis, the DNA was stained with 15  $\mu$ l of a 20- $\mu$ g/ml solution of ethidium bromide in distilled water (EtBr) and covered with a coverslip. Stained slides were analyzed by viewing at  $\times$ 200 with an epifluorescent microscope (excitation filter 510–560 nm green light, barrier filter 590 nm) with an attached charge-coupled device camera and image analysis software (Komet image analysis system, Kinetic Imaging, Liverpool, UK). The fluorescent head or nucleus diameter and the length ( $\mu$ m) of any accompanying trailing DNA tails resulting from strand breakage were measured for each nucleus analyzed. Measurements were made in five sectors on each slide, counting 5 to 10 nuclei in each sector, randomly positioning the lens above each sector, and counting left to right from the upper-left-hand corner of the field of view. Overlapping nuclei or tails were not counted. The image system calculates a large number of quantitative parameters for each nucleus, the most important being the total intensity of each comet (comet optical intensity), the percentage of DNA in the tail, and the tail moment (TM), which is the product of the %DNA in the tail  $\times$  the tail length/100. Tail moment was used to calculate DNA damage in the current study.

**Vitellogenin.** Both plasma and cytosolic vitellogenin were quantified using slot-blotting techniques as described by McCordle et al. [17], followed by semiquantitative measurements of densitometry. Cytosolic fractions were prepared while making microsomes as described previously. Vitellogenin protein was assayed using a polyclonal rabbit anti-English sole vitellogenin as the primary antibody and was obtained from the National Oceanic and Atmospheric Administration (NOAA) in Seattle (WA, USA) [18]. Vitellogenin was analyzed in blood plasma for 20% of the fish sampled, and hepatic vitellogenin was measured in the cytosolic fractions of all fish sampled. Fish were size classed and aged using previous methods (see [ftp://ftp.sccwrp.org/pub/download/PDFs/1994\\_95ANNUALREPORT/ar10.pdf](ftp://ftp.sccwrp.org/pub/download/PDFs/1994_95ANNUALREPORT/ar10.pdf)), and  $L = 335.3186(1 + e^{-0.0369(\text{Age} - 2.720)})$ . Estimates ranged from two to five years of age. Only sexually mature male and female animals were sampled for vitellogenin. Plasma and cytosolic protein was assayed using a 96-well plate reader with bovine serum albumin (BSA) as the standard. Protein samples were normalized to contain 50 and 30  $\mu$ g of protein per slot for plasma and cytosolic fractions, respectively. Serum/plasma was obtained by immediately spinning approximately 0.5 ml of ice-chilled blood collected with nonheparinized syringes from the dorsal aorta in a portable centrifuge for 2 min at 500 g at room temperature. Previous studies in our laboratory had indicated that no appreciable degradation of protein occurred following this protocol [17]. Samples were then placed in liquid nitrogen and eventually stored at -80°C until processed.

#### Liver histopathology

Livers of English sole ( $n = 147$ ), bigmouth sole ( $n = 114$ ), and hornyhead turbot ( $n = 98$ ) were removed on the survey vessel and necropsied. Following necropsy and removal, livers were immediately preserved in Dietrich's fixative. Samples were dehydrated in an alcohol series, cleared in Clear-Rite-3 (Kalamazoo, MI, USA), and embedded in paraffin. Sections were then cut at 4  $\mu$ m, placed on poly-*l*-lysine-coated slides, stained with Gill and Weigert's hematoxylin, and counterstained with eosin Y. Slides were interpreted at the Marine

Table 2. Polyaromatic hydrocarbons (PAHs) analyzed in sediment

Low-molecular-weight PAHs		High-molecular-weight PAHs
1,6,7-Trimethylnaphthalene	2-Phenyltridecane	Anthracene
1-Methylnaphthalene	3-Phenyltridecane	Benz[a]anthracene
1-Methylphenanthrene	4-Phenyltridecane	Benzo[a]pyrene
2-Methylnaphthalene	5-Phenyltridecane	Benzo[b]fluoranthene
Acenaphthene	7+6-Phenyltridecane	Benzo[e]pyrene
Acenaphthylene	2-Phenyltetradecane	Benzo[ghi]perylene
Biphenyl	3-Phenyltetradecane	Benzo[k]fluoranthene
Indeno[1,2,3-cd]pyrene	4-Phenyltetradecane	Chrysene
Naphthalene	5-Phenyltetradecane	Dibenz[a,h]anthracene
Perylene	6-Phenyltetradecane	Dibenzothiophene
C1-Naphthalenes	7-Phenyltetradecane	Fluoranthene
C2-Naphthalenes		Fluorene
C3-Naphthalenes		Phenanthrene
C4-Naphthalenes		Pyrene
2-Phenyldecane		C1-Fluorenes
3-Phenyldecane		C2-Fluorenes
4-Phenyldecane		C3-Fluorenes
5-Phenyldecane		C1-Phenanthrenes/anthracenes
2-Phenylundecane		C2-Phenanthrenes/anthracenes
3-Phenylundecane		C3-Phenanthrenes/anthracenes
4-Phenylundecane		C4-Phenanthrenes/anthracenes
5-Phenylundecane		C1-Fluoranthenes/pyrenes
6-Phenylundecane		C1-Chrysenes
2-Phenyldodecane		C2-Chrysenes
3-Phenyldodecane		C3-Chrysenes
4-Phenyldodecane		C4-Chrysenes
5-Phenyldodecane		
6-Phenyldodecane		

CYP1A expression, however, did not correlate with abundance values.

#### Population/community endpoints

To determine potential population/community effects, several diversity indices were measured at all sites where demersal fish were collected (Table 4). According to three of the diversity indices used (Shannon–Wiener, dominance, and evenness), the outfall station (T1) possessed higher diversity than the reference (T11) location (Table 4). The reference location (T11) possessed a higher Margalef diversity than the outfall (T1), but these results were not statistically significant. These results are indicative of a nondegraded community at the outfall station. In addition, English sole, hornyhead turbot, and bigmouth sole accounted for 8, 8, and 7% of the total fish biomass sampled. It is worth noting that the outfall (T1) possessed the highest mean biomass (18.9 kg) and that T6 pos-

sessed the lowest (4.9 kg). Margalef diversity was significantly inversely correlated to hornyhead turbot ( $r^2 = 0.53$ ;  $p = 0.01$ ) and English sole ( $r^2 = 0.65$ ;  $p = 0.02$ ) histopathological severity. Abundance, Shannon–Wiener values, Margalef diversity, and evenness did not correlate with any other biomarker examined. Diversity indices also failed to correlate to sediment chemistry at all study sites evaluated.

## DISCUSSION

#### Sediment chemistry

Higher sediment PCBs were observed at the outfall station (T1). However, evidence also exists that the impact of sediment-associated PCBs on flatfish is actually decreasing in this region of coastal Southern California. Total PCB concentrations of liver have decreased in pacific sanddab (*Citharichthys sordidus*) and longfin sanddabs (*Citharichthys xanthostigma*) in the Southern California Bight area from 1985 through 1994

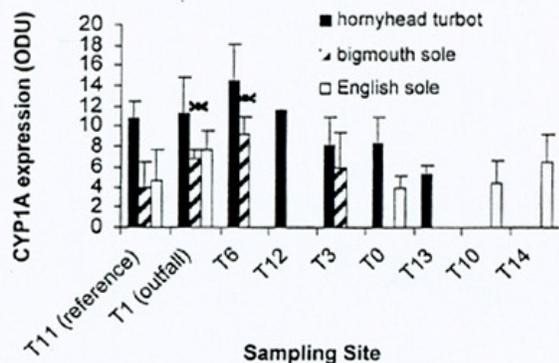


Fig. 3. The CYP1A expression in male and female flatfish species at various oceanic locations of Southern California (USA). \*  $p < 0.05$ . Values represent the mean and standard deviation of CYP1A expression in 12 to 22 animals per site; ODU = optical density units.

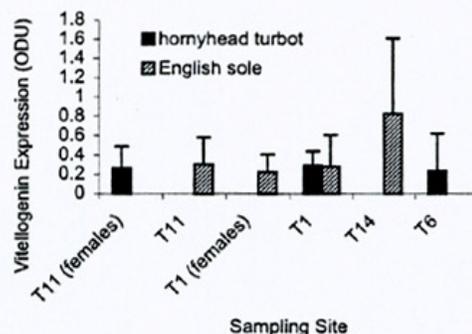


Fig. 4. Plasma vitellogenin expression in male and female English sole and hornyhead turbot at various oceanic locations of Southern California (USA). Values represent the mean and standard deviation of vitellogenin expression in 3 to 15 animals per site; ODU = optical density units.

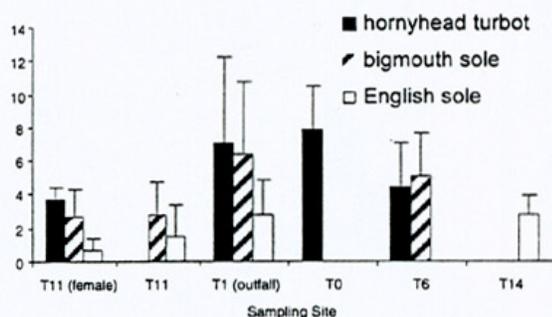


Fig. 5. Cytosolic (hepatic) vitellogenin expression in three species of male and female flatfish at various oceanic locations of Southern California (USA). Values represent the mean and standard deviation of cytosolic vitellogenin expression in 3 to 15 animals per site; ODU = optical density units.

[4]. The same study reported a 5- to 35-fold decrease in total PCB liver concentrations in Dover sole (*Microstomus pacificus*) between 1977 and 1994. Although the monitoring of municipal effluents is considered a point-source endeavor, it is important to note that historical regionwide impacts of PCBs have been shown to present less of a threat. Schiff [20] reported that approximately 42% of the sediments sampled in the Southern California Bight area were contaminated with respect to PCBs. Schiff [20] also reported that concentrations of PCBs were disproportionately greater in outfall areas of the Bight. Raco-Rands [21] estimated that 99% of PCBs and other constituents discharged from outfall areas were discharged between 1972 and 1988. This would indicate that the current sediment PCBs found within the outfall area were derived from historical inputs.

Mean high-molecular-weight sediment PAH concentrations were 115  $\mu\text{g}/\text{kg}$  and low-molecular-weight sediment PAHs 370

$\mu\text{g}/\text{kg}$  for the area immediately surrounding the outfall. During July 2000, concentrations of total PAHs ranged from 21 to 673  $\mu\text{g}/\text{kg}$ . These levels are comparable to the previous years surveyed. During July 1999, concentrations of PAHs ranged from 19 to 972  $\mu\text{g}/\text{kg}$  (<http://www.ocsd.com/main.htm>). Sediments sampled close to the outfall location historically have higher proportions of lower-molecular-weight PAH compounds, such as naphthalene (<http://www.ocsd.com/main.htm>). Philips et al. [22] also reported significantly elevated levels of sediment naphthalenes at Orange County's outfall (177  $\text{ng}/\text{g}$ ). Overall, although PAHs and PCBs were present in the sampling area, concentrations have been declining and were relatively low compared to other locations in the Southern California Bight.

#### Biochemical endpoints

The main objectives of this study were to assess the expression of various hepatic bioindicators of three species of fish (hornyhead turbot, English sole, and bigmouth sole) located at and around the Orange County municipal outfall and to determine whether these endpoints were related to sediment PCBs, to PAHs, or to detrimental effects on these fish species and their respective populations.

Significantly elevated levels of hepatic CYP1A were observed at T6 for hornyhead turbot and bigmouth sole. The CYP1A induction was also significantly elevated in English sole at T1 (outfall). Total sediment PCBs correlated to English sole CYP1A expression ( $r^2 = 0.97$ ;  $p = 0.037$ ). In contrast to PCB relationships, no correlations were obtained between CYP1A expression and sediment PAHs, which were relatively low. Previous studies [23,24] have demonstrated that CYP1A induction in various fish species was related to exposure to xenobiotic chemicals, such as PAHs and PCBs. Stein et al. [25] reported elevated hepatic mono-oxygenase activity from

Table 3. Major histopathological observations in English sole (ES), bigmouth sole (BS), and hornyhead turbot (HT) sampled in 10 stations off Orange County (CA, USA)<sup>a</sup>

Species	T11 (reference)	T1 (outfall)	T2	T6	T14	T10	T13	T12	T3	T0
ES	[60] 4/60 HCN (7%) 1/60 HD (2%) 1/60 S (2%) 1/60 SC (2%)	[60] 1/60 CL (2%) 3/60 HCN (5%) 1/60 HH (2%)	—	—	[6] 1/6 SC (17%)	[7] 0%	[2] 0%	[1] 0%	—	[11] 0%
BS	[20] 0%	[5] 0%	[1] 0%	[14] 0%	[2] 0%	[1] 0%	[1] 0%	[10] 0%	[60] 0%	—
HT	[4] 1/4 HH (25%)	[33] 1/33 BF (3%) 1/33 CCF (3%) 5/33 EF (15%) 1/33 HH (3%) 1/33 HR (3%)	[8] 0%	[7] 0%	—	—	[6] 1/6 EF (17%)	[20] 2/20 EF (10%)	[8] 0%	[12] 1/12 EF (8%) 1/12 LCA (8%)

<sup>a</sup> [n] = number of specimens examined per station; some specimens may have more than one type of histopathological condition, BF = basophilic focus, CCF = clear cell focus, CL = chronic lympho-mononuclear infiltrate, EF = eosinophilic focus, S = steatosis (fatty change), HCN = hepatocellular coagulative necrosis, HD = hydropic degeneration, HH = hepatocellular hyalinization, HR = hepatocellular regeneration, LCA = liver cell adenoma, SC = sinusoidal congestion.

Table 4. Population measurements and community diversity indices for July 2000 Orange County (CA, USA) Sanitation District sampling

Station	Species richness (spp./haul)	Abundance (no./haul)	Biomass (kg/haul)	J' <sup>a</sup>	Margalef diversity	75% dominance
T11 (reference)	20	636.0	11.001	0.73	4.45	4
T1 (outfall)	15.7	579.0	16.087	0.61	2.32	3.00
T6	13	94.0	4.951	0.81	2.66	5.00
T3	16	822.0	19.005	0.51	2.3	2.00
T14	15.5	678.0	16.131	0.71	2.25	4
T10	15	252.0	7.306	0.64	2.54	3.50
T12	12.7	417.0	6.508	0.99	2.9	5.00
T13	13.3	507	5.643	0.69	2.98	3.5

<sup>a</sup> J' = species evenness.

PAH- and PCB-contaminated sites in Puget Sound (WA, USA) in English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*), and rock sole (*Lepidopsetta bilineata*). Total sediment PAHs were 3,600 ng/g wet-weight sediment for the Duwamish Waterway (Seattle, WA, USA) and 21 ng/g (total PAHs) for Polnell Point (Puget Sound). Total sediment PAH levels at the outfall in our study were near sevenfold less (~500 ng/g). Collier et al. [26] also found significantly higher levels of CYP1A in English sole and starry flounder in a PAH- and PCB-contaminated area of Puget Sound when compared to a reference location. These results are further supported by other studies involving flatfish exposure to xenobiotic chemicals [27–29].

Levels of biliary FACs displayed no significant differences between sites. Levels were highest at the reference location for equivalents of benzo[a]pyrene, naphthalene, and phenanthrene. The higher levels present at the reference location (T11) suggest perhaps an alternative reference site for OCSD sampling schemes. It is important to note, however, that no correlations were observed between biliary FACs and CYP1A induction in English sole. These results surprisingly contrast previous studies with flatfish [8,25]. However, Eggins et al. [30] reported a lack of correlation between CYP1A and exposure to sediment PAHs as measured by 1-hydroxypyrene in bile, obtaining higher CYP1A induction in offshore areas and higher PAH exposure in inshore areas of the southern North Sea in a study with plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*). No significant correlation was observed between PAH compounds in sediment and FAC levels in English sole collected from those sites. This could possibly be due to differential exposure, mixture antagonism by other compounds, or feeding preferences of each species.

No statistically significant differences were observed in hepatic DNA damage for the sites examined, and no significant correlation was seen between DNA damage and the other hepatic or biliary indicators examined. Previous studies have indicated increased DNA damage at PAH-contaminated sites compared to clean reference locations [13,25,31]. Studies by Padrang et al. [13] indicated higher DNA damage in bullheads (*Ameiurus nebulosus*) in PAH- and PCB-contaminated sediment sites in three locations—Big Creek (western Lake Erie, Canada), Hamilton Harbor (western Lake Ontario, Canada), and the Detroit River (Canada)—when compared to reference locations. The DNA adducts have also been used extensively as an indicator of genotoxicity. Sediment PAH concentrations were relatively low in our study and may not have caused significant DNA damage. Hepatic xenobiotic DNA adducts were found to be significantly higher in English sole and starry

flounder sampled in the PAH-contaminated Duwamish Waterway compared to a reference station at Polnell Point near Puget Sound [25]. Stein et al. [25] confirmed that levels of DNA damage (PAH adducts) in the fish examined were generally related to the level of PAH contamination at each site. The DNA adducts were associated with hepatocellular nuclear pleomorphisms and megalocytic hepatitis in English sole from contaminated locations at Puget Sound [31]. It is likely that since limited impact was observed histologically, the minor amounts of DNA damage that were observed did not exceed thresholds necessary for higher-level effects.

The hepatic severity metric was inversely correlated with abundance for English sole. However, the histopathological evaluations revealed that for the three species examined, no statistical differences existed in hepatic severity between the outfall (T1) and a reference station. Consequently, hepatic severity in the English sole does not appear to be associated with the outfall effluent. Since no other biochemical indicator of chemical exposure was related to hepatic severity or any other histopathological endpoint, it is likely that no association exists with the chemical agents (PAHs or planar aromatic compounds) that induce the indicators examined in this study.

In the area surrounding the OCSD municipal outfall, male English sole, bigmouth sole, and hornyhead turbot were exposed to levels of estrogenic compounds sufficient enough to raise their plasma and hepatic levels of vitellogenin to that equivalent to sexually mature females. The effluents of municipal sewage treatment plants contain both synthetic and natural estrogens and xenoestrogens. These compounds can mimic estrogens in male and juvenile fish [32]. Purdom et al. [33] demonstrated that sewage effluent containing estrogenic substances induced vitellogenin synthesis in male trout. A recent study conducted in New York (USA) on the effects of municipal sewage effluent to sunshine bass indicated a 1730% (plasma) and 131% (hepatic) increase in male vitellogenin levels when compared to reference animals [17]. Several studies have documented increases in male vitellogenin levels in fish from marine waters. Allen et al. [34] reported higher plasma vitellogenin in male flounder in several estuaries. Lye et al. [35] found that male flounder (*P. flesus*) exposed to sewage effluent discharged into tidal stretches of rivers and estuaries of the United Kingdom contained unexpectedly high concentrations of plasma vitellogenin. Although vitellogenin was observed in Southern California flatfish, no apparent relationships existed with abundance or any other community metrics indicating populations of these particular species are unaffected by estrogen exposure. Reasons for this are unclear and warrant further study to determine the agents responsible for estrogenic

Table 5. Abundances of hornyhead turbot, bigmouth sole, and English sole from 1986 through 2001 at Orange County (CA, USA) Sanitation District sampling locations. The 1986 through 2000 abundances consist of the mean of both summer and winter sampling. The 2001 abundances are for winter sampling only. Stations displayed represent those stations where the three species have been collected consistently since 1986

	T1 (outfall)	T11 (reference)	T6	T3
<b>Hornyhead turbot</b>				
1986	74	11	9	31
1987	44	21	7	33
1988	31	20	13	18
1989	15	16	8	33
1990	19	24	22	32
1991	40	30	34	43
1992	29	11	16	32
1993	36	17	19	20
1994	34	15	7	26
1995	26	11	11	11
1996	18	13	2	9
1997	31	24	11	19
1998	8	12	3	24
1999	32	22	14	30
2000	29	43	18	30
2001	56	49	6	18
<b>Bigmouth sole</b>				
1986	26	39	62	49
1987	49	107	100	76
1988	50	88	48	49
1989	24	88	43	73
1990	17	87	34	60
1991	18	57	38	43
1992	34	37	41	10
1993	32	74	22	31
1994	39	45	12	14
1995	20	32	11	26
1996	10	22	3	29
1997	13	31	3	30
1998	3	4	4	9
1999	33	49	16	33
2000	17	124	26	123
2001	24	80	2	28
<b>English sole</b>				
1986	1	1		
1987	3	1		
1988	3	1		
1989	10	5		
1990	17	11		
1991	22	5		
1992	6	3		
1993	6	5		
1994	3	0		
1995	5	34		
1996	54	37		
1997	86	25		
1998	4	0		
1999	2	7		
2000	28	18		
2001	86	129		

activity and why expression of vitellogenin in the males is not altering populations.

#### Ecological measurements

The number of fish species, individuals, and fish biomass at the outfall is comparable to the other 55-m stations in the vicinity of the OCSD study area (Table 5). Throughout the past 16 years, population metrics and community diversity indices appear to be unaffected by the OCSD wastewater out-

fall and may in fact be enhanced because of the increase in food availability near the outfall (<http://www.ocsd.com>). Winter sampling from January 1986 to January 2001 at the outfall possessed the highest mean values for the Shannon–Wiener, Margalef, and evenness indices (<http://www.ocsd.com>). Mean indices and metric values from summer sampling dating back to August 1985 and continuing to August 2000 indicated that the outfall possessed higher diversity indices than reference locations. Margalef diversity was inversely correlated to histopathological severity in hornyhead turbot and English sole. This would suggest that the sites containing higher incidences of histopathological severity for these two species have lower overall diversity. However, no comparable relationship was observed in bigmouth sole.

No relationships were observed between biochemical endpoints of exposure, histopathological endpoints, or population metrics in this study indicating a relatively nonimpacted area. Few studies have attempted to correlate biochemical responses with endpoints observed at the population and community levels of biological organization [36]. Klopper-Sams et al. [37] conducted a study evaluating the effects of treated bleached-kraft effluent on CYP1A expression in mountain whitefish (*Prosopium williamsoni*) and longnose sucker (*Catostomus catostomus*). No relationship was observed between ethoxyresorufin-*O*-deethylase activity and various population parameters, including catch per unit effort, species composition, and abundance [37]. Adams et al. [38] reported that growth of redbreast sunfish (*Lepomis auritus*) was inversely related to ethoxyresorufin-*O*-deethylase activity at mercury- and PCB-contaminated sites. Schlenk et al. [36] obtained a significant inverse correlation between fish health index of centrarchids, hepatic CYP1A of *Micropterus salmoides*, and species richness. More studies are necessary to determine the effectiveness of using biomarkers to jointly evaluate animal health and population–community effects.

#### CONCLUSIONS

No significant increases were observed in liver histopathology or DNA damage in fish from the outfall sites. Likewise, biochemical indicators of exposure and effect were also not affected, with the exception of CYP1A induction in English sole at the outfall (T1) and hornyhead turbot and bigmouth sole at T6. In general, based on the historical population data of these three species of fish in the outfall area, no significant reductions have been observed in population sizes.

The only consistently elevated biomarker was vitellogenin in male flatfish. However, the lack of significant abundance diminishment in the three flatfish examined in this study over the past 15 years indicates that exposure to estrogens may not be affecting these populations. Although it would appear that the estrogenic chemicals present in the area surrounding the outfall are not causing higher-level population effects, more in-depth population and community-level studies are necessary to substantiate this claim. Future screening of estrogenic chemicals in fish and wastewater would be a wise decision for monitoring the status of the fish populations in this area of coastal Southern California.

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## REFERENCES

1. Southern California Coastal Water Research Project. 1973. The ecology of the Southern California Bight: Implications for water quality management. Technical Report 104. El Segundo, CA, USA.
2. Stull J, Baird R, Heeson T. 1986. Marine sediment core profiles of trace constituents offshore a deep wastewater outfall. *J Water Pollut Control Fed* 57:833-840.
3. Young D, McDermott D, Heeson T. 1976. DDT in sediments and organisms around southern California outfalls. *J Water Pollut Control Fed* 48:1919-1928.
4. Schiff K, Allen MJ. 2000. Chlorinated hydrocarbons in flatfishes from the Southern California, USA, Bight. *Environ Toxicol Chem* 19:1559-1565.
5. Olsgard F, Gray JS. 1995. A comprehensive analysis of the effects of offshore oil and gas exploration and production on the benthic communities of the Norwegian continental shelf. *Mar Ecol Prog Ser* 122:277-306.
6. Sumpter JP, Jobling S. 1995. Vitellogenesis as a biomarker of oestrogenic contamination of the aquatic environment. *Environ Health Perspect* 32:2498-2506.
7. Stegeman JJ, Lech JJ. 1991. Cytochrome P450 monooxygenase systems in aquatic species: Carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environ Health Perspect* 90:101-109.
8. Johnson LL, Casillas E, Sol S, Collier T, Stein J, Varanasi U. 1993. Contaminant effects on reproductive success in selected benthic fish. *Mar Environ Res* 35:165-170.
9. Beyer J, Sandvik M, Hylland K, Fjeld E, Egaas E, Aas E, Skare JU, Goksøyr A. 1996. Contaminant accumulation and biomarker responses in flounder (*Platichthys flesus* L.) and Atlantic cod (*Gadus morhua* L.) exposed by caging to polluted sediments in Sørfjorden, Norway. *Aquat Toxicol* 36:75-98.
10. Myers MS, Stehr CM, Olson OP, Johnson LL, McCain BB, Chan S-L, Varanasi U. 1994. Relationships between toxicopathic hepatic lesions and exposure to chemical contaminants in English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*), and white croaker (*Genyonemus lineatus*) from selected marine sites on the Pacific Coast, USA. *Environ Health Perspect* 102:2-17.
11. Baumann PC, Mac MJ, Smith SB, Harshbarger JC. 1991. Tumor frequencies in walleye (*Stizostedion vitreum*) and brown bullhead (*Ictalurus nebulosus*) in tributaries of the Laurentian Great Lakes. *Can J Fish Aquat Sci* 48:1804-1810.
12. Steinert SA. 1996. Contribution of apoptosis to observed DNA damage in mussel cells. *Mar Environ Res* 42:253-259.
13. Padrang R, Petras M, Ralph S, Vrzoc M. 1995. Alkaline single cell gel (Comet) assay and genotoxicity monitoring using bullheads and carp. *Environ Mol Mutagen* 26:345-256.
14. National Oceanic and Atmospheric Administration. 1993. Sampling and analytical methods of the National Status and Trends Program National Benthic Surveillance and Mussel Watch Projects 1984-1992: Overview and summary of methods, Vol 1. NOAA Technical Memorandum NOS ORCA 71. Silver Spring, MD, USA.
15. Rice CD, Schlenk D, Ainsworth J, Goksøyr A. 1998. Cross-reactivity of monoclonal antibodies against peptide 277-294 of rainbow trout CYP1A1 with hepatic CYP1A among fish. *Mar Environ Res* 46:87-91.
16. Krahn MM, Rhodes LD, Myers MS, Moore LK, MacLeod WD, Malins DC. 1986. Associations between metabolites of aromatic compounds in bile and the occurrence of hepatic lesions in English sole (*Parophrys vetulus*) from Puget Sound, Washington. *Arch Environ Contam Toxicol* 15:61-67.
17. McArdle M, Elskus A, McElroy A, Larsen B, Benson W, Schlenk D. 2000. Estrogenic and CYP1A response of mummichogs and sunshine bass to sewage effluent. *Mar Environ Res* 50:175-179.
18. Lomax DP, Roubal WT, Moore JD, Johnson LL. 1998. An enzyme-linked immunosorbent assay (ELISA) for measuring vitellogenin in English sole (*Pleuronectes vetulus*): Development, validation and cross reactivity with other pleuronectids. *Comp Biochem Physiol B* 121:425-436.
19. Krebs CJ. 1989. *Ecological Methodology*. HarperCollins, New York, NY, USA, p 654.
20. Schiff KC. 2000. Sediment chemistry on the mainland shelf of the Southern California Bight. *Mar Pollut Bull* 40:268-276.
21. Raco-Rands V. 1996. Characteristics of large municipal wastewater treatment facilities in 1994. In Cross J, Francisco C, Hallock D, eds, Southern California Coastal Water Research Project Annual Report 1994-95. Westminster, CA, USA, pp 10-20.
22. Phillips CR, Venkatesan MI, Bowen R. 1997. Interpretation of contaminant sources to San Pedro Shelf sediments using molecular markers and principal component analysis. In Eganhouse RP, ed. *Molecular Markers in Environmental Geochemistry*. American Chemical Society Symposium Series 671. Washington DC, pp 242-260.
23. Spies RB, Stegeman JJ, Hinton DE, Woodin B, Smolowitz R, Okihiro M, Shea D. 1996. Biomarkers of hydrocarbon exposure and sublethal effects in embiotocid fishes from a natural petroleum seep in the Santa Barbara Channel. *Aquat Toxicol* 34:195-219.
24. Collier TK, Stein JE, Goksøyr A, Myers M, Gooch JW. 1995. Biomarkers of PAH exposure in oyster toadfish (*Opsanus tau*) from the Elizabeth River, Virginia. *Mar Environ Res* 39:348-349.
25. Stein JE, Collier TK, Reichert WL, Casillas E, Hom T, Varanasi U. 1992. Bioindicators of contaminant exposure and sublethal effects: Studies with benthic fish in Puget Sound, Washington. *Environ Toxicol Chem* 11:701-714.
26. Collier TK, Anulacion BF, Stein JE, Goksøyr A, Varanasi U. 1995. A field evaluation of cytochrome P4501A as a biomarker of contaminant exposure in three species of flatfish. *Environ Toxicol Chem* 14:143-152.
27. Addison RF, Edwards AJ. 1988. Hepatic microsomal mono-oxygenase activity in flounder, *Platichthys flesus*, from polluted sites in Langesundfjord and from mesocosms experimentally dosed with diesel oil and copper. *Mar Ecol Prog Ser* 46:51-54.
28. Stegeman JJ, Woodin BR, Goksøyr A. 1988. Apparent cytochrome P450 induction as an indication of exposure to environmental chemicals in the flounder *Platichthys flesus*. *Mar Ecol Prog Ser* 46:55-60.
29. Goksøyr A, Husøy AM, Larsen HE, Klungsoyr J, Wilhelmsen S, Maage A, Brevik EM, Andersson T, Celander M, Pesonen M, Förlin L. 1991. Environmental contaminants and biochemical responses in flatfish from the Hvaler Archipelago in Norway. *Arch Environ Contam Toxicol* 21:486-496.
30. Eggens M, Bergman A, Vethaak D, van der Weiden M, Celander M, Boon JP. 1995. Cytochrome P4501A indices as biomarkers of contaminant exposure: Results of a field study with plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) from the southern North Sea. *Aquat Toxicol* 32:211-225.
31. Myers MS, Johnson LL, Hom T, Collier TK, Stein JE, Varanasi U. 1998. Toxicopathic hepatic lesions in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: Relationships with other biomarkers of contaminant exposure. *Mar Environ Res* 45:47-67.
32. Tyler CR, Joblin S, Sumpter J. 1998. Endocrine disruption in wildlife a critical review of the evidence. *Crit Rev Toxicol* 28: 319-361.
33. Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. 1994. Estrogenic effects of effluents from sewage treatment works. *Chem Ecol* 8:275-285.
34. Allen Y, Matthiessen P, Scott AP, Haworth S, Feist S, Thain JE. 1999. The extent of oestrogenic contamination in the UK estuarine and marine environments—Further surveys of flounder. *Sci Total Environ* 233:5-20.
35. Lye CM, Frid CLJ, Gill ME, McCormick D. 1997. Abnormalities in the reproductive health of flounder *Platichthys flesus*, exposed to effluent from a sewage treatment works. *Mar Pollut Bull* 34: 34-41.
36. Schlenk D, Perkins EJ, Hamilton G, Zhang YS, Layher W. 1996. Correlation of hepatic biomarkers with whole animal and population-community metrics. *Can J Fish Aquat Sci* 53:2299-2309.
37. Kloepper-Sams PM, Swanson SM, Marchant T, Schryer R, Owens JW. 1994. Exposure of fish to biologically treated bleached-kraft effluent. I. Biochemical, physiological and pathological assessment of rocky mountain whitefish (*Prosopium williamsoni*) and longnose sucker (*Catostomus catostomus*). *Environ Toxicol Chem* 13:1469-1482.
38. Adams SM, Crumby WD, Greeley MS Jr, Ryon MG, Schilling EM. 1992. Relationships between physiological and fish population responses in a contaminated stream. *Environ Toxicol Chem* 11:1549-1557.