Use of chemical markers to identify sources of fecal indicator bacteria in the lower Santa Ana River

James A. Noblet1, Diana L. Young, Eddy Y. Zeng* and Semsi Ensari2

ABSTRACT - The Santa Ana River and adjacent wetlands have been identified as potential sources of fecal indicator bacteria (FIB) to the surf zone at Huntington Beach, California, but it remained unclear whether sewage or non-sewage related contamination was the main concern. To address this issue, we collected and analyzed 54 water samples from three locations in the intertidal zone near the mouth of the Santa Ana River, California, for a suite of 10 fecal steroids and caffeine. The data were used to identify possible sources of FIB within the lower Santa Ana River watershed. The sampling times were chosen to assess the influence of daily and fortnightly tidal cycles. Steroid ratios were different from those found in raw sewage or the effluent plume from a local wastewater treatment plant, and were more influenced by the spring/neap tidal cycle than by the daily tides, or by station location. Multivariate statistical analysis showed that the concentrations of FIB were better correlated with cholesterol (CHOE) than with the typical sewage sterols. Conversely, coprostanol (COP) was found to correlate most strongly with turbidity, suggesting that it stemmed from tidal resuspension of bottom sediments. Moreover, the relative abundances of certain steroids suggested a diagenetic rather than a biogenic source for the COP content of the samples. The results implied that sewage was not a significant source of fecal steroids, and therefore perhaps FIB to the study area. Instead, birds may be one possible source of the intermittently high levels of FIB observed in the Santa Ana River and the nearby surf zone.

INTRODUCTION

Public beaches are an important economic, recreational, and cultural resource for southern California. In an effort to protect beach-goers from waterborne disease, state regulations require that water quality be monitored routinely at public beaches with 50,000 or more annual visitors (Assembly 1997-1998). Beach water quality monitoring programs are required to collect samples and measure three types of FIB - total coliforms (TC), fecal coliforms (i.e., *E. coli*, or EC), and the enterococcus group (ENT). The State of California has set uniform contact water quality standards for the three FIB. If the concentration of any FIB in a sample exceeds the respective standard, the local health officer is required to post warnings, or close the beach to swimmers if a sewage spill is suspected.

Huntington State Beach and city beaches in California have been particularly affected by the new regulations. There have been a total of 684 health advisories posted at Huntington State Beach and city beaches between July 1999 and April 2002, 84% of which were due to ENT exceeding the contact water quality standards (Grant et al. 2002). Huntington State Beach even received national attention in the summer of 1999, when a large section of the beach was closed to the public (Boehm et al. 2002) due to persistently high levels of all three FIB groups. In response, the Orange County Sanitation District (OCSD; Fountain Valley, CA) conducted a comprehensive survey and inspection of the local sewage infrastructure, but no significant sewage leaks were identified (Grant et al. 2001). The lack of an obvious sewage source spawned a number of studies to identify possible mechanisms for sewage outfall plume transport to the coastal zone (Boehm et al.)

*Present address: Guangzhou Institute of Geochemistry, Chinese Academy of Science, P.O. Box 1131, Guangzhou 510640, China
1California State University-San Bernardino, 5500 University Parkway, San Bernardino CA 92407-2397
2Henry Samueli School of Engineering, University of California, Irvine, CA 92691
2002), gain an understanding of the temporal variability of surfzone FIB concentrations (Boehm et al. 2002), and identify other possible sources of fecal contamination (Grant et al. 2001). In the latter study, Grant et al. (2001) did find compelling evidence that a local constructed wetland, Talbert Marsh, was a net source of ENT to the surfzone. Moreover, the study identified birds and urban runoff as the most likely sources of FIB, particularly ENT, to the marsh, which in turn acted as a reservoir and secondary source of ENT to the local surf zone.

The results of the study by Grant et al. (2001) prompted a similar but expanded follow-up study on the sources and dynamics of fecal indicators in the lower Santa Ana River watershed. As one component of the larger follow-up study, the objective of the research described herein was to use selected chemical markers for identifying possible sources of FIB in the lower Santa Ana River watershed, and thus potentially to nearby areas of Huntington Beach. Caffeine (CAF) and a suite of fecal steroids, including CHOE (5-cholesten-3β-ol), COP (5β-cholestan-3β-ol), epicoprostanol (eCOP, 5β-cholestan-3β-ol), cholesterol (CHOA, 5α-cholestan-3β-ol), α-cholestanone (aONE, 5α-cholestan-3-one), β-cholestanone (bONE, 5β-cholestan-3-one), campesterol (CAM, 24-methyl-5-cholesten-3β-ol), β-sitosterol (βSIT, 24-ethyl-5-cholesten-3β-ol), stigmasterol (STIG, 24-ethyl-5,22-cholestadiene-3β-ol), and stigmastanol (STAN, 24-ethyl-5α-cholestan-3β-ol) were selected for investigation. These compounds (particularly COP) have been recognized and used as chemical markers of sewage contamination for decades (Tabak et al. 1971, Sheldon and Hites 1978, Hatcher and McGillivary 1979, Walker et al. 1982, Takada and Eganhouse 1998, Maldonado et al. 2000, Seigener and Chen 2002). CAF, thought to be a unique marker for sewage effluent (Standley et al. 2000, Simpson et al. 2002), is highly water soluble and present primarily in the dissolved phase of aqueous samples. In contrast, fecal steroids are large hydrophobic molecules that bind readily to suspended particles and sediments, and are thus suitable for source tracking in solid samples.

The present study focused on the use of steroid ratios, instead of individual steroid concentrations, to elucidate source information, because several previous studies have shown the utility of measuring a suite of C_{27} - C_{29} sterols, stanols, and stanones for source tracking of fecal contamination (Venkatesan and Santiago 1989, Grimalt et al. 1990, Leeming et al. 1996, Standley et al. 2000). Use of individual steroids in source tracking may lead to erroneous conclusions because fecal steroids are not necessarily of anthropogenic origin. These compounds also are produced in the digestive tract of many higher animals, including birds, and both land and marine mammals (Venkatesan et al. 1986, Venkatesan and Santiago 1989, Leeming et al. 1996, Standley et al. 2000). In addition, COP and other 5α and 5β stanols can be produced by diagenetic processes in anoxic sediments (Grimalt et al. 1990). Finally, to facilitate the interpretation of fecal steroid data, turbidity, chlorophyll, total suspended solids, TC, EC, and ENT were also measured concurrently with the selected chemical markers.

**METHODS**

**Sample Collection**

Three locations in the Santa Ana River (SAR) estuary were sampled during June and July of 2001. One station was located just below and west of the Pacific Coast Highway bridge (W2), and the two other stations were located at the entrance of two isolated sloughs, a lower slough (W4) and an upper slough (W5) that are subject to tidal fluxes of seawater (Figure 1). Six sampling excursions were conducted within the time frame encompassing two spring-neap tidal transitions. During each excursion, three samples were collected from each station at different tidal stages, i.e., near the end of the flood tide, the beginning of the ebb, and the base of the ebb or slack period (Figure 2). Overall, 54 ~18 L samples were collected by a field crew using multiple grabs with a stainless steel bucket and poured into pre-cleaned five gallon glass bottles. All glass bottles were cleaned just prior to sampling using a laboratory glassware detergent, washed with ~4 M nitric acid, and rinsed with ultra-pure water and analytical grade methanol. One bottle was filled with ultra-pure water in the laboratory, and carried along with the field crew during sampling as a field blank. Samples were returned to the laboratory and stored at 4°C until filtered.

**Sample Extraction**

All the SAR samples were filtered within 24 h of collection with acid washed and combusted 142 mm diameter (0.7 μm pore size) pure glass TCLP filters, using a nitrogen (chromatographic grade) pressurized filtration system. Filtrates were collected in 4 L
amber glass bottles and stored at 4°C until extracted. Only 4 L of each filtrate sample (five gallons) were retained, and the remaining filtrate was discarded. Filtrate samples were extracted and analyzed as soon as possible, but always within 24 h after filtration. In contrast, filters containing suspended solids were placed in individual glass jars with Teflon®-lined closures, and stored frozen (-20°C) for up to one year until analyzed. Thus, the filtrate samples were analyzed in the order they were received from the field, whereas the filters were pulled from the freezer in no particular order for analysis. This mode of processing had the effect of randomizing the order in which the suspended solid samples were analyzed, and thus presumably mitigating any temporal bias in the analytical results.

Each filtrate sample (4 L) was extracted using 90 mm Empore C_{18} solid-phase extraction (SPE) disks, following the procedure of Standley et al. (2000). Loaded filters were dried in individual glass desiccators after Capangpangan et al. (1996), weighed, and extracted using supercritical fluid extraction (SFE). The suspended solids concentration was calculated from the filter mass difference and the sample volume. The SFE extractor consisted of a 10 mL stainless steel extraction cartridge, layered from the bottom with a glass fiber filter, about 2 mm of granular copper (20-30 mesh), and the dried filter containing suspended particles. The extraction was performed using an SFX-220 Supercritical Fluid Extraction System (ISCO, Lincoln, NE) with heated restrictors. Just prior to extraction, samples were spiked with 5 mL of a 1.0 ppm recovery surrogate solution (androstanol, 5α-androstan-3β-ol) and 300 mL of acetone as a polarity modifier. Samples were subject to a 10-min static extraction, followed by a 30 mL dynamic extraction, at 80°C and 365 atm, with the restrictors at 100°C, and methylene chloride solvent collection.

**Instrumental Analysis**

The SPE and SFE extracts were spiked with cholesterol-d_{6} (Cambridge Isotope Laboratories, Andover, MA) as an internal standard, taken to dryness under a nitrogen stream. The residue was then derivatized for 10 min at 70°C in 0.1 mL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1 % trimethylchlorosilane (MSTFA +1 % TMCS, Pierce, Rockford, IL). The derivatized extracts were immediately analyzed for caffeine (dissolved phase only) and steroids on a Varian 3800 gas chromatography.
graph with a Saturn 2000 ion trap mass spectrometer (Varian Inc., Walnut Creek, CA). Chromatographic separation was provided by a 60 m X 0.32 mm i.d. (0.25 µm film thickness) DB-XLB column (J&W Scientific, Folsom, CA), temperature-programmed from 60°C (held for 0.5 min) to 320°C (held for 15 min) at a rate of 20°C /min. Ultra-high-purity helium was used as carrier gas at a constant flow of 1.3 mL/min. Split/splitless injection was programmed as follows: 1:50 for 0.01 min; off for 5 min; and 1:50 afterwards. The mass spectrometer was operated at the electron ionization mode with 50 µamps of emission current, and spectra were scanned from 50 to 500 m/z at an ionization storage level of 35 m/z and an ionization time factor of 100%.

Analytical Performance

Preliminary experiments were conducted to evaluate the analytical methods. CAF and steroid standards were spiked into 4 L samples of ultrapure water at 25 ng/L, and extracted with SPE disks using the procedure described above. The recoveries for CAF ranged from 72% – 120% (n = 6) with an average recovery of 97 ± 17%. The average recovery for the entire suite of steroids (n = 6) was 108 ± 11%. The performance for actual sample analysis was monitored by using a recovery surrogate, androstanol (5α-androstan-3β-ol). Recoveries of androstanol from the dissolved phase sample analyses, including field blanks and duplicates (n = 63), ranged from 64.3 – 139 % with a mean recovery of 91 ± 13%.

During the interim period between analysis of the dissolved and particulate phase samples, experiments were conducted to determine the optimum conditions for SFE of the particle-laden filters. SFE performance was optimized using both a spiked inert material, reagent grade Celite 545-AW (Supelco Inc., Bellefonte, PA), and also a reference sediment, IAEA-408, which had certified concentrations for six of the sterol target analytes (IAEA, Vienna, Austria). Again, SFE recoveries in the actual sample analyses were monitored using androstanol as a recovery surrogate. Recoveries for all the SFE filter extractions (n = 68) ranged from 44 – 152%, with a mean of 89 ± 24%. Because this study focused primarily on steroid ratios, none of the data presented herein were adjusted for recoveries.

The lowest concentration calibration standard for all analyses was 0.1 mg/L, which corresponds to a minimum quantitation limit (MQL) of ~2.5 ng/L for the dissolved phase samples. The reporting limit for the particle phase was dependent on the sample mass, which varied significantly. However, for the average suspended solids sample mass of 320 mg, the corresponding MQL would be ~30 ng/g.

Analysis of Potential Source Samples

Samples of both untreated raw sewage and treated wastewater were obtained from the OCSD that discharges treated wastewater via a sewage outfall to the coastal ocean off Huntington Beach, CA. In-situ treated effluent samples were collected from the outfall plume using a CTD-Rosette Sampler (Sea Bird Electronics, Bellevue, WA). The raw sewage was diluted 1:10 with deionized water and the treated effluent was analyzed as received, both of which were stored at 4 °C and analyzed within 24 h of receipt. In addition, samples of fresh (i.e., still wet) bird feces was collected from the field area, combined into a single composite sample, and kept frozen until analyzed. For analysis, a small aliquot of thawed bird feces (~22 mg) was dissolved in 1 L of distilled water. All samples were processed by SPE and analyzed as described above.

Microbiological and Water Quality Analyses

Samples were collected hourly at each sampling station by the University of California-Irvine (UCI) field crews and transported to the UCI laboratory within 6 h. Samples (~20 mL) were immediately analyzed for TC, EC, and ENT using the Colilert® and Enterolert™ defined substrate tests (IDEXX Laboratories, Westbrook, ME). For turbidity, 60 mL samples were analyzed using a DRT-15CE Portable Turbidimeter (HF Scientific, Toronto, Canada). Stations W4 and W5 were also outfitted with water quality sondes, YSI Model 6900XL, that estimated chlorophyll concentration from in-situ measurements of fluorescence (YSI, Inc., Yellow Springs, OH).

Data Evaluation

Three types of data analysis were used to evaluate the chemical marker data. First, steroid ratios, such as the percentage of COP (COP/Σsteroids), the sum of COP and eCOP relative to total steroids ((COP+eCOP)/Σsteroids), and the ratios of the 5b to 5a epimers for the stanols and stanones, i.e., COP/(COP+CHOA) and bONE/(aONE+bONE) were calculated. Because COP has been identified as a major component of fecal steroids in sewage contaminated samples, values of COP/Σsteroids and (COP+eCOP)/Σsteroids may be proportional to the extent of sewage contamination. In addition,
Grimalt *et al.* (1990) found that plotting \( \text{COP}/(\text{COP}+\text{CHOA}) \) against \( \text{bONE}/(a\text{ONE}+\text{bONE}) \) provided a unique method for distinguishing between sewage and non-sewage derived pollution in complex environmental systems. They found that stanol and stanone ratios greater than 0.7 implied an *in vivo* production of COP, and thus were indicative of sewage pollution.

Second, selected individual and grouped chemical marker ratios and concentrations were compared graphically with the contemporaneous FIB data. Finally, multivariate statistical methods were used in order to quantify the relationships between the chemical marker and FIB data. The data analyses included cluster analysis and Pearson product moment correlation analysis performed using MINITAB Release 13.32 (Minitab, State College, PA). Pearson correlation values were calculated for the relationship between two different sums of steroids and the FIB.

**RESULTS**

**Distribution of Chemical Markers**

The target chemical marker compounds were widely distributed in the samples analyzed, with all but eCOP detected in either the particulate or dissolved phase of at least 42 out of 54 SAR samples (Table 1). CHOE was the most ubiquitous and abundant of the steroids, the only analyte detected in all the samples from both the particulate and dissolved phases, and its concentrations were substantially higher than all other analytes. CHOA, bSIT, STIG, and STAN were also detectable in almost all the samples (from either particulate or dissolved phase). Conversely, CAF was detected only in the dissolved phase, and eCOP was detected only in the particulate phase. Note that CAM was added as a target analyte for the particulate phase.

The distribution data in Table 1 allowed us to calculate the ratios of analyte concentrations in the particulate \( (C_p) \) to the dissolved phase \( (C_w) \) over a range of suspended solid concentrations from 19 to 71 mg/L. The log-transformed ratios of \( C_p/C_w \) for all appropriate steroids were fairly invariant, as indicated by the small standard deviations and 95% confidence intervals relative to the mean values (Table 2). These results were used to evaluate the quality and variability of the data.

**Steroid Ratios**

The average COP/\( \Sigma \)steroids values in suspected sources and SAR samples exhibited several interesting features (Figure 3). First, both the particulate and dissolved phase samples had COP/\( \Sigma \)steroids ratios that were markedly lower than either raw sewage or treated effluent samples from the OCSD. Second, the average COP/\( \Sigma \)steroids ratio in the particulate samples was comparable to that of seagull feces and slightly lower than that of duck feces reported in the literature (Leeming *et al.* 1996). Finally, all the field samples had higher relative COP contents than the bird fecal sample collected at the field area.

Ratios of \( \frac{\text{COP}+\text{eCOP}}{\Sigma \text{steroids}} \) and suspended solids in all SAR samples were plotted as a function of date, station, and diurnal tidal cycle (Figure 4). The highest \( \frac{\text{COP}+\text{eCOP}}{\Sigma \text{steroids}} \) ratios occurred toward the end of the ebb tides (i.e., slack). Moreover, there was no apparent correlation between the \( \frac{\text{COP}+\text{eCOP}}{\Sigma \text{steroids}} \) ratios and concentrations of suspended solids.

The \( \frac{5\beta}{(5\alpha+5\beta)} \) ratios for the SAR samples (both the particulate and dissolved phases) and suspected sources were calculated and plotted as stanols and stanones (Figure 5A). Suspected sources investigated included OCSD raw sewage and treated effluent, offshore OCSD outfall plume (Station 2205, 35 m depth), and SAR bird feces. Ratios for samples collected from the offshore OCSD outfall plume were identical to those for raw sewage. All of the sewage related samples were found to plot in the upper right corner of the graph (i.e., > 0.7 for both the stanol and stanone ratios). This result is in agreement with several other studies that have used these parameters for source identification, which found that sewage samples typically are very near unity for both ratios (Grimalt *et al.* 1990). In contrast, the SAR samples are spread across the bottom of the graph, and covering the full range of possible values from 0 to 1. All SAR samples were below 0.7 for the stanol ratio, and all but eight samples were below 0.7 for the stanone ratio. The bird fecal sample collected from the SAR field area plotted very near the origin, and thus the local birds are clearly differentiated from the sewage sources of fecal contamination.

The ratios for the particulate phase of the SAR samples in Figure 5A were expanded to explore the possible reasons for the range of stanone ratios observed. The data do not exhibit any type of pattern related to the station location (Figure 5B) or
diurnal tidal stage (Figure 5C), but do show a pattern related to the lunar tidal cycle (Figure 5D). The stanone ratio was generally higher during the neap tides and decreased during the spring tide. However, the pattern broke down on the second spring tide of the study period (Figure 5D), which raises concerns as to whether the previously observed pattern was indicative of an actual environmental process.

Comparison of Fecal Indicator Bacterial and Chemical Marker Data

A comparison of the FIB data with several chemical marker ratios and concentrations was performed (Figure 6). Over the timeframe of the study, there was only a single sampling interval when there was a large spike in all three FIB (Figure 6A). Of all the possible chemical marker parameters, only the sum of the most abundant sterols in bird feces, primarily CHOE, showed a comparable spike in the data (Figure 6B). Interestingly, the concentrations of COP and CAF, presumably more traceable to sewage contamination than the other marker compounds, did not exhibit any comparable spike (Figure 6C). There were three large spikes in the TC toward the end of the study period (Figure 6A), the second of which was coincidental with a large spike in the turbidity data (not shown). However, the turbidity data were found to more significantly correlate with sewage steroid data than the FIB data, as identified by the cluster analysis presented below.

Table 1. Distribution of the target chemical marker compounds in 54 Santa Ana River samples analyzed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Particulate (ng/g-dry wt)</th>
<th>Dissolved (ng/L)</th>
<th>Total (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (min, max)</td>
<td>Stddev</td>
<td>Detected</td>
</tr>
<tr>
<td></td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>0.10 (nd, 0.49)</td>
<td>0.11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.009 (nd, 0.396)</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>14.7 (1.79, 87.1)</td>
<td>1.87</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2.35 (0.17, 7.18)</td>
<td>0.76</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>1.33 (nd, 5.16)</td>
<td>1.29</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>3.99 (nd, 4.77)</td>
<td>0.76</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>3.88 (nd, 17.28)</td>
<td>3.5</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>0.64 (nd, 2.45)</td>
<td>0.58</td>
<td>27.1</td>
</tr>
<tr>
<td></td>
<td>1.01 (nd, 5.82)</td>
<td>1.02</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>0.94 (nd, 5.69)</td>
<td>0.97</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>25.4 (3.52, 114.2)</td>
<td>20.1</td>
<td>492</td>
</tr>
</tbody>
</table>

*particulate = solid particulate matter with size > 0.7 μm; dissolved = filtrates with size < 0.7 μm.
*stddev = standard deviation.
*Number of samples in which the compound was present at detectable levels.
*nd = not detected at the designated reporting levels.
*na = not analyzed for in the respective phase.

Table 2. Quantitative evaluation of the partitioning behavior of selected steroid compounds, as characterized by log (Cp/Cw) with Cp and Cw being the concentrations in the particulate and dissolved phases, respectively.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Sample size</th>
<th>Mean log (Cp/Cw)</th>
<th>stddev</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>coprostanol (COP)</td>
<td>11</td>
<td>4.04</td>
<td>0.39</td>
<td>0.23</td>
</tr>
<tr>
<td>cholesterol (CHOE)</td>
<td>54</td>
<td>4.52</td>
<td>0.36</td>
<td>0.1</td>
</tr>
<tr>
<td>cholestanol (CHOA)</td>
<td>50</td>
<td>4.91</td>
<td>0.38</td>
<td>0.1</td>
</tr>
<tr>
<td>α-cholestanone (aONE)</td>
<td>1</td>
<td>4.27</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>β-cholestanone (bONE)</td>
<td>16</td>
<td>4.35</td>
<td>0.35</td>
<td>0.17</td>
</tr>
<tr>
<td>β-sitosterol (bSIT)</td>
<td>44</td>
<td>5.3</td>
<td>0.37</td>
<td>0.11</td>
</tr>
<tr>
<td>stigmastanol (STIG)</td>
<td>42</td>
<td>4.41</td>
<td>0.27</td>
<td>0.08</td>
</tr>
<tr>
<td>stigmasterol (STAN)</td>
<td>46</td>
<td>4.04</td>
<td>0.42</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*stddev = standard deviation.
*CI = confidence interval.
*n/a = not applicable.
Figure 3. Ratios of coprostanol/total steroids (COP/Σ steroids) in suspected sources and field samples. Those sources marked with an asterisk (*) are from the literature. Error bars are the 95% confidence intervals (n = 54).

Figure 4. Ratios of sum of coprostanol and epicoprostanol to total steroids, (COP+eCOP)/Σ steroids, and suspended solids (> 0.7 µm) data plotted as a function of date, station, and diurnal tidal stage.

Statistical Analyses
The dendrograms produced by the cluster analyses show the degree of similarity among the parameters included in the analysis (Figure 7). In general, all of the parameters exhibited a high degree of similarity (>67%). Even so, higher levels of similarity among certain groups of parameters are readily apparent. For example, COP, eCOP, and bONE, are closely clustered (Figure 7, top), consistent with the perception that these steroids are sewage derived. Perhaps the most interesting result of this analysis is the observation that the FIB data do not cluster with the sewage steroids (i.e., COP, eCOP, and bONE). Rather, the FIB group most closely with CHOE, and then with the plant sterols bSIT and STIG (Figure 7, bottom). Other interesting results were that COP paired with turbidity (Figure 7, bottom), and that CAF was on an isolated branch of the dendrogram, and did not pair with any of the other parameters (Figure 7, top).

Based on the results of the cluster analyses and the steroid composition of potential sources, two groupings of steroids were selected as additional parameters for the statistical analysis. One group of sewage related steroids, COP+eCOP+bONE (sum1), and another group of bird fecal steroids, CHOE+CHOA+ bSIT (sum2). The bird steroids were the three most abundant steroids measured in the bird feces sample. These two sums were evaluated relative to the FIB data (log transformed prior to analysis) using Pearson Product Moment Correlation Analysis. There were significantly moderate correlations between log EC ($r = 0.536, p < 0.001$), log ENT ($r = 0.637, with p < 0.001$) and sum2. A somewhat lower correlation was found between log ENT ($r = 0.398, p < 0.01$) and sum1. Considering the correlation between log TC and log EC ($r = 0.553, p < 0.001$), the strengths of these correlations are not negligible. Interestingly, COP did not generate any significant correlations with the FIB data.

DISCUSSION
Source Assessment for Fecal Steroids
The ratios of the $5β/(5α+5β)$ stanols and stanones clearly showed that the SAR samples did not have the steroid profile typical of raw sewage, treated effluent or the OCSD outfall plume (Figure 5A). These ratios are effective in differentiating between potential sources because they provide information about the relative contributions of two
different production pathways for the $5\alpha$ and $5\beta$ stanols. The stanols are produced \textit{in vivo} by transformation of CHOE to a cholestenone, and then to $5\alpha$ or $5\beta$ cholestanone, followed by reduction to COP or CHOA (Grimalt et al. 1990, Takada and Eganhouse 1998). In contrast, in-situ formation of stanols in anaerobic sediments occurs by direct reduction of the 5-6 double bond of CHOE. Since the $5\alpha$ epimer is thermodynamically more stable, its formation is favored by the \textit{in-situ} reduction. In humans and some marine mammals, formation of the $5\beta$ epimer is highly favored (Venkatesan and Santiago 1989). Hence, the ability to distinguish between sources is a function of the ability to determine the relative contributions from \textit{in-vivo} and \textit{in-situ} production of the fecal steroids.

However, it has been shown that CHOA can be produced biogenically by a number of aerobic organisms, such as phytoplankton, zooplankton, and macrophytes (Grimalt et al. 1990). Therefore, in areas of high productivity, the utility of the stanol ratio may be reduced. Since the equivalent stanone ratio would not be affected by the biogenic production of the $5\alpha$ stanol, Grimalt et al. (1990) suggested that it be used as a complimentary parameter for distinguishing sewage pollution from other sources of fecal steroids.

The low stanol ratios for the SAR samples (Figure 5) implied an \textit{in-situ} origin for the fecal steroids detected in this study. However, the significant variability in the stanone ratio may suggest at least some \textit{in-vivo} contribution to the fecal steroid pool. Another possibility is that low levels of diagenetically produced coprostanol could become suspended during vigorous tidal flows, and then oxidized under aerobic conditions back to the stanone. Laboratory incubation experiments using radiolabeled compounds have demonstrated that interconversion of stanols and stanones does occur (Grimalt et al. 1990). In addition, the available fate data on COP in natural waters suggests that its half-life is less than 10 d under aerobic conditions (Takada and Eganhouse 1998). The fact that COP, eCOP, and bONE correlate with turbidity (Figure 6, bottom) also supports the idea of an \textit{in-situ} diagenetic source for these steroids.

Another aspect of the data is the relatively high levels of eCOP that occurred intermittently over the duration of the study (Table 1). COP/eCOP ratios derived from Table 1 varied from 0.88 to infinity (i.e., eCOP = 0). The measured levels of eCOP were surprising since it is known to be only a trace component of sewage steroids, and was not observed in any significant amount in any of the potential sources. Venkatesan and Santiago (1989) found that

---

**Figure 5.** Ratios of $5\beta/(5\alpha + 5\beta)$ stanols versus stanones in samples from the Santa Ana River and selected potential fecal sources (A), where ratios above 0.7 on one or both axes are typical for sewage sources (dashed lines), and in particulate samples from the Santa Ana River, plotted by sampling station (B), diurnal tidal cycle (C), and lunar tidal cycle (D).
COP/eCOP ratios less than one are indicative of marine mammal contributions. However, given the nature of the study area, contributions from marine mammals were unlikely. A similar pattern of intermittently low COP/eCOP ratios was observed in a study of several sites on the west coast of Florida, where again a marine mammal source was deemed unlikely (Sherblom et al. 1997). Also noteworthy, Eganhouse et al. (1988) found the COP/eCOP ratio in anaerobic digester sludge was 0.9 to 9, while the effluent solids ranged from 23 to 38. The results caused these investigators to question the utility of using the COP/eCOP ratio to differentiate between wastewater and natural contributions. It is likely that eCOP is the product of multiple redox cycles (stanol <=> stanone) occurring in the intertidal zone,
which is also consistent with our hypothesis on the formation of bONE. However, very little is known about the stability and reactions of these compounds under various redox conditions, and further experimental work would be necessary to verify this hypothesis.

Additional Evidence for Non-Sewage Sources of Fecal Steroids

Several additional pieces of evidence are present in the fecal steroid data that support the hypothesis that the fecal steroids in the study area are primarily of non-sewage origin. First, there is the agreement of the chemical marker data obtained in this study with previous studies in similar environmental settings. For example, Venkatesan and Kaplan (1990) found that in Santa Monica Bay, California, COP comprised 50-80% of the total steroids near sewage outfalls, but dropped to about 3% at stations distant from the source. Also, Phillips et al. (1997) conducted a study in 1994 which measured fecal steroids in sediments at two locations in the Santa Ana River and one location in Talbert Marsh. That study found sediment COP concentrations of 67 to 380 ng/g, which are comparable to our data for the suspended solids phase, which ranged from below detection to 487 ng/g (Table 1). These data suggest that the local sediments may be the source of the suspended solids, and that the COP content of the sediments has not changed significantly since 1994.

Additional evidence can be drawn from an estimate of the equilibrium state of the system can be made using the steroid partitioning data (Table 2). Since the organic carbon content of the particulate phase was not determined, a nominal value of 1% was assumed for this assessment. Takada and Eganhouse (1998) estimated the log octanol-water partition coefficient (log $K_{ow}$) for COP to be 6.5 to 7.5 from the water solubility of CHOE. Assuming that the organic carbon-normalized partition coefficient ($K_{oc}$) is approximated by $K_{oc} = 0.41 K_{ow}$ (Mackay et al. 1992), then the log ($C_p/C_w$) for the steroids would be about 4.11 to 5.11 at equilibrium. This is in general agreement with the measured average values from this study, which ranged from 4.04 (±0.39) to 5.30 (±0.37) (Table 2). These results imply that the system appears to be at approximate equilibrium between the dissolved and particulate phases, which suggests that the suspended solids and the associated fecal steroids detected in this study may have been derived from tidal scouring and resuspension of local bottom sediments rather than from an offshore source.

Among the parameters examined, only the summed concentration of the three most abundant sterols found in the bird feces collected at the field area was found to coincide with the single high FIB event (Figure 6B). The simultaneous sharp increase in bird fecal steroid and FIB concentrations is consistent with a previously reported hypothesis that birds may be a possible source of FIB to the watershed (Grant et al. 2001).

It should be noted that birds are not the only possible source of the FIB based on the fecal steroid data. Interestingly, dogs have fecal steroid composition very similar to that of birds (Leeming et al. 1997). In order to distinguish between these potential FIB sources, Leeming et al. (1997) used the ratio of fecal coliforms to Clostridium perfringens. The feces from birds and humans have virtually no C. perfringens spores (<0.01%), whereas domestic pets (dogs and cats) contain nearly equal amounts of both chemicals.
C. perfringens and fecal coliforms. Thus, this additional analysis could potentially be used in future studies to distinguish between FIB contributed by birds and domestic pets.

**LITERATURE CITED**


**ACKNOWLEDGEMENTS**

The authors are grateful to Professor S.B. Grant for providing the original impetus for this work. Also, we thank D. Tsukada for help in processing the samples, and A. Bolkhovitinov for GC/MS analyses. Lastly, we are very grateful to all the UCI students who performed the sample collection.