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**Steroid estrogens in ocean sediments**

A.J. Feitz*, O. Braga, A.I. Schäfer and G.A. Smythe

*Centre for Water and Waste Technology, School of Civil and Environmental Engineering, University of New South Wales, Sydney, NSW, 2052, Australia.

Bioanalytical Mass Spectrometry Facility, Faculty of Medicine, University of New South Wales, Sydney, NSW, 2052, Australia.

Civil, Mining and Environmental Engineering, University of Wollongong, NSW, 2522, Australia.

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*Corresponding author. Andrew Feitz: Tel: +61 2 9385 5082, Fax + 61 2 9313 8624, E-mail: andrew.feitz@unsw.edu.au

**Abstract**

Ocean sediment samples were taken adjacent and 7km from a deep ocean outfall (average depth 80m), the discharge point of an enhanced primary sewage treatment plant. All samples contained steroid estrogens at nanogram per gram levels with higher concentrations at the 7km sampling site. The concentration of estrone ranged from (0.16 - 1.17 ng/g), 17β-estradiol (0.22 – 2.48 ng/g) and the synthetic 17α-ethinylestradiol (<0.1 – 0.5 ng/g). The values detected correspond with estimates based on the proportion of estrogens sorbed to particles in the effluent and the expected proportion of sewage origin particles in the ocean sediments. The results suggest that estrogens associated with the particulate fraction aggregate on contact with high ionic strength seawater and settle to the seafloor after discharge through deep ocean outfalls.

**Keywords:** estrone, estradiol, ethinylestradiol, sediment, wastewater, sewage

1. Introduction

Understanding the fate of estrogens in the environment is key to understanding the potential for human released estrogens to induce abnormal reproduction in aquatic environments (Purdom et al., 1994; Desbrow et al., 1998; Routledge et al., 1998). Estrogens have been detected in rivers (Kuch and Ballschmitter, 2001; Snyder et al. 2001; Alder et al., 2001), lakes (Matsui et al., 2000; Shen et al., 2001; Snyder et al., 2004) and marine samples (Atkinson et al., 2003; Tashiro et al., 2003) and the primary sources are considered to be from sewage treatment plants and agricultural runoff or discharge (Tashiro et al., 2003; Kołodziej et al., 2004). There is now increasing evidence that estrogens such as estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are sorbing to riverbeds (Peck et al., 2004; Williams et al., 2003; de Alda et al., 2002; Holthaus et al., 2002), lake (Mibu et al., 2004) and estuarine (Thomas et al., 2004, Thomas et al., 2001) sediments where degradation, especially for EE2 under anaerobic conditions, is slow (Ying and Kookana., 2003; Ying et al., 2003; Jürgens et al., 2002). The release and fate of estrogens into the marine environment is little studied but of considerable interest as they appear to be relatively stable and show limited degradation even under aerobic conditions (Ying et al., 2003). Their affect on coral reefs is of particular concern (Atkinson et al., 2003). When estrone concentrations are greater than 300 pg/L, there is the potential of net uptake and potential accumulation in the reef benthos (Atkinson et al., 2003). Estrogens in the coastal marine environment may affect reproductive biology through embryonic development (Hathaway and Black, 1969), altered enzymatic activities (Ghosh and Ray, 1993a; Ghosh and Ray, 1993b) or cellular damage or apoptosis (Wiens et al., 1999; Viaengro et al., 2000). Much more information is required to characterise the presence of human-derived estrogens in marine environments and to determine their potential effects on the marine ecosystem. This paper reports on preliminary measurements of estrogens in ocean sediments.

2. Materials and Methods

2.1. Ocean sediment sampling

Ocean sediment samples were collected near the discharge point from a large coastal enhanced primary sewage treatment plant (STP). The STP is located in eastern Sydney and services domestic sewage (75%) and industrial wastewater (25%). It provides enhanced primary treatment (i.e. with FeCl₃ addition) for an average flow of 480 ML/day with ultimate disposal by deep ocean discharge (3.6km offshore, average 80m deep). A Motor Vessel (MV) Oceanographer was used as a stable platform, from which a “Smith McIntyre” grab (capacity approximately 5L) (Fig. 1) was deployed to collect sediment samples. Samples were collected from 2 locations (2 samples per location), adjacent to the Malabar deep ocean outfall and approximately 7km south of the outfall (Fig. 2). The vessel was manoeuvred to hold its position until the grab had reached the seafloor and a sediment sample was taken. In order to ensure samples are as representative as possible, the angle and speed at which the grab was lowered to the seafloor, was controlled and maintained for all the sub-sites. The grab was lowered to approximately 3 m above the seafloor and then released to collect the sample. In setting the angle and speed at which the grab is lowered, consideration was given to two things: maximising the volume of the sediment sample retrieved, and minimising the bow wave generated from the grab moving through the water column. This method of controlling the grab fall rate has been shown elsewhere to reduce the loss of the fine surface material (Bloomsquish, 1992). The samples were transferred to a 0.5L glass bottle and stored at ~20°C prior to analysis.
2.2. Standard Preparation

Estronone (E1), estradiol (E2), ethinylestradiol (EE2), and estrone-2, 4,16,16-d4 (d4-E1) were obtained from Sigma (Aldrich, Sydney, Australia). The d4-E1 was used as internal standard. Stock solutions of individual non-deuterated standards and deuterated internal standard were prepared by dissolving known amounts of 0.16mg/ml. Working standard solutions were obtained by further diluting stock solutions with water to obtain final concentrations of 0.5pg/µl. The stock solution of internal standard was further diluted with water to obtain a final concentration of 100pg/µl. Methanol and acetone/nitrite HPLC grade were obtained from Ajax Finechem, Sydney, Australia. Other solvents were of analytical grade, and they were used as supplied (Ajax Finechem, Sydney, Australia). Mill-Q water was used for all experimental procedures.

2.3. Preparation of sediment samples

Analysed were extracted from the sediment samples using liquid phase extraction (LPE). Prior to sample preparation, internal standard was added. Samples were then frozen with liquid nitrogen and then dried in a vacuum refrigerator for 24 hours at -70°C. The dried pellets were weighed and then dissolved in a mixture of 100ml acetone/hexane (50:50). The solution-sample slurry was then stirred and sonicated for 30 minutes. The supematant was then filtered through Whatman No. 1 glass fibre filter paper (Whatman, Sydney, Australia), the filter rinsed with a mixture of acetone/hexane (50:50), followed by solvent evaporation at 80°C in a water bath. The residues were dissolved in acetone. Analyses were then extracted from water samples by solid phase extraction (SPE) using the LC-18 SPE cartridges filled with 1.0 g of C18 (Supelco, Sydney, Australia). After fitting the SPE cartridge into a 12-Port Vacuum Manifold (Supelco, Sydney, Australia), the SPE was sequentially conditioned with 2x10 mL methanol, 1x10 mL Milli-Q water. Sample loading was achieved by passing standards and water samples through the LC-18 SPE cartridge. After sample loading, cartridges were dried in a vacuum desiccator for 30 to 40 minutes. Elution of the analytes was achieved by using 2.5 mL methanol and 0.1 mL of Milli-Q water. The collected solution was dried under vacuum and reconstituted to 1 mL with acetone before derivatisation and analysis.

2.4. Effluent sample preparation and centrifugation

Before effluent samples were processed, internal standard was added. After the addition of various quantities of NaCl, high-speed centrifugation was used to separate the solids from the water. Separation of the solid particles was achieved by centrifuging 2L samples at 10,000 rpm for 1 hour at 0°C. The water layer was removed for further solid phase extraction (see above) before derivatisation and analysis using GC-MS.

2.5. Sample Derivatisation for GC-MS Analysis

The derivatisation was carried out using a modified version of the method used by Nakamura et al. (2001) for the pentafluoroethylpropyltrimethylsilyl derivative. To the acetone extract, 100 µL of 10% aqueous potassium carbonate and 10 µL of pentafluoroethylpropyltrimethylsilyl reagent were added, and the solution was stirred for 30 minutes. The supernatant was removed and the collected solution was then added to the vial and kept at room temperature for 30 minutes. The toluene was added to 1 mL before analysis.

Gas Chromatography Mass Spectrometry Conditions

All GC-MS analyses were carried out using an Agilent 5890 gas chromatograph interfaced to an Agilent 5997B MS Engine (Agilent Technologies, Ryde, Australia). Chromatographic separations were performed with an HP-5MS capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). The GC oven temperature was programmed at 150°C for 1.5 min and then 36°C per minute to 310°C, final hold 70 min. The GC-MS interface heater, the ion source, quadrupole, and injection port temperatures were maintained at 260, 240, 100 and 260°C, respectively. Pulse splitless injection was used with a pulse pressure of 241 kPa (1.1 min) and purge time delay of 8 min. The MS analyses were performed with an electron-capture negative-ion (ECNI) source, using methane as reagent gas (Ultrapure grade, Matheson Gas Products Inc.) and selected ion monitoring mode. The (M-) ion and (M-TMS+) ions were monitored for all compounds with a dwell time of 100 ms per single ion. The injection volume was 1.0 µL. The standards containing all the estrogens (non-deuterated and deuterated) at accurate defined concentrations were derivatized as described above. Quantification was carried out by calculation of the response factors (RF) based on the area of the non-deuterated and deuterated estrogens standards. These ratios were converted to concentrations using a linear regression equation, which was used to assign the unknown concentrations. Signals for method limit of detection (LOD) and limit of quantification (LOQ) was set at 3- and 6-fold height of noise, respectively.

3. Results and discussion

Marine sediments taken near the ocean outfall were found to contain all three estrogens (Table 1; Fig. 3). The concentration of estrogens 7 km from the outfall were often considerably higher than immediately adjacent the outfall (Fig. 3). Previous studies monitoring total organic carbon (TOC) and particle size in the sediments indicate a slight increase in TOC and presence of finer particles at the 7 km sampling site compared to that at the outfall (AWT, 2000). Substantially higher TOC and a greater presence of fine particles was found 3 to 5 km from the outfall, suggesting that higher concentrations of particles and hence estrogens may be located in this region.

Based on silver enrichment data in marine sediments approximately 1-2 km from the outfall, it is estimated that sewage particles account for approximately 2% to the surficial sediment (top 10 cm) (Matthai et al., 2002). The majority of particles comprise of natural contributions from settling marine particulate matter and resuspended sediment. Using the 2% figure as a guide and assuming that there is no desorption of estrogens from the particles on contact with the seawater, the estimated concentration in the surficial sediments is close to the measured values (Table 2). It is expected that there will be a large fraction of estrogens attached to fine particles (Holthaus et al., 2002; Holbrook et al., 2004) and such particles are likely to aggregate on contact with higher inclusive strength waters and show greater settleability. Indeed, after adding various concentrations of salt to the STP effluent samples to induce aggregation, the concentration of E1 in particular in the aqueous sample (i.e. without pellet) decreased markedly without increasing salt concentration (Fig. 4). EE2 was not detected in any of the centrifuged liquid samples.

These preliminary results suggest that estrogens attached to particles in the effluent and, on contact with higher inclusive strength seawater, are aggregating and settling to the sea floor. As the measured concentration is similar to the estimated concentration in the marine sediments suggests that the degradation in the sediments is slow. The mean seawater flux 1-2 km from the outfall is 0.23 g/m²/d (Matthai et al., 2002), which is in the range where sulphate reduction and estrogens are likely to be equally important in oxidizing organic matter (Canfield, 1989). Fe and Mn porewater profile for marine sediments samples taken near the outfall indicate that near the sediment-water interface there is a thin oxic layer but at depths greater than 1-2 cm in the sediments the presence of elevated dissolved phase Mn and Fe indicates suboxic to anoxic organic matter reduction (Matthai et al., 1998). Anoxic conditions in marine sediments, in comparison to aerobic conditions, are not conducive to degradation of estrogens and the half-lives for E2 and E3S biodegradation are greater than 70 days (Ying and Kookana, 2005).

The poor removal of estrogens during primary clarification is well known (Holbrook et al., 2004; Ternes et al., 1999; Anderson et al., 2003). With effectively no removal of E1 and E2 during
treatment at the STP plant (Braga et al., 2005), it is important to consider the potential impacts of such a large load of estrogens on the receiving environment. The high levels of dilution obtained during ocean disposal may prove sufficient for the prevention of endocrine effects in marine animals, although picomolar concentrations have been shown to affect the development of aquatic organisms (Routledge et al., 1998; Schoenmakers et al., 1981; Harries et al., 1997). Discharges into large bays or harbours where flushing is limited could prove problematic (Atkinson et al., 2003). The presence of trace levels of estrogens in marine sediments needs to be considered in the context that estrogens are one of many classes of compounds that exhibit estrogenic behaviour that are likely to be found in the sediments (e.g. PAHs and organochlorines (Leeming et al., 1991)). There are almost certainly other contaminants in the marine sediments, present at higher concentrations, but with lower estrogenic activity in the sediments. Further studies are required to determine the extent that estrogens present at nanogram per gram concentrations in the sediments affect marine invertebrates, the base of the aquatic food chain, and whether the presence of estrogens poses a risk to ecosystem function.

4. Conclusions
Steroid estrogens were found in deep ocean sediment samples at nanogram per gram concentrations. The presence of the synthetic 17α-ethinylestradiol in the sediments confirms that the estrogens (at least 17α-ethinylestradiol) are of human origin. While there are likely to be other compounds that exhibit estrogenic behaviour in the sediments, steroid estrogens have high estrogenic activity and their presence may affect the sediment ecology. Further studies are needed to determine whether estrogens, at low concentrations detected, affect the development of marine invertebrates and whether there is potential for estrogen bioaccumulation in the marine environment.

5. Acknowledgements
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6. References


Figure captions

Figure 1: Smith McIntyre grab sampler
Figure 2: Sediment sample collection locations
Figure 3. Concentration of estrogens in sediment samples taken 0km and 7km from the deep ocean outfall. Error bars indicate standard error of the mean.
Figure 4. Estrogen content in the separated liquid sample after the addition of NaCl to STP effluent samples and high speed centrifugation. EE2 was not detected in any liquid samples after centrifugation.

Table caption

Table 1. Measured and expected concentration of estrogens assuming no desorption from effluent sewage particles upon ocean disposal.

Figure 2

Figure 3
Table 1

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Mean concentration in treated effluent (ng/L)</th>
<th>Mean fraction adsorbed to particles (%)</th>
<th>Estimated concentration on particulates (ng/g)</th>
<th>Estimated concentration in surficial sediments (ng/g)</th>
<th>Measured concentration in surficial sediments (ng/g)</th>
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</thead>
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<tr>
<td>E1</td>
<td>54</td>
<td>24</td>
<td>96</td>
<td>1.9</td>
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<tr>
<td>E2</td>
<td>14</td>
<td>43</td>
<td>45</td>
<td>0.9</td>
<td>0.22 – 2.48</td>
</tr>
<tr>
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<td>&lt;5</td>
<td>100</td>
<td>19</td>
<td>0.4</td>
<td>&lt;0.1 - 0.5</td>
</tr>
</tbody>
</table>

* data from Braga et al (2005)

* assuming suspended solids concentration is 135 mg/L (SWC, 2002)

* assuming EE2 concentration in treated effluent of 2.5 ng/L

* assuming sewage particles contribute 2% to the total surficial sediments (Matthai et al., 2002)