ESTROGENIC AND ANTI-ESTROGENIC ACTIVITY IN WASTEWATER EFFLUENT: 
FATE DURING SOIL AQUIFER TREATMENT

by
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**Introduction**

Soil aquifer treatment (SAT) is used in Tucson, Arizona to polish wastewater effluent during infiltration for local groundwater recharge. The Sweetwater Recharge Facilities, which receive effluent from the Roger Road Wastewater Treatment Plant (RRWTP), have been in operation since 1991. Recharged water is, in turn, used as a non-potable source for landscapes and irrigation. Alternatively, RRWWTP effluent is discharged to the Santa Cruz River, which is effluent dominated during most of the year. Most of the discharged water infiltrates to groundwater within 30 miles of the outfall.

Exposure to wastewater effluent is of concern due to potential adverse effects on wildlife and humans. Fish exposed to wastewater effluent can display both male and female sexual characteristics (1). This effect was attributed to hormones and hormone mimics found in treated wastewater.

Because local water demand exceeds the natural rate of groundwater replenishment and the Tucson allotment of Central Arizona Project Water, it is necessary to find an alternative water source to meet local long term water supply requirements. Before reclaimed water can be used for the purpose, health effects resulting from the reuse of wastewater must be determined. Several studies have shown that the removal of organics with soil depth during infiltration is efficient, and that loss of organics depends on both the soil organic matter and biological activity (2, 3). Much of the benefit of SAT in terms of organic removal occurs in the first few feet of sediment.

In this study, the potential for removal of endocrine disrupting chemicals via infiltration and storage underground was examined. To assess the importance of wastewater treatment processes for attenuating estrogenic compounds in wastewater, samples were taken from various points during the recharge of wastewater and analyzed for estrogenic activity using an *in vitro* method based on estrogen-dependent protein synthesis in *S. cervisiae* (4). When exposed to an estrogenic compound, a receptor-estrogen complex forms that is capable of binding to the estrogen response element (ERE) within the promoter region of the *lac Z* gene. Production of β-galactosidase follows. β-galactosidase is secreted into the medium, and reacts with chlorophenol red β-D-galactopyranoside (CPRG). The metabolized CPRG is red in color; unreacted CPRG is yellow. Estrogenic activity is measured as color production by light absorption at 570 nm.

**Experimental**

**Site Description**

Samples were collected from the City of Tucson’s Sweetwater Recharge Facilities (SRF) (Figure 1) in southern Arizona. This site consists of eight recharge basins (RB1-8) that receive chlorinated secondary effluent from the RRWTP. The SRF began operation in 1991, and provides short-term (6-12 months) aquifer storage for reclaimed water that is ultimately recovered for non-potable applications. Recharge basin RB-1 contains two monitoring wells located at 4.5 m (MW-5) and 37 m (WR-199A) below land surface.

**Sample Preparation**

Pond water samples were collected from RB-8, MW-5, and WR-199A. Samples were filtered using 0.45 µm cellulose nitrate filters (Millipore) on the day of collection. The RB-8 sample consisted of ponded secondary effluent during a period of water infiltration. Hydrophobic organics were extracted from the filtered samples using C-18 disks (3M Empore). Retained organics were eluted with two 10-mL portions of 100% ethyl alcohol. Eluates were dried under
nitrogen, and the dry residual materials were re-dissolved in Nanopure water to yield concentration factors of 200×.

Yeast Estrogen Screen (YES) Bioassay
The YES bioassay of Routledge and Sumpter (4) was used to measure estrogenic and anti-estrogenic activity in RB-8, MW-5 and WR-199A water samples. The published procedure was modified as described by de Boever et al. (5). The modified procedure for measurement of total estrogenic activity was carried out by serially diluting each (200×) sample concentrate across 10 wells in a 96-well plate (Costar).

The recombinant strain of S. cerevisiae used here was provided by John Sumpter of Brunel University, Oxbridge, U.K. Yeast cells were grown in the medium to OD_{630} = 1.0cm^{-1}. The culture was then diluted in the same medium and added to each well of the 96-well plate containing sample dilutions. Plates were then incubated for 24 hours at 32°C for growth of S. cerevisiae and estrogen-dependent expression of lacZ. At that point, a solution of cycloheximide/CPRG (chlorophenol red β-D-galactopyranoside) was added to each test well. After an additional 24 hours for β-galactosidase-dependent color development, absorbance was measured at 570 nm (β-galactosidase activity) and 630nm (turbidity).

The positive control series was developed in a similar manner to yield test concentrations of 17α-ethynyl Estradiol (EE₂) ranging from 1.0×10^{-7} M to 5.0×10^{-12} M. IC_{50} was defined as the concentration of EE₂ that produced a half-maximal test response (Figure 2). Results derived from environmental samples were converted to an equivalent concentration of EE₂ based on:

$$EE_2(\text{equivalent}) = \frac{IC_{50,EE_2}}{(FS)(CF)}$$

where FS is the volume fraction of sample in the dilution that produced a half-maximal test response and CF is the sample concentration factor (here 200×).

Agonist/Antagonist Bioassays
Estrogen agonist/antagonist activities were evaluated using the YES bioassay by determining the effect of sample organics on the positive (EE₂) control series. The dilution series and the remainder of the procedure was as described. Agonist effects were evident if the environmental sample raised the lower limb of the S-shaped EE₂ curve. Anti-estrogens depressed the upper limb of the positive control curve (Figure 2). Sample additions (constant over a specific EE₂ dilution series) produced final sample concentrations factors of 5, 10, or 20× in the test mixtures. To show that the modified YES bioassay can measure anti-estrogenic activity trans-4-hydroxytamoxifen (4-OHT, Calbiochem) was added at final concentrations from 1.0×10^{-7} M to 1.0×10^{-6} M to the EE₂ positive control series. Tamoxifen is widely regarded as a partial antagonist of estrogen activity. That is, estrogen-dependent response is blocked by tamoxifen in certain human tissues (e.g., breast) although tamoxifen is an estrogen agonist in uterine tissues (6, 7, 8). To assure ourselves regarding the ability of our cells to respond to anti-estrogens like 4-OHT and trans-tamoxifen, we reproduced those studies prior to carrying out work with environmental samples that were hypothesized to contain anti-estrogenic activity. 4-OHT proved to be anti-estrogenic at concentrations ranging from 1.0×10^{-7} M to 1.0×10^{-6} M (Figure 3).

Results and Discussion
Previous work by our group using an in vitro hER-β competition-binding assay (data not shown) indicated that estrogenic activity is significantly attenuated during soil aquifer treatment of chlorinated secondary effluent at the Sweetwater Recharge Facilities in Tucson, Arizona. Here, the yeast-based reporter gene assay was used to validate previous measurements using the binding assay. Initial results using the YES assay indicated that estrogenic activity was not detected in wastewater effluent and at the shallow monitoring well (MW-5), which collects water at a depth of approximately fifteen feet below land surface (Figure 4). Water collected at the deep monitoring well (WR-199a, approximately 120 ft below land surface), however, exhibited estrogenic activity equivalent to 0.68 nM EE₂ (Table 1). To better understand these unexpected results, the YES assay was modified to allow for the detection of anti-estrogens in aqueous samples.

Results from the modified YES assay indicate that estrogenic activity and anti-estrogenic activity are both present in wastewater effluent. Organic concentrates derived from RB-8 (Figure 5) and MW-5 (not shown) depressed the response of the reporter gene assay to EE₂. Turbidity data (λ = 630nM, not shown) indicated that yeast cell numbers in test wells were independent of the level of supplementary organic provided from RB-8 or MW-5 as well as the concentration of EE₂. That is, the observed loss of β-galactosidase activity was unrelated to sample-dependent inhibition of cell growth. Results support the hypothesis that these waters contained anti-estrogens. Conversely, when the EE₂ reporter gene controls were supplemented with organics from the WR-199A sample (Figure 6), the assay response was clearly amplified in wells containing relatively low levels of EE₂ (≤4x10⁻¹⁰ M). The simplest explanation is that antagonist compounds present in the RB-8 and MW-5 samples were substantially removed by percolation to the local aquifer, unmasking the effect of residual estrogen mimics that remained in the WR-199A sample. It is evident that estrogen agonists survived infiltration in concentrations that were sufficient to produce a response in the reporter gene assay. The shape of the EE₂ curves, when supplemented with the WR-199A concentrate, suggests that the effects of EE₂ and concentrate agonists within the reporter-gene assay were additive.

The fate and transport of estrogens and anti-estrogens differ during basin detention and subsequent percolation. Pond samples were collected over a four-day period from Recharge Basin 8 and tested using the YES assay. The results showed a simultaneous decline in estrogenic activity and increase in anti-estrogenic activity as a function of increasing basin detention time (Figure 8). The mechanism responsible for the decrease in estrogenic activity in the pondwater may be biodegradation, photo-oxidation, algal activity, or some combination of these.

**Acknowledgments**
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Figure 1. Sweetwater Recharge Facilities site plan. A portion of the chlorinated effluent is infiltrated for temporary storage and recovery in the local unconfined aquifer. (Based on graphic provided by the City of Tucson).

Figure 2. Agonist and antagonist responses in a positive control (EE₂) curve from the yeast-based reporter gene assay. Presence of estrogen agonists will cause an increased response in the lower limb of the curve. Presence of antagonists will cause a decreased response in the upper limb of the curve, as shown.
Figure 3. Antagonist experiment with 4-OHT. Anti-estrogenic activity was observed at $3.0 \times 10^{-7}$ M 4-OHT and higher concentrations. Results were dose-dependent. Results with trans-tamoxifen (not shown) were similar, although anti-estrogenic effects were not observed at dosages below $6.0 \times 10^{-7}$ M.

Figure 4. Reporter-gene activity as a function of volume fraction derived from sample concentrates in assay mixtures consisting of yeast, growth media and the (200×) concentrated sample. $\beta$-galactosidase activity was measured colorimetrically $A_{570} - R(A_{630})$ after a 24-hr incubation period. Results show that only the WR-199A concentrate was estrogenic.
Figure 5. Reporter-gene activity as a function of EE<sub>2</sub> concentration and contribution of RB-8 concentrate to assay mixtures. Results show that the RB-8 concentrates depressed the assay response at relatively high levels of EE<sub>2</sub> addition. β-galactosidase activity was measured colorimetrically \( A_{570} - R(A_{630}) \) after a 24-hr. incubation period.

Figure 6. Reporter-gene activity as a function of EE<sub>2</sub> concentration and contribution of the WR-199A concentrate to assay mixtures. The WR-199A concentrate contributed significantly to the assay response at relatively low levels of EE<sub>2</sub> addition. β-galactosidase activity was measured colorimetrically \( A_{570} - R(A_{630}) \) after a 24-hr. incubation period.
Figure 7. Reporter-gene activity of ponded secondary effluent (RB-8) as a function of time. Samples were collected over a four day period. After four days of infiltration, RB-8 Pond has lost estrogenic activity, as indicated by absorbance data.

Table 1. Summary of estrogenic activities determined using the competitive binding and reporter-gene assays. Values are reported as equivalent concentrations (EEQs) of 17α-ethinyl estradiol (EE₂).

<table>
<thead>
<tr>
<th>Sample or compound tested</th>
<th>DOC² (mg/L)</th>
<th>IC₅₀ (nM)</th>
<th>Equivalent Concentration (nM)¹</th>
<th>EE₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Binding Assay</td>
<td>Reporter-Gene assay</td>
<td>Binding Assay¹</td>
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<tr>
<td>E₂</td>
<td></td>
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<tr>
<td>EE₂</td>
<td>10</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB-8</td>
<td>20.7</td>
<td>-</td>
<td>-</td>
<td>4.7</td>
</tr>
<tr>
<td>MW-5</td>
<td>13.9</td>
<td>-</td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td>WR-199A</td>
<td>1.93</td>
<td>-</td>
<td>-</td>
<td>0.22</td>
</tr>
</tbody>
</table>

¹Calculated using:

\[ 
EE₂ - EQ = \frac{IC₅₀}{(FS) \times (CF)} 
\]

²DOC was measured in water samples that were pre-filtered using a 0.45 µm Millipore filter.
References


