

# **Report for 2003AZ12B: Attenuation of Estrogenic Activity in Reclaimed Water and Stormwater During Impoundment in Natural Systems**

- Conference Proceedings:
  - Bjolseth, I.M., Quanrud, D.M., Karpiscak, M.M., Ela, W.P., Lansey, K.E., and Arnold, R.G. 2003. Fate of Estrogenic Activity during Wetland Treatment of Wastewater Effluent and Stormwater Runoff. In: Proceedings, 11th Biannual Symposium on Groundwater Recharge, Phoenix, AZ, June 5-7, 2003.
- Dissertations:
  - Bjolseth, I.M. 2004. Fate of Estrogenic Activity during Wetland Treatment of Wastewater Effluent and Stormwater Runoff. Unpublished M.S. thesis. Department of Chemical and Environmental Engineering. The University of Arizona. Tucson, Arizona.

Report Follows

## **A. Problem and Research Objectives:**

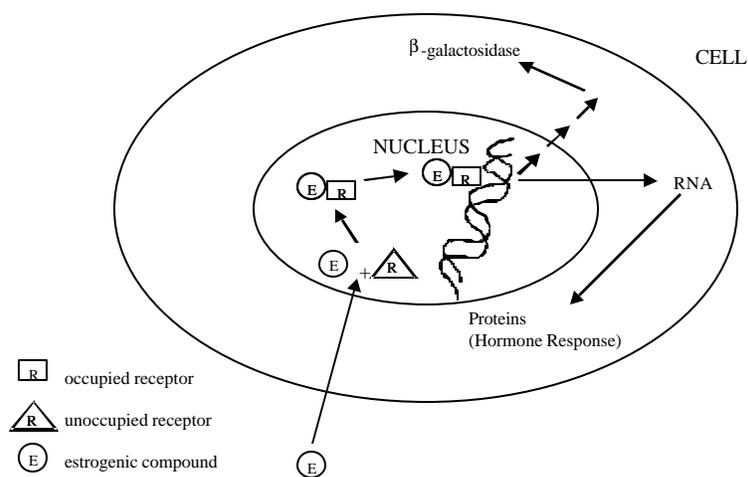
The growing demand for water in Arizona and the semiarid southwestern United States prompts consideration of alternative water sources, including reclaimed water and stormwater runoff. On the other hand, caution regarding acceptable uses and use-dependent treatment requirements preceding water reuse is warranted, in part due to discoveries regarding the presence of endocrine disrupting compounds (EDCs) in domestic wastewater effluent. Among the EDCs measured in treated wastewater, estrogen, estrogen metabolites, and anthropogenic estrogen mimics are responsible for most observable ecological effects. Furthermore, there has been speculation regarding the effects of chronic human exposure to estrogenic compounds, such as elevated incidence of breast and prostate cancers and decline in sperm quality. There is a critical need to examine the fate of estrogenic compounds during wastewater treatment and the efficacy of effluent polishing techniques as methods for limiting estrogenic activity in reclaimed water.

Previous work in our laboratories at the University of Arizona indicates that local municipal wastewater contains ecologically significant levels of estrogenic activity. This activity is typically attenuated by 50-60 percent during secondary (biological) wastewater treatment at the Roger Road Wastewater Treatment (RRWWTP) (trickling filter) and the Ina Road Wastewater Pollution Control Facility (activated sludge). Locally available polishing techniques for improving effluent quality include wetlands treatment. However, the efficacy of constructed wetlands for removing estrogenic activity in treated effluent has not been established. This project was intended to provide a starting point for evaluation of constructed wetland technology as a treatment method for removing EDCs from wastewater effluent. A second objective was to examine the presence of estrogenic activity in stormwater runoff and to evaluate whether impoundment of runoff resulted in changes of estrogenic activity.

*Related research.* A variety of compounds with estrogenic properties are only partly removed during conventional wastewater treatment (Huang and Sedlak, 2000). Residual estrogens can produce changes in the overt sexual characteristics of exposed fish and elevated vitellogenin (an egg precursor protein) levels in exposed males (Folmar *et al.*, 1996; Harries *et al.*, 1996). Such observations have led to widespread speculation that exposure to estrogenic pollutants in the environment is responsible for recently observed increases in several types of human cancers and worldwide declining sperm levels in men. In fact, the effect of exposure to EDCs on human health is not known with certainty, and relevant epidemiological data is not likely to arise in the near future.

The endocrine system regulates numerous critical cellular activities by producing and controlling the concentrations of hormones. Cells respond to hormones at exceptionally low concentrations, commonly < 1 nM. Hormones are normally recognized at the cellular level through complexation reactions with intracellular or membrane-bound chemical receptors. Soluble hormone/receptor complexes bind to specific DNA sequences, stimulating gene expression. Estrogen, for example, is produced in mature ovarian follicles and transported through the bloodstream to elicit response in distant cells that produce estrogen receptors. The DNA binding regions for these compounds are called estrogen response elements (ERE; [Figure 1](#)). In this manner, estrogens, or perhaps estrogen-mimicking chemicals, regulate many aspects of sexual development and function, reproduction, etc. Estrogen agonists and antagonists

both bind to the estrogen receptor. In the case of antagonists, the receptor complex is unable to initiate gene transcription.



**Figure 1.** Basis of response to estrogens among regulated cells. Steps: (i) estrogen binds to estrogen receptor to produce estrogen/receptor complex; (ii) complex binds to specific DNA sequences (estrogen receptor elements) to initiate transcription/translation. The figure omits detailed aspects of estrogen response physiology.

Due to the difficulty and expense of measuring steroid hormones in complex aqueous-phase matrices at relevant (ng/L) levels, there have been relatively few measurements from which environmental fate and transport can be determined. From the recent USGS survey of United States streams (Kolpin *et al.*, 2002), it is apparent that natural and synthetic estrogens survive conventional wastewater treatment, at least in part. A number of *in vitro* bioassays have been devised to screen chemicals for estrogenic effects. In a few instances, those same tests have been used to measure estrogenicity in complex mixtures of chemicals including domestic wastewater and wastewater effluent (Tanaka *et al.*, 2001; Holbrook *et al.*, 2002; Turney *et al.*, in press). Such studies have begun to yield evidence regarding the probable fate of estrogenic compounds during conventional wastewater treatment. Previous efforts by our research group to account for changes in aqueous-phase estrogenic activity during wastewater treatment and subsequent polishing steps have led to the following summary observations (Conroy *et al.* (submitted), Turney *et al.* (in press), Quanrud *et al.* (2002a), and Quanrud *et al.* (2002b)):

- i. *From 40 to 70% of the soluble, aqueous-phase estrogenic activity in raw domestic wastewater is removed during secondary wastewater treatment.* Fractional removals depend somewhat on the efficiency of organic conversion during biochemical treatment steps.
- ii. *Effluent polishing via soil-aquifer treatment (percolation and temporary underground storage) can reduce estrogenic activity by an order of magnitude.* Local soil characteristics impact this result. Most reduction in estrogenic activity occurs in the top few feet of basin soils. Turney *et al.* (in press) measured estrogenic activity in secondary effluent from the Roger Road Wastewater Treatment Plant before and after percolation through about 120 feet of unconsolidated

sediments at the Sweetwater Recharge Facilities. Results indicate that >95% of the residual estrogenic activity in treated wastewater is removed after percolation to the local unconfined aquifer. The dominant removal mechanism, at least over time scales of days to weeks, is thought to be adsorption to sediments.

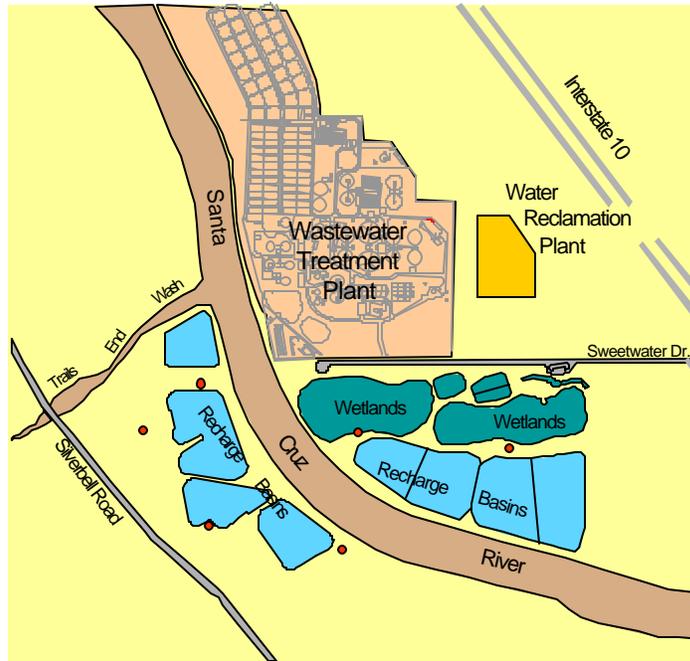
iii. *Estrogenic activity in secondary effluent is attenuated by >65% after transport along a 23-mile reach of the Santa Cruz River near Tucson, Arizona.* In effluent-dependent Santa Cruz River, in which there was no dilution or groundwater/surface water exchange over the reach sampled, estrogenic activity was significantly attenuated over a transport distance of 23 miles. The estimated travel time was on the order of a day. Processes responsible for downstream water quality improvements are poorly understood but may involve photodegradation reactions or sorption onto streambed sediments.

*Project objectives.* We examined the fate of estrogenic activity in wastewater effluent, backwash water, and stormwater runoff during passage through two local surface water service impoundments (described below). The central hypothesis was that wetland treatment and impoundment of wastewater/runoff would lower the levels of estrogenic activity present in these waters. It was hypothesized that attenuation of estrogenic activity occurs during wetland treatment via a combination of mechanisms including biodegradation, sorption, and photodegradation reactions.

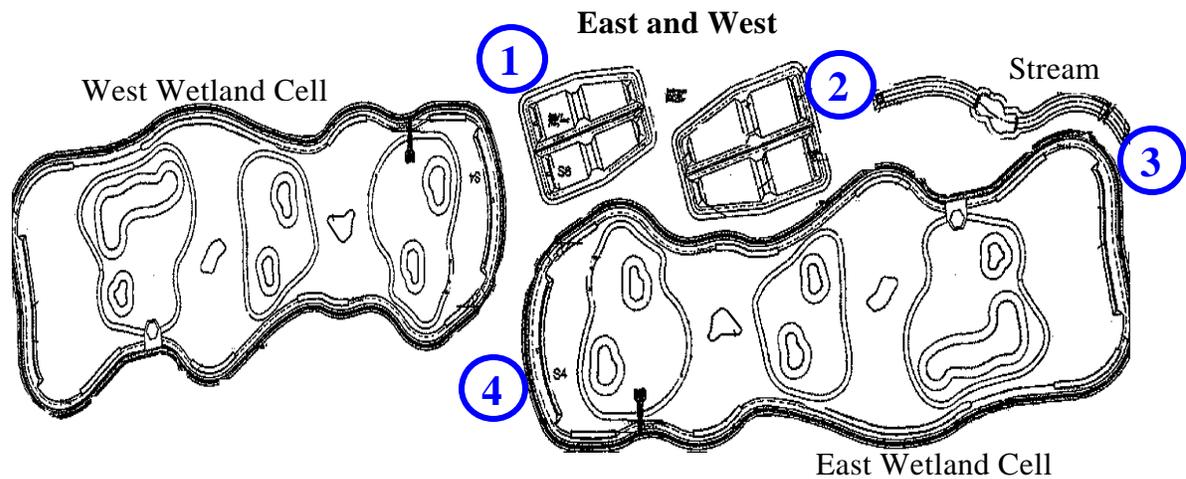
## **B. Methodology:**

### *Field sites*

Two local field sites in the City of Tucson, Arizona, were utilized in this research: the Sweetwater Wetlands and the Tucson (Ajo) Detention Basin (TADB), also known as the Kino Wetlands. The Sweetwater Wetlands is a part of the Sweetwater Recharge Facilities (SRF) (Figure 2), located west of Interstate 10 near Prince Road. The SRF is owned and operated by the City of Tucson. The wetland component is comprised of 1.8 acres (0.7 ha) of settling basins, a stream system and 15 acres (6.1 ha) of wetland cells (Figure 3). The dominant vegetation in the settling basins and wetland cells includes several different species of bulrush and cattail. The Sweetwater Wetlands receive a mixture of secondary effluent from the RRWTP and backwash water from the City of Tucson Reclaimed Water Plant. The facility has been in operation since 1997. The research team has access to the SRF via an ongoing research project studying water quality changes (including fate of estrogenic activity) during soil aquifer treatment.



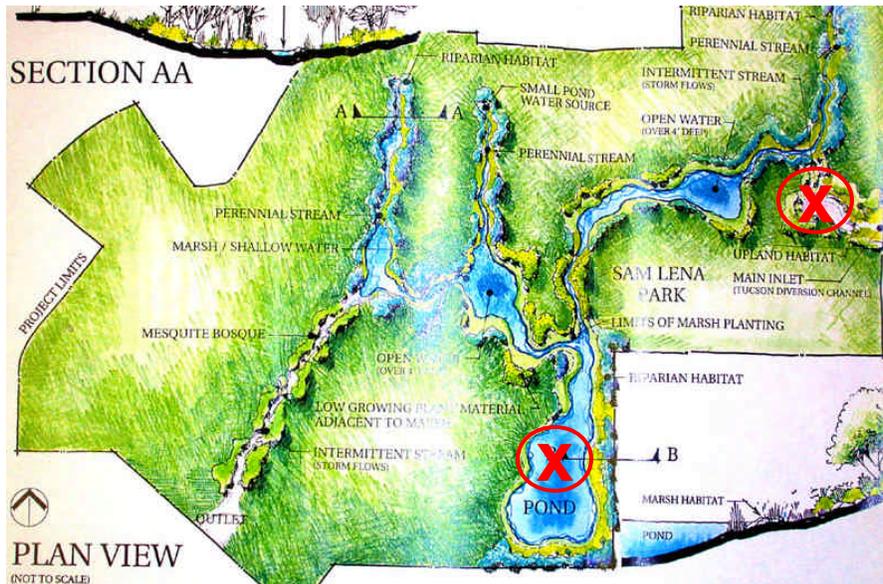
**Figure 2.** Site map for the Sweetwater Recharge Facilities, Tucson, Arizona.



**Figure 3.** Sweetwater free-water-surface wetland cells. Samples were obtained from (1) the inlet to the settling basins, (2) the outlet of the settling basins, (3) the outlet of the stream, and (4) the outlet of the wetland.

The TADB project (Figure 4) is a constructed stream and marsh system located in the south-central portion of the City of Tucson just north of Ajo Way and west of County Club Road. The project was completed in 2002 and is operated by the Pima County Department of Transportation and Flood Control District. The wetland system is designed to receive, treat, and detain urban stormwater runoff. The facility consists of four stream components that feed into three marshes (total area of 8.4 acres) and a 7-acre pond. The marshes are approximately one half emergent vegetation and one half open water.

The riparian system received its first stormwater flows in September 2002. The stormwater is detained in the large pond and is pumped to the inlets of the streams to maintain the riparian system. The operational scheme of this system allows examination of the effect of extended detention time on attenuation of estrogenic activity in stormwater. The research team received permission from Chris Bartos, Pima County Kino Sports Complex, to conduct the research at this site.



**Figure 4.** Site map of the Tucson Ajo Detention Basin. Samples were obtained at the marked locations: near the inlet to the system and at the big pond.



**Figure 5.** The 7-acre pond at the Tucson Ajo Detention Basin. View is looking to the south; photo was taken January 17, 2003. Tucson Electric Park is in the background.

Work was conducted over a 14-month period. Three sets of samples were collected from the Sweetwater wetlands; at the TADB, nine sets of samples were obtained during this study. Sampling locations at the Sweetwater wetlands (Figure 3) included the two influent water sources (backwash water, secondary effluent); the inlet and outlet of the east settling basin; and the inlet and outlet of the

east free-water-surface wetland cell. At the TADB, stormwater runoff samples were collected at the inlet to the facility and at the 7-acre pond. The sampling schedule at the TADB was designed to take advantage of new runoff derived from local storm activity in 2003. We adjusted the sampling program to permit evaluation of the fate of estrogenic activity in stormwater runoff during impoundment in the 7-acre pond.

Methods for quantifying estrogenic activity *in vitro* include competition binding, reporter gene expression, and cell proliferation assays. These assays (Table 1) differ in terms of effort and demand for technical skill and their sensitivities to aqueous phase 17 $\beta$ -estradiol (E2, natural female estrogen hormone). In this project, samples were analyzed using the competition binding and reporter gene assays to evaluate estrogenic activity in waters from the two constructed wetland/impoundment sites.

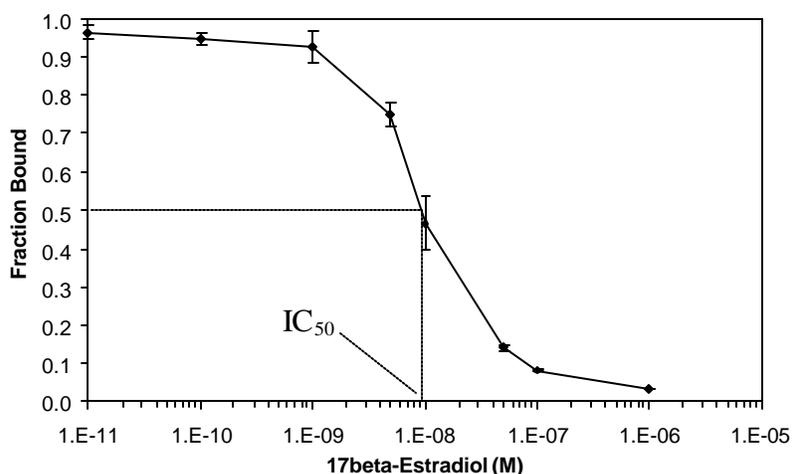
**Table 1.** Comparison of *in vitro* assays for chemical estrogenicity based on sensitivities and effort. Sensitivity data are the lowest concentrations of 17 $\beta$ -estradiol that produce an estrogenic response.

Bioassay	Representative Sensitivity		Time Required (days)	Reference
	(ng/L)	(nM)		
1. <i>In Vitro</i> competition Binding assay	500	~2.0	1	Bolger <i>et al.</i> , 1998
2. <i>In Vitro</i> Gene Expression (reporter-gene assay)	100	0.5	1-3	Routledge and Sumpter, 1996 Coldham <i>et al.</i> , 1997
3. <i>In Vitro</i> Cell Proliferation assay	~3	0.01	5-7	Soto <i>et al.</i> , 1995

Competition Binding Assay. The competition receptor-binding assay is relatively fast and straightforward. The receptor-binding assay used here follows procedures established for the Estrogen Receptor- $\beta$  Competitor Assay (Bolger *et al.*, 1998). In this method, estrogenic activity is measured by displacement of a fluorescent ligand bound to human estrogen receptor (hER- $\beta$ ). Compounds capable of binding to the receptor (e.g., 17 $\beta$ -estradiol) displace the fluorescent estrogen. Displacement is detected by fluorescence polarization. Method detection limits for 17 $\beta$ -estradiol are  $\sim 10^{-9}$  M. A drawback of this type of assay is that receptor-binding assays do not differentiate between endocrine system agonists (estrogen mimics) and antagonists (those that block endocrine system response).

Fluorescence polarization was measured with a Beacon 2000 variable temperature fluorescence polarization system (PanVera, Model P2300) using an excitation wavelength of 360nm and a fluorescence detection wavelength of 530nm at 25°C. The instrument provides a numerical indication of the degree of light polarization at the fluorescent wavelength. The fluorescence polarization method is fully described in Turney *et al.* (in press). The IC<sub>50</sub> for 17 $\beta$ -estradiol varied between  $8.3 \times 10^{-9}$  –  $1.1 \times 10^{-8}$  M (2.26 – 2.99  $\mu$ g/L) (Figure 6). The “IC<sub>50</sub> sample” was derived experimentally (the volume

fraction that produced 50% ES2 displacement), and the concentration factor (200x) resulted from the C18 extraction procedure.



**Figure 6.** Competition binding assay response for 17β-estradiol.

Yeast Estrogen Screen (YES) Assay. The estrogen-inducible expression system used in this study is comparable to the method described in Routledge and Sumpter (1996) to study endocrine disruption by municipal wastewater and effluent-impacted surface waters in the United Kingdom. The method responds to compounds capable of passing through the yeast cell envelope and binding to hER-β. The complex binds to the ERE in the recombinant yeast strain, per above. In this approach, estrogenic activity is measured via expression of *lacZ* fused to a human estrogen response element sequence in *Saccharomyces cerevisiae*. Consequent β-galactosidase activity is measured colorimetrically based on conversion of chlorophenol red β-D-galactopyranoside to a red product. Although the assay has been used extensively to measure estrogenicity among synthetic compounds (Routledge and Sumpter 1996, Harris *et al.* 1997, Beresford *et al.* 2000), its application for measurement of estrogenicity in complex matrices has been more limited (Holbrook *et al.*, 2002).

Whole-water samples were collected in muffled glass bottles, immediately filtered (0.45 μm, Millipore) upon return to the laboratory, and stored at 4°C until further processing. Hydrophobic organics in whole-water samples were concentrated by extraction onto Empore C-18 disks (3M) and elution in ethanol. The alcohol solution was evaporated to dryness and the nonvolatile residuals were resuspended in water or buffer to achieve nominal concentration factors of 100-300 or greater. Sample concentrates were analyzed for estrogenic activity using one or more of the *in vitro* assays described above. Results were expressed as an equivalent concentration of 17β-estradiol, after accounting for the concentration factor used.

### **C. Principal Findings and Significance:**

The working hypothesis in this study was that impoundment of secondary effluent or stormwater runoff in wetlands would lower the concentrations of organics responsible for estrogenic activity. Hypothesis

testing involved sampling and analysis at the Sweetwater Wetlands (secondary effluent) and the Tucson Ajo Detention Basin (stormwater runoff).

*Estrogenic activity in wastewater effluent (Sweetwater Wetlands)*

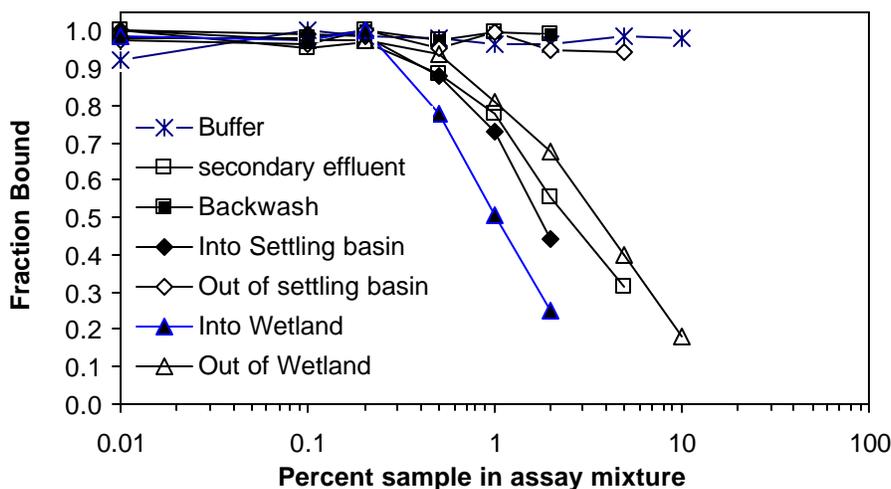
The water entering the inlet of the settling basin at the Sweetwater Wetlands consists of a mixture of backwash water from the City of Tucson’s Reclaimed Water Plant and secondary effluent from the Roger Road Wastewater Treatment Plant. The mixing ratio during all three sampling events in this study was 3:1 (secondary:backwash). Estrogenic activity was not detected in the backwash water itself (Table 3), however, binding assay results for samples taken at the inlet to the settling basin, consisting of the secondary/backwash mixture, consistently exhibited higher levels of estrogenic activity than secondary effluent alone (Table 3, Figure 7). It was hypothesized that this seemingly anomalous result was due to the presence of anti-estrogenic activity in the backwash water. The presence of anti-estrogenic activity in backwash water is a topic for additional study.

**Table 3.** Summary of estrogenic activities at Sweetwater Wetlands sampling points. Data shown represent equivalent E2 equivalent concentrations (nM).

<b>Sample Date and Location</b>	<b>Binding Assay</b>	<b>YES Assay</b>
<i>February 5, 2003</i>		
Filter backwash water	Non detect	-
Secondary effluent	1.8	-
Into settling basin	2.5	-
Out of settling basin	Non detect	-
Into east wetland cell	4.5	-
Out of wetland	1.1	-
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<i>August 29, 2003</i>		
Filter backwash water	Non detect	Non detect
Secondary effluent	7.5	$1.6 \times 10^{-1}$
Into settling basin	20.0	$9.1 \times 10^{-2}$
Out of settling basin	3.0	$1.3 \times 10^{-2}$
Into east wetland cell	7.5	$2.5 \times 10^{-1}$
Out of wetland	1.8	Non detect
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<i>February 5, 2004</i>		
Filter backwash water	-	Non detect
Secondary effluent	-	$3.8 \times 10^{-2}$
Into settling basin	-	$1.6 \times 10^{-2}$
Out of settling basin	-	$4.7 \times 10^{-3}$
Into east wetland cell	-	$1.3 \times 10^{-1}$
Out of wetland	-	$2.0 \times 10^{-3}$

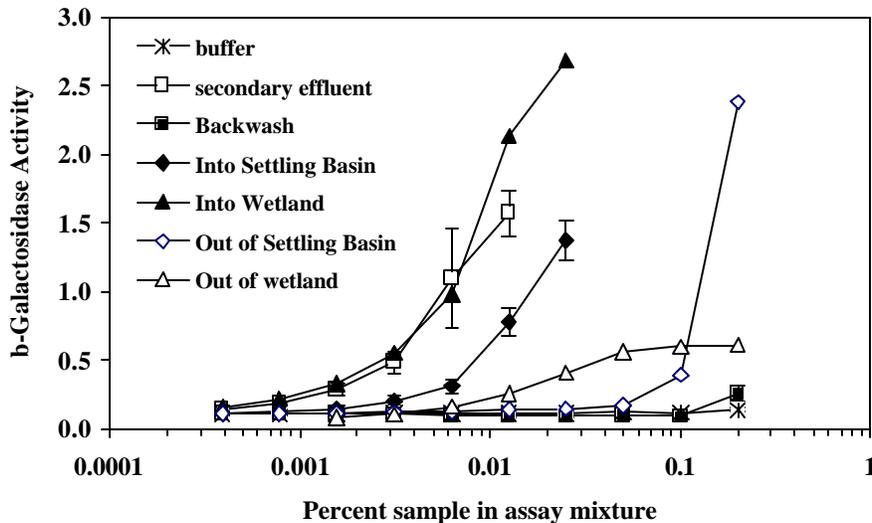
In all three sampling events (Table 3), estrogenic activity decreased substantially (by about 50 percent) during passage of the secondary/backwash mixture through the settling basin but then increased substantially again through the connecting stream (Figure 7). This result was consistently observed in

both assays (Table 3). The reasons for an increase in estrogenic activity after passage through the stream segment are unknown and are an appropriate subject for additional study. Estrogenic activity then consistently decreased during the six-day residence time in the East free-water-surface wetland cell. There was no apparent seasonal variability in removal efficiency of estrogenic activity during wetland treatment. These results were observed using both assay methods.



**Figure 7.** Competitive binding assay measurements of estrogenic activity among water samples derived from Sweetwater surface wetland sampling points collected on February 5, 2003. Ordinate values are the fraction of a fluorescent marker that is bound to hER- $\beta$ . Estrogenic compounds, as defined by this assay, are those that can displace the marker compound.

Samples obtained during August 2003 and February 2004 from the Sweetwater Wetlands were examined for estrogenic activity using both the competitive binding (Figure 7) assay and the YES assay (Figure 8). Results from the two assays were in reasonable qualitative agreement and showed that the stream effluent (wetland influent) was more estrogenic than the stream influent and also that wetland treatment is a reasonable polishing procedure for removing residual estrogenic compounds from secondary effluent. However, the huge drop in estrogenic activity across the wetlands (> 80% reduction; indicated by the YES assay) was unexpected. The measured estrogenic activity (in terms of  $17\beta$ -estradiol equivalent concentration) was significantly higher in the binding assay than in the YES assay. We believe that the low response of the YES assay to wetlands effluent was due to a combination of the presence of anti-estrogenic activity (antagonists) along with removal of estrogenic compounds (agonists) during wetland treatment.



**Figure 8.** YES assay measurements of estrogenic activity among water samples from the Sweetwater surface wetlands collected on August 29, 2003. The presence of estrogenic compounds in the concentrated samples stimulates synthesis of  $\beta$ -galactosidase in the strain of yeast employed.  $\beta$ -galactosidase activity is then measured based on light absorbance at 570 nm.

#### *Estrogenic activity in urban stormwater runoff (Tucson Ajo Detention Basin)*

This aspect of the study was designed to create perspective relative to levels of estrogenic substances in wastewater effluent and urban stormwater runoff. We compared estrogenic activity in stormwater runoff to that of secondary effluent before and after treatment at the Sweetwater Wetlands. We also examined hypotheses regarding first flush effects and the effect of stormwater storage on estrogenic activity at the TADB.

A total of nine sampling events were performed at the TADB during this study; results from measurements for estrogenic activity from the inlet and the 7-acre (big) pond are provided in [Table 4](#). Measurements were obtained using the competitive binding and reporter gene bioassays.

Estrogenic activity was consistently detected in samples from the TADB when using the binding assay and the magnitude of response was 10-25% of that seen in wetland samples of wastewater origin ([Table 4](#) and [Figure 9](#)). Results from the reporter gene assay were equivocal in this regard; estrogenic activity was detected in only one set of stormwater runoff samples (collected on 03-30-04) from the TADB.

In order to address the discrepancy in results among the two assay techniques, a modified reporter gene assay was used to look for the presence of anti-estrogenic activity in stormwater runoff. The presence of compounds with anti-estrogenic activity (antagonists) would lower the response in the YES assay and increase the response in the binding assay. The YES (reporter gene assay) only responds to

compounds with estrogenic activity (agonists) where as the binding assay responds to all compounds capable of binding to the human estrogen receptor (agonists plus antagonists). The investigators have previously used the modified YES assay to examine the fate of anti-estrogens in wastewater effluent during soil aquifer treatment (Conroy *et al.*, submitted).

**Table 4.** Estrogenic activity in stormwater samples collected at the Ajo Detention Basin in Tucson during 2003-2004. Samples represent estrogenic activity in either the 7-ac pond itself or in stormwater runoff that was influent to the pond on the date shown. Estrogenic activity is reported as the equivalent 17 $\beta$ -estradiol concentration (nM).

Sampling Location and date	E2 Equivalent Conc. (nM) (binding assay)	E2 Equivalent Conc. (nM) (YES assay)
Influent (02-13-03)	3.8	-
Big pond (02-13-03)	0.75	-
Influent (02-26-03)	Non detect	-
Big pond (02-26-03)	1.3	-
Influent (06-13-03)	-	Non detect
Big Pond (06-13-03)	-	Non detect
Influent (08-13-03)	0.75	Non detect
Big pond (08-13-03)	0.45	Non detect
Influent (10-02-03)	0.45	Non detect
Big pond (10-02-03)	1.1	Non detect
Influent (10-23-03)	0.40	Non detect
Big pond (10-23-03)	0.43	Non detect
Influent (11-14-03)	0.38	Non detect
Big pond (11-14-03)	0.38	Non detect
Influent (02-19-04)	-	Non detect
Big pond (02-19-04)	-	Non detect
Influent (03-30-04)	-	$1.0 \times 10^{-1}$
Big pond (03-30-04)	-	$1.0 \times 10^{-1}$

Anti-estrogenic activity was detected in the stormwater runoff entering the TADB (Figure 9). In the modified YES assay, the presence of anti-estrogenic compounds in the stormwater runoff results in a depression of the upward (right hand) limb of the positive control (EE2) curve. The amount of depression increased with increasing addition of runoff concentrate (Figure 9). This result is in contrast to the agonist response seen for “fresh” secondary effluent obtained from the Roger Road Wastewater Treatment Facility (Figure 10) in which there is an additive response to the left hand portion of the EE2 positive control curve. The presence of anti-estrogenic activity in stormwater runoff is consistent with observed results in which estrogenic activity was detected using the binding assay and not detected using the reporter gene assay.

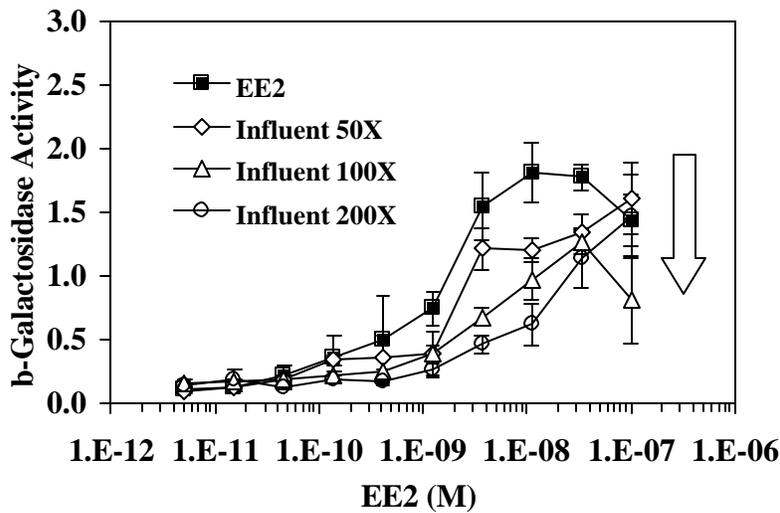


Figure 9. Anti estrogenic activity (antagonism) detected in rainfall runoff entering the TADB facility. Anti-estrogenic activity is indicated by the downward deflection (arrow) of the right hand side of the EE2 + Influent curves. The amount of antagonism increases with increasing additions of stormwater runoff concentrate added to the EE2 positive control.

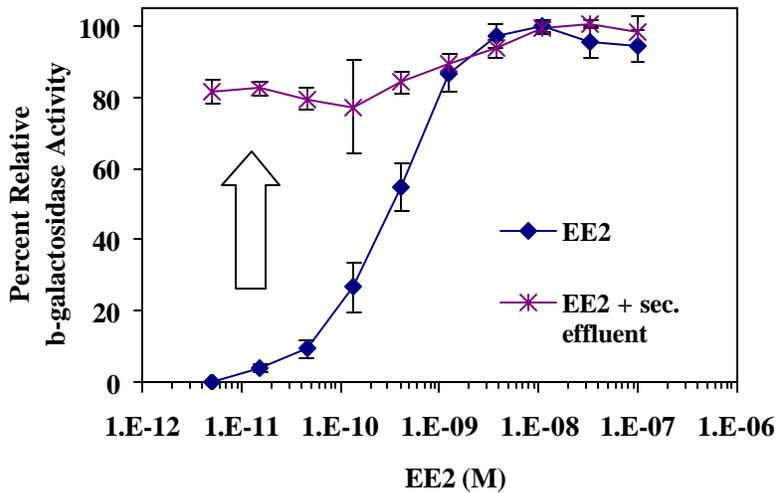


Figure 10. Estrogenic activity (agonism) detected in fresh secondary effluent from the Roger Road Wastewater Treatment Plant. Agonism is indicated by the upward deflection (arrow) of the left hand side of the EE2 + sec. effluent curve.

Data gathered during this study also support the preliminary conclusion that estrogenic activity in stormwater runoff is attenuated over time during detention in the 7-acre pond at the TADB. On more than one occasion, the pond water exhibited a lower level of estrogenic activity than new influent stormwater runoff when samples were collected shortly after storm events. For example, on February

26 and August 13 in 2003, estrogenic activity was higher in the influent stormwater than in the detained water in the big pond. Both sample events were conducted within 24 hours after a >0.50 inch rainfall event in the area. This preliminary observation supports the hypothesis that estrogenic activity is attenuated over time during impoundment. Mechanisms responsible for attenuation of estrogenic activity may include photodegradation, volatilization, and sorption to sediments. Elucidation of attenuation mechanisms is another topic for additional study.

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