Executive Summary:

This report provides an update for our current status regarding the AWI 07-18 proposal “Comparison of Estrogenic Compound Removal Efficiency from POTWs across Arizona.” This project involves a collaboration between the Arizona state universities (University of Arizona, Arizona State University and Northern Arizona University) and the Arizona Department of Health Services to evaluate the estrogen removal efficiencies of 6 Publicly Owned Treatment Works (POTWs). The goal is to determine if different wastewater treatment trains vary in the effectiveness of removal of chemicals in the environment that are or mimic estrogens. A second goal of this project is to develop a lateral flow assay that will perform as a sensor of specific estrogenic chemicals. This sensor should provide a rapid, field ready, inexpensive test of chemicals in wastewater effluent samples.

Our findings indicate that there is little correlation among assay methodologies for evaluating removal efficiencies from the treatment facilities. This lack of correlation among methods is probably related to matrix effects found in the sludge and influent. Such methodological problems make conclusions regarding overall removal efficiency using any one assay technique problematic. Evaluation of the liquid effluent, however, did provide similar qualitative results across plants with some facilities having higher estrogen output than others regardless of the methods used to evaluate the results. Although this measure of estrogen output does not answer the original question of overall facility efficiency, it does provide answers as to which facilities may be releasing more estrogenic chemicals into the environment, though it does so without consideration how much estrogenic input is coming into each facility. Therefore, this result of effluent estrogenicity cannot be used to evaluate how well each facility functions to remove estrogens. Furthermore, our results provide proof-of-concept that a lateral flow assay is a feasible alternative to other more complex assays for evaluating individual compounds though more work needs to be completed to lower the sensitivity of the assay and to validate it.

Introduction:

Projections for the Tucson and Phoenix areas suggest that regional water demands cannot be completely satisfied using renewable resources even when Colorado River allotments are completely utilized (Figure 1). Even in rural Northern Arizona, there is great concern about whether growth-fueled demand will exceed renewable water resources. Water supply sustainability in these and other high-growth areas of the Southwest depends on water conservation measures and water reclamation/reuse. Water suppliers in Tucson, Phoenix and Flagstaff already use or plan to use treated wastewater as a water resource. Only the uses that will be permitted and respective use-dependent degrees of treatment remain to be established. There are no federal regulations beyond
those dealing with potable water quality requirements to guide state and local governments through water reuse regulatory processes.

Conventional treatment processes fail to eliminate many trace organic contaminants from municipal wastewater, including some that disrupt normal endocrine function in animals. Feminization of males causing skewed sex ratios among fish have been observed near wastewater outfalls (eg. Figure 2) in many parts of the world, including the United States. Many of these abnormalities serve as markers indicating the wastewater treatment process is not removing estrogen-like compounds. Optimal treatment – that which protects environmental health in an economically efficient manner – has not been agreed upon in those situations. Further, when wastewater effluent contributes to the potable water supply, water quality issues may be particularly contentious. In the Tucson and Flagstaff areas, USGS analysis of wells suggests that contaminants in local wastewater effluents can be leaked into the groundwater systems. Last, considering other toxicological endpoints beyond estrogenicity in reclaimed waters may become useful in the future to determine where to focus future research efforts. The hypothesis to be tested in this study is that POTWs differ among each other and temporally in their estrogenic compound removal efficacy. The proposed project specific aims are to 1) provide baseline data for removals of estrogens and estrogen activities and toxic components among Arizona POTWs in their present state, and 2) develop a rapid, inexpensive assay for two common estrogenic compounds that can serve as markers for whole-sample estrogenic activity. The long-term goal of this project is to provide water managers with a rapid, inexpensive tool for evaluating treatment efficacy.

Why should different assay methodologies be used to evaluate estrogens levels in wastewater effluent and removal efficiencies of different treatment facilities? Each method uses a very different biological or chemical means of evaluating estrogens. The yeast estrogen screen (YES)
assay determines the ability of any compound to act like an estrogen because it incorporates the estrogen receptor into the assay and records binding of compounds that are estrogens or mimic them. The advantage of this system is that anything that acts like an estrogen may be detected; the disadvantages are that real biological systems have more than one type of estrogen receptor, and the YES assay may not allow for direct interpretation of results into real organisms that may be exposed to the effluent. Further, in the assay presented here, the YES system will not evaluate any antiestrogenic activity that may also be contained within chemicals in the water or sludge.

In contrast, the Enzyme-Linked Immunosorbent Assay (ELISA) test uses a very specific antibody as opposed to the estrogen receptor used in the YES assay to capture just one very specific estrogenic compound at a time (in this report, 17β-estradiol or E2 and 17α-ethynyl estradiol or EE2). The high specificity of the assay allows for evaluation of how that one compound may be removed by differing treatment processes, but is subject to matrix effects that may impact how the antibody interacts with the compound being measured.

Liquid chromatography/mass spectroscopy (LC/MS) technology can now detect and quantify many estrogenic compounds down to the nanogram per liter level required for environmental occurrence, fate and transport studies. This methodology uses enhanced chemical separation and extraction procedures to allow quantitation to these low levels, but is also subject to interference by matrix effects. In contrast to the YES screen, but similar to the ELISA, this methodology can determine the concentration of individual compounds, but because of the number of target analytes is limited, non-target estrogenic chemicals that might be potent and apparent using a YES screen would remain unseen in an LC/MS analysis.

The Approach Used: This project expands on earlier collaborative work by the University of Arizona and Northern Arizona University to establish influent-to-effluent loss of estrogens/estrogenic activity at Arizona POTWs. Six facilities were selected to provide a range of unit operations and organic carbon removal efficiencies in both larger urban areas and smaller, more rural municipalities. POTWs in Phoenix, Tucson, Flagstaff, Prescott, and Payson were sampled once. Measurements included influent, effluent, and biosolids concentrations of 17β-estradiol (E2, the primary estrogen in vertebrate animals), 17α-ethynyl estradiol (EE2, an active ingredient in oral contraceptives) and total estrogenic activity. The YES screen assay was used at the U of A, and NAU used the ELISA for E2. Furthermore, NAU furthered the development of an inexpensive lateral flow assay sensor for E2 and EE2 that will produce a rapid (under 10 min), convenient evaluation of E2 and EE2 in wastewater. Funds also supported final LCMS/MS validation of these assays. Once available and further validated, these assays will provide POTWs managers with a rapid assessment of their facilities’ ability to remove these marker compounds. Determinants of estrogen removal efficiencies were investigated by examining correlations between treatment process selection/operational data and estrogen removal efficiency across assays. Once assays are further validated, such relationships may be used to prescribe processes or process-dependent efficiencies that will prepare reclaimed water for specific applications including indirect potable reuse.

The principal investigators are indebted to the participating municipalities for their cooperation and support of this project. The continuing cooperative assistance of Pima County Wastewater
Management and the City of Flagstaff is especially noted. Although education and training benefits were not the primary project objectives, AWI funds were used to support student education in environmental engineering and science at participating universities. We also thank ADHS for all of their support in this project for providing the LCMS/MS analysis. Student participants benefitted from an unusual opportunity to interact with sanitary engineering professionals at operational facilities. Workforce development activities including the training of four undergraduates (Larissa Jatho, Geertje Tulipani, Melissa Malvar, and Fiyori Melaki) and three graduate students (Bingfeng Dong, Sandra Tseske, and Beatriz Estrada) in this project.

This report below follows the Scope of Work Document Outline that was organized in consultation with Chuck Graf, AWI Associate Director at the ADEQ. Each step is outlined below and the current status is reported. Where appropriate, we have interpreted the data and made conclusions. In summary, this project had two main objectives: 1) sampling of wastewater treatment plants to determine estrogenic compound removal efficiency and 2) sensor development for estrogenic compounds. Ten separate work items were listed in the original Scope of Work for the first objective; outcomes and final results for each work item are discussed below. For the second objective, sensor development activities were conducted for two estrogen, 17α-ethynylestradiol (EE2) and 17β-estradiol (E2); a full description of each of these projects is also provided below.

**Objective A. WWTP Estrogenic Compound Removal Efficiency**

**Work Item A.1. Due 2/1/07: In consultation with AWI and ADEQ, hold organizational meeting to plan project and compile a list of candidate WWTPs for sampling.**

a. Wastewater: Sampled WWTPs should represent several of the common tertiary unit treatment processes used in Arizona (funding will limit sampling to two sampling periods that will include no more than 4-6 plants).

b. Biosolids: Sampled WWTPs should represent the key biosolids treatment processes used in Arizona, including processes that generate Class A, Class B, and “exceptional quality” (if available) biosolids. Only the final sludge will be sampled for this project.

**Result.** The sites were chosen in consultation with Chuck Graf of AWI/ADEQ. They are listed below in Table 1. Six facilities were chosen and sampled at the listed dates.
Table 1. List of facilities sampled during the first sampling cycle.

<table>
<thead>
<tr>
<th>Wastewater Treatment</th>
<th>Contact Person</th>
<th>Telephone number</th>
<th>E-mail Address</th>
<th>Date Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randolph Park, Tucson</td>
<td>Arturo Norzagaray</td>
<td>520-319-0125</td>
<td></td>
<td>4/24/2007</td>
</tr>
<tr>
<td>New Ina Road, Tucson</td>
<td>Dave Bartos</td>
<td>520-579-6042</td>
<td></td>
<td>4/24/2007</td>
</tr>
<tr>
<td>American Gulch Water Reclamation Facility, Payson</td>
<td>David Millien</td>
<td>928-474-5257</td>
<td><a href="mailto:ops@npgcable.com">ops@npgcable.com</a></td>
<td>7/11/2007</td>
</tr>
<tr>
<td>Sundog, Prescott</td>
<td>Allen Davidson</td>
<td></td>
<td>allen.davidson@cit yoprescott.net</td>
<td>6/13/2007</td>
</tr>
<tr>
<td>Rio de Flag, Flagstaff</td>
<td>Ron Benford</td>
<td>928-556-1301</td>
<td><a href="mailto:rbenford@ci.flagsta">rbenford@ci.flagsta</a> ff.az.us</td>
<td>6/7/2007</td>
</tr>
<tr>
<td>91st Avenue, Phoenix</td>
<td>Jim Coughenour</td>
<td>602-495-7982</td>
<td><a href="mailto:Jim.coughenour@phoenix.gov">Jim.coughenour@phoenix.gov</a></td>
<td>6/27/2007</td>
</tr>
</tbody>
</table>

**Work Item A.2.** Prepare a summary of the sampling approach and compile a list of analyses to be performed during each sampling period.

**Result.** This task was completed and reported to AWI on 2/14/07 (see attached Memorandum).

**Work Item A.3.** ADHS will provide technical support in developing techniques to validate the E2 and EE2 levels in samples and facilitate training of an NAU student on the techniques. (Report on activities in items A.10 and A.14).

**Result.** ADHS developed the techniques to validate the E2 and EE2 levels in the samples. ADHS has a standing offer to train interested NAU, ASU and UA staff and students on the operation of the Applied Biosystems liquid chromatography tandem mass spectrometer (LCMS/MS) at the State Laboratory. The results are within the expected ranges for both E2 and EE2 in wastewater samples. These results were compared to those found using the ELISA and the YES.

**Work Item A.4.** Perform sampling during first half of grant year.

**Result.** As of July 11, 2007, all treatment facilities listed in Table 1 were sampled. Process flow schematics of all facilities, indicating the sampling points at each, are presented in Fig. 3a-f below:
a. Ina Road, Tucson

b. Randolph Park, Tucson

c. Sundog Plant, Prescott
Fig. 3a-f. Schematics of all the facilities sampled. The red triangles in each figure represent where influent, effluent, and sludge samples were collected.
**Work Item A.5.** Prepare a short description of the toxicological assay work completed by Dr. Plewa at the University of Illinois.

Result. The report was completed on March 17, 2007 and is attached.

**Work Item A.6.** Extract and evaluate samples with YES screen and ELISA and validate ELISA with LCMS/MS.

Result: Extraction: Extraction and evaluation of all the samples was completed across all assays. Data and interpretation are presented in Work Item 7 below. Fig. 4a-c illustrates how samples were processed following collection of liquid and solids.

a. Influent solids and liquids

b. Effluent liquids
c. Sludge liquids and solids

Figure 4a.-c. Flow charts for handling and extraction of samples from wastewater treatment facilities.

**Result: Lateral Flow Assay Sensor Development:** We made significant progress on the development of the lateral flow assay with the help of one NAU undergraduate (Fiyori Melaki) and two German exchange undergraduate students (Larissa Jatho and Geertje Tulipani). The sensitivity of the assays for E2 and EE2 is between 1 and 10 nM, as determined by LCMS/MS analysis performed by the ADHS Laboratory. This sensitivity is enough to evaluate E2 and EE2 concentrations in extracted and concentrated samples, but is not yet sensitive enough to evaluate unextracted and unconcentrated samples. Details are provided in Section B below.

**Work Item A.7. Prepare table of sampling results and hold data discussion meeting.**

**Result.** Quantitative results for all assays have been received. The results are presented below in several tables. Table 2 below provides the absolute molar (liquids) or moles/g (solids) values for E2, EE2 and total estrogenicity for each assay at each treatment facility. Evaluation of the data demonstrates that there were often significant differences in the absolute value for the same samples across the different assay methods.
Table 2. Molar (liquid) or moles/g (solids) quantities of E2 and EE2 or estrogenicity in EE2 equivalents in samples assayed from different treatment facilities. ww= wet weight; dw=dry weight.

<table>
<thead>
<tr>
<th>Treatment Facility Date of Collection and Sample Type</th>
<th>Molarity or moles/g ELISA</th>
<th>Molarity or moles/g YES</th>
<th>Molarity or moles/g LC/MS E2</th>
<th>Molarity or moles/g LC/MS EE2</th>
<th>Molarity or moles/g LC/MS E2 + EE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randolph Park Influent Liquid</td>
<td>2.49E-10</td>
<td>6.71E-11</td>
<td>1.05E-10</td>
<td>1.61E-11</td>
<td>1.21E-10</td>
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<tr>
<td>Randolph Park Influent Solid</td>
<td>4.66E-11</td>
<td>0.00E+00</td>
<td>1.13E-12</td>
<td>2.00E-11</td>
<td>2.11E-11</td>
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<tr>
<td>Randolph Park Effluent Liquid</td>
<td>3.58E-10</td>
<td>5.51E-11</td>
<td>1.17E-11</td>
<td>1.77E-11</td>
<td>2.94E-11</td>
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<tr>
<td>Randolph Park Sludge Liquid</td>
<td>3.46E-10</td>
<td>0.00E+00</td>
<td>4.18E-12</td>
<td>3.61E-12</td>
<td>7.78E-12</td>
</tr>
<tr>
<td>Randolph Park Sludge Solid ww</td>
<td>6.69E-11</td>
<td>0.00E+00</td>
<td>2.97E-13</td>
<td>5.37E-12</td>
<td>5.67E-12</td>
</tr>
<tr>
<td>Randolph Park Sludge Solid dw</td>
<td>9.58E-12</td>
<td>0.00E+00</td>
<td>2.96E-12</td>
<td>5.27E-11</td>
<td>5.56E-11</td>
</tr>
<tr>
<td>Ina Road Influent Liquid</td>
<td>3.07E-10</td>
<td>1.13E-10</td>
<td>1.36E-10</td>
<td>1.44E-12</td>
<td>1.37E-10</td>
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<tr>
<td>Ina Road Influent Solid</td>
<td>9.89E-11</td>
<td>0.00E+00</td>
<td>2.10E-12</td>
<td>9.62E-12</td>
<td>1.17E-11</td>
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<tr>
<td>Ina Road Effluent Liquid</td>
<td>1.75E-10</td>
<td>2.76E-11</td>
<td>8.83E-12</td>
<td>1.38E-12</td>
<td>1.02E-11</td>
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<tr>
<td>Ina Road Sludge Liquid</td>
<td>9.33E-10</td>
<td>5.51E-12</td>
<td>7.87E-11</td>
<td>8.39E-13</td>
<td>7.95E-11</td>
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<tr>
<td>Ina Road Sludge Solid ww</td>
<td>6.09E-11</td>
<td>0.00E+00</td>
<td>6.95E-12</td>
<td>3.41E-12</td>
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<tr>
<td>Ina Rd Sludge Solid dw</td>
<td>1.42E-10</td>
<td>3.95E-10</td>
<td>3.26E-11</td>
<td>2.18E-11</td>
<td>5.43E-11</td>
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<tr>
<td>Sundog Influent Liquid</td>
<td>2.70E-10</td>
<td>2.61E-10</td>
<td>1.13E-10</td>
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<td>1.13E-10</td>
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<tr>
<td>Sundog Influent Solid</td>
<td>1.35E-10</td>
<td>0.00E+00</td>
<td>0.00E+00</td>
<td>5.77E-11</td>
<td>5.77E-11</td>
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<td>Sundog Effluent Liquid</td>
<td>2.39E-10</td>
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<td>Sundog Sludge Liquid</td>
<td>5.78E-10</td>
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<td>1.53E-10</td>
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<td>Sundog Sludge Solid dw</td>
<td>1.81E-10</td>
<td>7.69E-10</td>
<td>0.00E+00</td>
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<tr>
<td>Rio de Flag Influent Liquid</td>
<td>4.60E-10</td>
<td>2.10E-10</td>
<td>1.17E-10</td>
<td>5.44E-12</td>
<td>1.23E-10</td>
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<tr>
<td>Rio de Flag Effluent Liquid</td>
<td>4.49E-10</td>
<td>6.29E-11</td>
<td>1.52E-11</td>
<td>1.39E-12</td>
<td>1.66E-11</td>
</tr>
<tr>
<td>Rio de Flag Primary Sludge Liquid</td>
<td>1.27E-09</td>
<td>2.46E-10</td>
<td>3.93E-10</td>
<td>4.95E-11</td>
<td>4.43E-10</td>
</tr>
<tr>
<td>Rio de Flag Prim. Sludge Solid dw</td>
<td>4.12E-10</td>
<td>7.19E-10</td>
<td>1.05E-11</td>
<td>2.98E-11</td>
<td>4.03E-11</td>
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<tr>
<td>Rio de Flag Waste Sludge Liquid</td>
<td>9.36E-10</td>
<td>4.00E-10</td>
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<td>0.00E+00</td>
<td>2.30E-10</td>
</tr>
<tr>
<td>Rio de Flag Waste Act. Sludge Solid dw</td>
<td>2.08E-10</td>
<td>1.99E-09</td>
<td>5.02E-11</td>
<td>8.99E-12</td>
<td>5.92E-11</td>
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<td>91st Ave Influent Liquid</td>
<td>2.32E-10</td>
<td>6.41E-10</td>
<td>5.92E-11</td>
<td>3.26E-12</td>
<td>6.25E-11</td>
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<tr>
<td>91st Ave Influent Solid</td>
<td>5.52E-10</td>
<td>2.38E-11</td>
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<td>1.75E-11</td>
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<td>91st Ave Effluent Liquid</td>
<td>1.91E-10</td>
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<td>1.06E-11</td>
<td>1.06E-11</td>
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<td>91st Ave Sludge Solid</td>
<td>1.93E-10</td>
<td>1.92E-10</td>
<td>2.40E-12</td>
<td>2.99E-11</td>
<td>3.23E-11</td>
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<td>Payson Influent Liquid</td>
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<td>8.34E-10</td>
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<td>7.84E-12</td>
<td>7.84E-12</td>
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<td>Payson Influent Solid</td>
<td>9.19E-12</td>
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<td>1.14E-12</td>
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<td>Payson Effluent Liquid</td>
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<td>4.96E-12</td>
<td>1.18E-12</td>
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<td>7.40E-12</td>
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<td>Payson Sludge Liquid</td>
<td>2.39E-11</td>
<td>7.54E-12</td>
<td>0.00E+00</td>
<td>7.53E-12</td>
<td>7.53E-12</td>
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<td>Payson Sludge Solid</td>
<td>9.05E-12</td>
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<td>2.55E-12</td>
<td>1.49E-11</td>
<td>1.75E-11</td>
</tr>
</tbody>
</table>
In order to determine whether qualitatively, the assays provided similar relative results, the values for the assays were compared using pair-wise regression analysis for all samples types assayed within each facility. The following assays were compared: a) ELISA E2 to YES, b) ELISA E2 to LC/MS E2, and c) YES to LC/MS E2 + EE2. The last comparison was made because the YES assay screens for total estrogenicity, which may reflect significant impact from E2 and EE2, both highly estrogenic compounds.

The results of these analyses are presented in Table 3. The regression analysis demonstrates that only a couple of assays provided similar qualitative results within each sample, and that these results were not consistent across treatment facilities.

Table 3. Regression analysis comparisons between assays within each treatment facility. The values presented are the correlation coefficients. Cells with grey fill are those where a significant correlation was found.

<table>
<thead>
<tr>
<th>Facility</th>
<th>ELISA vs YES R-square</th>
<th>ELISA vs LC/MS E2 P-value</th>
<th>YES vs LC/MS Total P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randolph Park</td>
<td>0.338</td>
<td>0.084</td>
<td>0.472</td>
</tr>
<tr>
<td>P-value</td>
<td>0.226</td>
<td>0.057</td>
<td>0.132</td>
</tr>
<tr>
<td>Ina Road</td>
<td>0.122</td>
<td>0.272</td>
<td>0.074</td>
</tr>
<tr>
<td>P-value</td>
<td>0.498</td>
<td>0.288</td>
<td>0.603</td>
</tr>
<tr>
<td>Sundog R-square</td>
<td>0.123</td>
<td>0.752</td>
<td>0.001</td>
</tr>
<tr>
<td>P-value</td>
<td>0.562</td>
<td>0.057</td>
<td>0.969</td>
</tr>
<tr>
<td>Rio de Flag R-square</td>
<td>0.270</td>
<td>0.890</td>
<td>0.093</td>
</tr>
<tr>
<td>P-value</td>
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<td>0.558</td>
</tr>
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<td>91st Ave. R-square</td>
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<td>0.000</td>
<td>0.973</td>
</tr>
<tr>
<td>P-value</td>
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<td>0.980</td>
<td>0.014</td>
</tr>
<tr>
<td>American Gulch R-square</td>
<td>0.643</td>
<td>0.702</td>
<td>0.001</td>
</tr>
<tr>
<td>P-value</td>
<td>0.103</td>
<td>0.076</td>
<td>0.955</td>
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</table>

Furthermore, using a Friedman’s Repeated Measures Analysis of Variance, we determined whether the assays provided overall quantitative differences within facilities across assays by comparing the overall levels across all samples and assays within each facility. Those results are provided in Figs 5a-f and Table 4. Overall, the ELISA and YES assays returned higher levels of estrogen or estrogenicity than did the LC/MS assays although even this result was not completely consistent across facilities. This result is to be expected, though, since both ELISA and YES assays may be sensitive to the presence of other endocrine disrupting compounds.
Fig. 5 a-f. Average amount of E2 measured compared across assays for each treatment facility. P-values comparing estrogen/estrogenicity levels across assays are a) 0.003, b) 0.026, c) 0.056, d) 0.0007, e) Sample size was too small for analysis, f) 0.07.
We calculated the percent removal for each facility both for the total influent and effluent loads and just for the liquid portion of the influent and effluent for each assay. These results are presented in Table 4, indicated as percent removals.

Table 4. Percent removals calculated for each facility across all assays. Higher values suggest that the facilities have better removal efficiencies. Color codes are explained below. N/A: Not available because at least one value was not measurable in the assays.

<table>
<thead>
<tr>
<th>Facility</th>
<th>Overall Estrogen Removal, Sludge and Liquid (%)</th>
<th>Estrogen Removal, Influent Liquid to Effluent Liquid (%)</th>
<th>Overall Estrogen Removal, Sludge and Liquid (%)</th>
<th>E2 Removal, Influent Liquid to Effluent Liquid (%)</th>
<th>Overall EE2 Removal, Sludge and Liquid (%)</th>
<th>EE2 Removal, Influent Liquid to Effluent Liquid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randolph Park</td>
<td>28%</td>
<td>28%</td>
<td>-27%</td>
<td>90%</td>
<td>-2%</td>
<td>4%</td>
</tr>
<tr>
<td>Ina Road</td>
<td>54%</td>
<td>76%</td>
<td>47%</td>
<td>92%</td>
<td>41%</td>
<td>4%</td>
</tr>
<tr>
<td>Sundog</td>
<td>9%</td>
<td>89%</td>
<td>11%</td>
<td>92%</td>
<td>94%</td>
<td>2%</td>
</tr>
<tr>
<td>Rio de Flag</td>
<td>-36%</td>
<td>73%</td>
<td>6%</td>
<td>78%</td>
<td>88%</td>
<td>N/A</td>
</tr>
<tr>
<td>91st Ave</td>
<td>99%</td>
<td>100%</td>
<td>34%</td>
<td>100%</td>
<td>236%</td>
<td>-224%</td>
</tr>
<tr>
<td>American Gultch</td>
<td>99%</td>
<td>99%</td>
<td>45%</td>
<td>50%</td>
<td>N/A</td>
<td>15%</td>
</tr>
</tbody>
</table>

The percent removal data demonstrate a great deal of variability across assays. For example, overall plant removal (total load in treated wastewater and sludge produced by the plant as a percent of total load in solids and liquid present in influent to the plant) for the Rio de Flag facility ranges from -36% with the YES assay (suggesting greater levels of estrogens leaving the facility than entering) to 78% removal efficiency of estradiol (E2) based on results from the LC/MS assay. To statistically ask whether each assay provided a similar result within each treatment facility, we ranked the facilities from 1-6 with 6 representing the treatment facility with the lowest overall percent removal of estrogen. For visualization purposes in Table 4, in each vertical column of assay results we color ranked the facility percent removals: red being the least percent removal, orange the next, gold third, bright yellow fourth, yellow green fifth, and green sixth or most removal. The results show no correlation in percent removals across assays. From these data we conclude that there is no association among assays for evaluating fractional removal. This lack of consistency across assays suggests that each assay may be sensitive to interference from other compounds in the samples, and these interferences are different for the different types of assays.

Also, another confounding factor must be considered in interpreting the removal efficiency results. The liquid effluent and sludge samples represent the culmination of unit processes involving extensive mixing over long residence and treatment times. Relative temporal
uniformity in analytical results is to be expected. On the other hand, influent samples represent highly time-variable, non-uniform conditions. Thus, comparison of a number of single, randomly collected influent samples to more homogenized post-treatment samples will produce a range of apparent removal efficiency values, and could easily produce a result showing a greater concentration of a constituent in effluent than in influent. Frequent sampling of influent during the daily regimen of rising and falling flows, in conjunction with mass loading calculations based on flow-duration statistics, would help to better quantify the overall removal efficiency, but such an approach was beyond the scope and budget of this project.

Last, we evaluated the wastewater effluent liquid levels of estrogens across different facilities to determine which facility may be releasing the most and least amount of estrogens into the environment. The data are presented in Table 5.

Table 5: Amounts of E2 (ELISA and LC/MS), EE2 (LC/MS) or total estrogenicity (YES) in nanograms per liter (ng/l) in the effluent liquid from all facilities. Color coding used to visually rank the level of estrogen within each assay among all facilities: red is the highest concentration, orange is second highest, gold is third highest, bright yellow is fourth highest, yellow green is fifth highest, and green is lowest.

<table>
<thead>
<tr>
<th>Treatment Facility</th>
<th>ELISA</th>
<th>YES</th>
<th>LC/MS E2</th>
<th>LC/MS EE2</th>
<th>LC/MS Total</th>
<th>Rank Pts</th>
</tr>
</thead>
<tbody>
<tr>
<td>American Gulch, Payson</td>
<td>4.7</td>
<td>1.5</td>
<td>0.3</td>
<td>1.8</td>
<td>2.2</td>
<td>10</td>
</tr>
<tr>
<td>Ina Road, Tucson</td>
<td>47.6</td>
<td>8.2</td>
<td>2.4</td>
<td>0.4</td>
<td>2.8</td>
<td>14</td>
</tr>
<tr>
<td>91st Ave, Phoenix</td>
<td>52.1</td>
<td>0.5</td>
<td>0.0</td>
<td>3.1</td>
<td>3.1</td>
<td>14</td>
</tr>
<tr>
<td>Sundog, Prescott</td>
<td>64.9</td>
<td>9.6</td>
<td>2.2</td>
<td>0.3</td>
<td>2.5</td>
<td>14</td>
</tr>
<tr>
<td>Rio de Flag, Flagstaff</td>
<td>122.1</td>
<td>18.6</td>
<td>4.1</td>
<td>0.4</td>
<td>4.6</td>
<td>26</td>
</tr>
<tr>
<td>Randolph Park, Tucson</td>
<td>97.4</td>
<td>16.3</td>
<td>3.2</td>
<td>5.2</td>
<td>8.4</td>
<td>27</td>
</tr>
</tbody>
</table>

For visualization purposes, in Table 5, for each assay we color ranked all facilities’ estrogen concentration in the treated wastewater discharged from the plant. Each red represents the highest output, and was assigned a 6 for the purpose of the last ranking column. Each orange, the next highest output, was assigned a 5. Each gold was assigned a 4 and each yellow a 3. Each yellow green, which represents the second lowest output, was assigned 2, and green, which represents the lowest output of estrogens, was assigned a 1. Thus, the lowest score in the Rank Points column correlates with the facility that yielded the lowest concentration of estrogens in the treated wastewater in consideration of all of the analytical methods used in this study. On this basis, the estrogenic activity of treated wastewater from the Payson facility is relatively less than from the Rio de Flag and Randolph Park facilities, with the other facilities falling in between.

**Work Item A.8.** Reevaluate facilities sampled to determine whether they should all be sampled in second half. Determine if different sites should be added or substituted.

**Result.** Since the results demonstrated that there were inconsistencies across assays, during the second half of the project period we decided to evaluate where in the process there might be matrix impacts affecting the results. These data are currently under evaluation and will be presented as an addendum to this report at a later date.
Work Item A.9. Determine feasibility of adding additional plants should funds become available.

Result. As noted in the previous item, a evaluation of the source of matrix interferences needs to be done before further sampling should be considered. That evaluation is underway and will be presented as an addendum to this report at a later date.

Work Item A.10. Write mid-program report for AWI.


Conclusions, Objective A: No single treatment facility showed consistent removal efficiencies across assays. This result makes it difficult to determine at this time which facility is providing the greatest removal efficiency. However, a general consistency across facilities for the different assays allowed the facilities to be ranked in terms the relative estrogenicity level measured in the discharged, treated wastewater. This result does not take into account the amount of estrogen in the influent and therefore cannot be used to base conclusions on removal efficiency. Presumed matrix interferences are believed to be the source of the inconsistent results across assays. An evaluation is being performed to examine this issue and will be submitted as an addendum to this report. The second phase of this objective called for follow up testing of the wastewater treatment facilities sampled during the first phase of this project. However, this second phase of sampling was not performed. Until the matrix interference effects are understood, further testing to determine removal efficiencies would not be fruitful.

Objective B. Sensor Development

Work Item B.1. Prepare a brief description of work to date on developing the rapid E2 and EE2 ELISA tests and sensors.

Result. This task was completed and reported to AWI on 2/14/07 (see attached Memorandum).

Work Item B.2. Drs. Propper, Vail and Ingram will meet monthly to evaluate progress on assay development.

Result. Drs. Propper and Vail met monthly to discuss the progress of the assays. The project results are presented below under Work Item 3.

Work Item B.3. Prepare update of work done to develop the ELISA tests and sensors; include in item A.10.

Current Status:

Project 1: Development of a Lateral Flow Assay for the Detection of 17α-Ethynylestradiol in Wastewater. The aim of this research was to develop an alternate detection method for 17α-ethynylestradiol (EE2). This compound is a known endocrine disruptor, and this project initiated the development of a rapid, inexpensive, yet sensitive method of detection of
EE2 in wastewater as an alternative to lab-based methods such as gas chromatography/mass spectrometry. A lateral flow assay for E2 and/or EE2 is an antibody-capture assay that functions much like the well known pregnancy tests. Liquid containing E2 or EE2 is moved across a strip by capillary action. The strip contains reagents that allow for the detection and quantification of E2 or EE2 in the liquid sample. The result output is a line across the strip where the intensity of the line is proportional to the amount of E2 or EE2 in the sample.

An extensive series of preliminary experiments were conducted to optimize assay reagent concentrations and assay running parameters. Standard techniques of immunochromatographic assay development were employed. The assay was constructed in a “competitive” format such that the detected signal from the assay is inversely proportional to the amount of free EE2 present in the wastewater sample. Subsequent to assay optimization, wastewater samples from the Randolph Park and Ina Road treatment facilities were used to test the method for proof-of-concept.

The following description represents assay parameters after optimization.

**Project 1 Methods.** EE2 conjugated to bovine serum albumin (BSA-EE2; Sigma E1583) was striped onto Millipore HiFlow 07502 Nitrocellulose using a Matrix 1600 Reagent Dispensing Module at a concentration of 100 µg/ml in 50 mM sodium borate buffer at a dispense rate of 10 cm/sec; sample volume 0.5 µl/cm. Treated nitrocellulose was dried and cut into “test strips” 5 cm x 3 mm, with the BSA-EE2 stripe perpendicular to the long axis of the test strip, at a distance of 2 cm from the (distal) end.

Calibration curves were created by testing serial dilutions of EE2 in HEPES – BSA buffer, pH 6.8. Ten-fold concentrations of EE2 from 100 pM to 100 mM were used as calibration standards, and included a blank. All concentrations for the standard curve were measured in triplicate. Curve-fitting was performed using Prism v3.0.

Polyclonal rabbit anti-EE2 (Abcam) was used as the ‘primary’ antibody, diluted in sodium borate buffer at 1:12,000 from purchased stock. 60 µl of a given EE2 standard and 60 µL primary antibody dilution were mixed and incubated for 20 minutes. 100 µl of the incubated mixture was pipetted into a well of a standard 96-well microtiter plate. The proximal end of a test strip was immersed into the microtiter well and the reagents were allowed to adsorb up the test strip across the reaction stripe until reaching the distal end of the strip (10 minutes). The test strip was removed and dried at room temperature for 10 minutes. During the drying step, 60 µl of 5 nm colloidal gold, surface functionalized with Protein A, was diluted 1:70 from 50 O.D. stock in HEPES-BSA buffer and added to 60 µl of polyclonal goat anti-rabbit IgG diluted 1:400 from purchased stock. This step allowed the secondary antibody to become conjugated to the colloidal gold through antibody binding by Protein A. 100 µL of the colloidal gold-antibody solution was pipetted into a clean microtiter well and the proximal end of the dried test strip immersed into it for 10 minutes. The test strip was removed from the well, dried at room temperature for 20 minutes, and any resulting binding of colloidal gold-secondary antibody to the primary antibody (bound to the BSA-EE2 stripe) was determined using an Alpha-Innotec FluorChemSP camera and image analysis software. Measurements of non-specific binding were also performed using
test strips made with BSA in the absence of conjugated EE2. All dilutions and other parameters were as described above.

**Project 1 Results.** Calibration curves were generated separately for the two different wastewater treatment plants tested (Randolph Park and Ina Road). Figure 6 shows the calibration curve for Randolph Park samples. The IC$_{50}$ (the concentration at one-half of the maximum value) was $4.93 \times 10^{-8}$ M; $R^2 = 0.97$.

![Figure 6: Calibration Curve – Randolph Park](image)

Figure 7 shows the calibration curve used for Ina Road samples. The IC$_{50}$ = $1.7 \times 10^{-8}$ M; $R^2 = 0.96$.

![Figure 7: Calibration Curve – Ina Road](image)
Five samples from each treatment plant were extracted with 20%, 50%, and 80% methanol, and spiked with 10 nM EE2. Treatment plant samples were measured in triplicate at dilutions of 1:20 and 1:200. It was determined that 1:200 dilutions were below the detection limit of the assay, so results from 1:20 dilutions only are shown in Table 6, reported in moles per liter EE2.

<table>
<thead>
<tr>
<th>[M]</th>
<th>80%MeOH/20%H₂O</th>
<th>50%MeOH/50%H₂O</th>
<th>20%MeOH/80%H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent liquid</td>
<td>2.82•10⁻⁸ ± 1.49•10⁻⁸</td>
<td>4.34•10⁻⁸ ± 3.47•10⁻⁹</td>
<td>9.67•10⁻⁹ ± 3.48•10⁻⁹</td>
</tr>
<tr>
<td>Influent solid</td>
<td>5.58•10⁻⁹ ± 3.09•10⁻⁹</td>
<td>2.38•10⁻⁷ ± 1.48•10⁻⁷</td>
<td>1.67•10⁻⁸ ± 2.17•10⁻⁸</td>
</tr>
<tr>
<td>Effluent liquid</td>
<td>1.59•10⁻⁸ ± 1.51•10⁻⁸</td>
<td>1.18•10⁻⁸ ± 3.60•10⁻⁸</td>
<td>1.86•10⁻⁸ ± 1.33•10⁻⁸</td>
</tr>
<tr>
<td>Sludge liquid</td>
<td>1.89•10⁻⁸ ± 5.00•10⁻⁹</td>
<td>1.28•10⁻⁸ ± 1.22•10⁻⁸</td>
<td>9.22•10⁻⁹ ± 6.69•10⁻⁹</td>
</tr>
<tr>
<td>Sludge solid</td>
<td>1.24•10⁻⁹</td>
<td>3.24•10⁻⁹ ± 3.36•10⁻¹⁰</td>
<td>9.06•10⁻¹⁰</td>
</tr>
</tbody>
</table>

Table 6: EE2 concentrations determined by immunochromatography for Randolph Park

Samples from Ina Road were treated identically to the Randolph Park samples. Results are shown below in Table 7, reported in moles per liter EE2.

<table>
<thead>
<tr>
<th>[M]</th>
<th>80%MeOH/20%H₂O</th>
<th>50%MeOH/50%H₂O</th>
<th>20%MeOH/80%H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent liquid</td>
<td>1.06•10⁻⁸ ± 9.08•10⁻⁹</td>
<td>1.34•10⁻⁸ ± 1.02•10⁻⁸</td>
<td>3.94•10⁻⁸ ± 6.90•10⁻⁹</td>
</tr>
<tr>
<td>Influent solid</td>
<td>2.70•10⁻⁸ ± 5.37•10⁻⁹</td>
<td>3.14•10⁻⁸ ± 1.48•10⁻⁸</td>
<td>2.48•10⁻⁸ ± 1.70•10⁻⁸</td>
</tr>
<tr>
<td>Effluent liquid</td>
<td>1.43•10⁻⁸ ± 5.09•10⁻⁹</td>
<td>2.20•10⁻⁸ ± 1.20•10⁻⁸</td>
<td>3.86•10⁻⁸ ± 3.52•10⁻⁸</td>
</tr>
<tr>
<td>Sludge liquid</td>
<td>2.05•10⁻⁸ ± 1.29•10⁻⁸</td>
<td>8.61•10⁻⁹ ± 4.84•10⁻⁹</td>
<td>9.70•10⁻⁹ ± 9.26•10⁻⁹</td>
</tr>
<tr>
<td>Sludge solid</td>
<td>1.60•10⁻⁸ ± 8.44•10⁻⁹</td>
<td>9.44•10⁻⁹ ± 1.00•10⁻⁸</td>
<td>2.94•10⁻⁸ ± 2.25•10⁻⁸</td>
</tr>
</tbody>
</table>

Table 7: EE2 concentrations determined by immunochromatography for Ina Road

EE2 removal efficiency, based only on the difference in EE2 concentrations between liquid influent and liquid effluent was determined to be 43% for Randolph Park, and -18% for Ina Road. These results differ from those found for EE2 using the LCMS/MS technology shown. Table 4 shows that LCMS/MS % removal efficiency for EE2 from Randolph Park and Ina was 4% for both facilities. Again, more work needs to be conducted to identify which assay is subject to matrix effects.

Project 2: Development of a Lateral Flow Assay for the Detection of 17β-Estradiol in Wastewater. The aim of this research was to develop an alternate detection method for 17β-estradiol (E2). Like EE2, this compound is known endocrine disruptor, and this project initiated
the development of an immunochromatographic assay based on the same rationale as the EE2 assay.

An extensive series of preliminary experiments were conducted to optimize assay reagent concentrations and assay running parameters. Standard techniques of immunochromatographic assay development were employed. The assay was constructed in a “competitive” format such that the detected signal from the assay is inversely proportional to the amount of free E2 present in the wastewater sample.

Subsequent to assay optimization, wastewater samples from Randolph Park and Ina Road treatment facilities were used to test the method.

The following description represents assay parameters after optimization.

**Project 2 Methods.** Assay development and optimization were performed in a similar way to that described for the EE2 assay in Project 1. For this assay, the following parameters were determined:

1) BSA-E2 on test strip: 100 µg/ml
2) Primary antibody (monoclonal mouse anti-estradiol (Chemicon) dilution from purchased stock: 1:160,000
3) Secondary antibody (polyclonal goat anti-mouse (Chemicon) dilution from purchased stock: 1:1000
4) Protein a – colloidal gold conjugate; 1:70
5) E2 calibrator concentrations: 2²⁻⁰M, 1µM, 200 nM, 100 nM, 20 nM, 10 nM, 2 nM, 1 nM, 200pM and 0M (blank).

**Project 2 Results.** A single calibration curve was generated for the two different treatment plants tested (Randolph Park and Ina Road). Figure 8 shows the calibration curve used. The IC$_{50}$ was $2.54 \times 10^{-9}$ M; $R^2 = 0.87$ (due to attempted fit of the outlier).

![Figure 8: Calibration curve for E2 concentrations and blank (outlier).](image)
Wastewater treatment plant samples from Randolph Park and Ina Road were then analyzed using this method. Samples from both facilities were prepared at a final dilution of 1:20 and spiked with a final E2 concentration = 500pM. Table 7 shows the results from the Randolph Park samples from three different methanol extractions. Results are reported in moles/liter E2.

Table 8

<table>
<thead>
<tr>
<th>[M]</th>
<th>80%MeOH/20%H2O</th>
<th>50%MeOH/50%H2O</th>
<th>20%MeOH/80%H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent liquid</td>
<td>8.11<em>10^8± 3.74</em>10^8</td>
<td>6.65<em>10^8± 4.25</em>10^8</td>
<td>1.52<em>10^6± 1.09</em>10^6</td>
</tr>
<tr>
<td>Influent solid</td>
<td>2.66<em>10^-7± 2.18</em>10^-8</td>
<td>4.28<em>10^-7± 1.06</em>10^-7</td>
<td>3.00<em>10^-7± 8.14</em>10^-8</td>
</tr>
<tr>
<td>Effluent liquid</td>
<td>1.52<em>10^-7± 4.61</em>10^-9</td>
<td>1.18<em>10^-7± 8.06</em>10^-8</td>
<td>2.24<em>10^-7± 1.34</em>10^-7</td>
</tr>
<tr>
<td>Sludge liquid</td>
<td>7.38<em>10^-8± 3.52</em>10^-8</td>
<td>2.54<em>10^-7± 2.63</em>10^-8</td>
<td>8.03<em>10^-8± 2.34</em>10^-8</td>
</tr>
<tr>
<td>Sludge solid</td>
<td>9.69<em>10^-8± 8.27</em>10^-8</td>
<td>9.46<em>10^-7± 3.89</em>10^-7</td>
<td>6.70<em>10^-7± 4.22</em>10^-7</td>
</tr>
</tbody>
</table>

Table 9 shows the results from the Ina Road samples from three different methanol extractions. Results are reported in moles/liter or moles/g (solids) E2.

Table 9

<table>
<thead>
<tr>
<th>[M]</th>
<th>80%MeOH/20%H2O</th>
<th>50%MeOH/50%H2O</th>
<th>20%MeOH/80%H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent liquid</td>
<td>6.15<em>10^-9± 2.33</em>10^-9</td>
<td>4.10<em>10^-8± 2.48</em>10^-9</td>
<td>4.95<em>10^-8± 1.68</em>10^-9</td>
</tr>
<tr>
<td>Influent solid</td>
<td>N/A</td>
<td>1.04<em>10^-7± 1.43</em>10^-8</td>
<td>6.89<em>10^-8± 7.64</em>10^-9</td>
</tr>
<tr>
<td>Effluent</td>
<td>2.94<em>10^-8± 2.08</em>10^-8</td>
<td>3.81<em>10^-8± 5.97</em>10^-9</td>
<td>2.46<em>10^-8± 7.83</em>10^-10</td>
</tr>
<tr>
<td>Sludge</td>
<td>3.67<em>10^-7± 1.35</em>10^-7</td>
<td>2.69<em>10^-7± 9.30</em>10^-8</td>
<td>2.93<em>10^-7± 8.48</em>10^-8</td>
</tr>
<tr>
<td>Sludge solid</td>
<td>2.38<em>10^-7± 1.10</em>10^-7</td>
<td>1.22<em>10^-7± 3.50</em>10^-8</td>
<td>1.62<em>10^-7± 2.93</em>10^-8</td>
</tr>
</tbody>
</table>

Removal efficiencies for Randolph Park are 98% for Influent liquid to Effluent liquid (only 80%MeOH/20%H2O values used). Removal efficiencies for Ina Road are 95% for Influent liquid to Effluent liquid (only 80%MeOH/20%H2O values used). These compare to 90 and 94 percent from the LCMS/MS E2 result respectively, and -26% and 43% for the ELISA. Again, matrix effects may explain the differences.

Conclusions, Objective B: The results of this study demonstrate proof-of-concept for the lateral flow assays for both E2 and EE2. The assays are sensitive enough for detection of extracted E2 and EE2 in wastewater influent and effluent. Because of the matrix issues associated with the other assays in this project, we did not use this assay to currently evaluate the percent removal efficiencies, but will use these results to further validate the functionality of this rapid assay.
during the next round of funding. Therefore, this assay will be further evaluated for sensitivity to matrix effects, and optimized to lower the limit of detection so as to functionalize the assay for direct quantification of estrogens without extraction. The new AWI funding to Quanrud and colleagues, AWI Project 08-36, will provide funds to further support the development of this promising assay.