Project Final Report

CONTINUED INVESTIGATION OF CONTAMINANTS OF EMERGING CONCERN DISCHARGED FROM ONSITE SYSTEMS WITH EMPHASIS ON ENDOCRINE DISRUPTING COMPOUNDS

A pilot project to investigate the use of Yeast Estrogen Screen (YES) for detecting estrogenicity of septic tank percolate

PROJECT #13-01/319
2013-2016

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BUREAU OF WATER RESOURCES
AND
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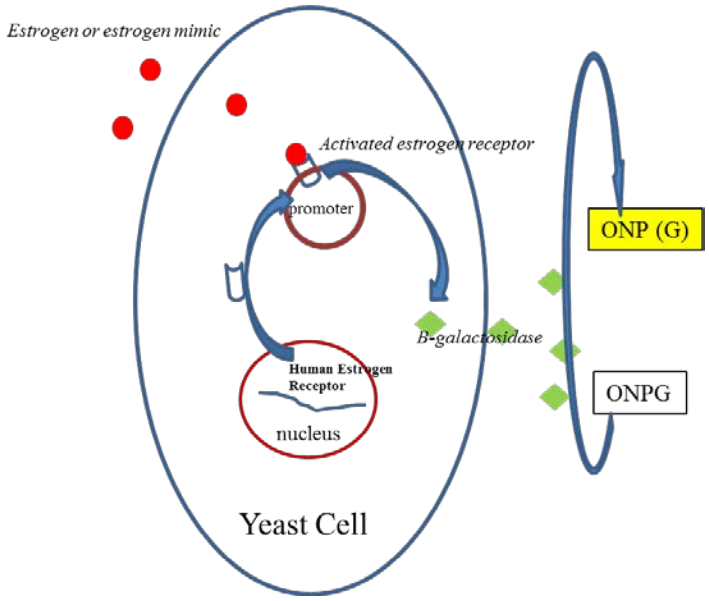

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and
The Environmental Protection Agency, REGION 1

Contents

PROJECT TITLE: CONTINUED INVESTIGATION OF CONTAMINANTS OF EMERGING CONCERN DISCHARGED FROM ONSITE SYSTEMS WITH EMPHASIS ON ENDOCRINE DISRUPTING COMPOUNDS.

Executive Summary

Previous work by Barnstable County Department of Health and Environment and others(Swartz et al. 2006, Standley et al. 2008, Schaider et al. 2010) suggests that Endocrine Disrupting Compounds (EDC) emanating from onsite septic systems have the potential to impact both human and wildlife/ecosystem health. The expense and, in some cases, the lack of analytical methodology make it impractical to monitor for the range of compounds in this category. This study investigated the possibility of using a modification of a Yeast Estrogen Screen (YES) test for the purpose of providing a coarse measure of compounds influencing estrogen physiology. The modification had the potential for reducing the cost and complexity of more standard YES assays by eliminating the solid-phase extraction part of the procedure and allow the direct introduction of environmental samples to the modified yeast growth mixture.

Hands-on instruction by authors of supporting work(Colosi and Kney 2011) was provided over the study period, and these investigators were regularly consulted during the project. Despite this level of collaboration, however we were unable to achieve an acceptable level of reproducibility required to assure quality of the data. We posit that percolate from onsite septic systems, the focus of our investigation, may contain factors that confound the simple analyses. The authors of supporting work indicate that high nitrate and phosphate values typical in percolate beneath onsite septic system drainfields modifies the normal growth of the yeast and would consequently may affect the activity of the reported gene activity which is the measure of estrogenicity.

Despite our inability to quantify estrogenicity using this method, the reader should understand that YES assays in general are widely used with success on both environmental and food product testing(Hermanowicz and Wozei 2002)(Beck et al. 2006)(Routledge and Sumpter 1996)(Bazin et al. 2013)(Mazur and others 2000). In the majority of studies, the solid phase extraction with subsequent evaporation and analyte reconstitution was used and produced acceptable reproducibility and adequate recoveries. Since these steps add significantly to the costs of the sample, our attempt here was to see if the YES without these costly steps could produce acceptable results. We can only conclude that, although the authors of the originating work and others, notably Balsiger et al.(Balsiger et al. 2010) who reference the relative ease of this type of assay still rely on a stability of conditions and expertise of analytical staff that we did not have in this project.

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Introduction

Endocrine Disrupting Compounds (EDC) constitute a wide variety of compounds that are shown to be of both public health and environmental consequence (Snyder et al. 2003). These compounds are part of a larger broad class of Contaminants of Emerging Concern (CEC) but are particularly insidious due to their impact on endocrine physiology of some animals at extremely low (ng/L) concentrations. Among the best documented impacts of EDC are those observed to occur in fish populations. For instance, fish populations exposed to estrogenic compounds as low as 5–6 ng/L have exhibited feminization of males through the production of vitellogenin, causing intersex in male fish and altered oogenesis in females which ultimately caused the collapse of that specie's population(Kidd et al. 2007). Investigations on Cape Cod have shown similar concentrations of endocrine-disrupting compounds originating from onsite septic systems entering ponds and lakes, thus having the potential for producing similar ecological impact in these receiving surface waters (Standley et al. 2008)(Swartz et al. 2006). Since in Barnstable County the dominant mode of wastewater disposal is the onsite septic system, it is imperative that we understand those factors that attenuate EDC in order to optimize septic system designs for their removal.

Research regarding EDC fate and transport has generally been hampered by three main factors: the expense and complexity of EDC chemical assays, the lack of analytical techniques to assay many known EDC, and our lack of knowledge and understanding regarding the full suite of chemical compounds that can cause endocrine disruption. To address this problem, a number of biological assays have been developed that expose mammalian cells (Villeneuve et al. 2007)(Hilscherova et al. 2002), fish (Jobling and Sumpter 1993)(Sabo-Attwood et al. 2007)(Panter et al. 1998), insects (Soin and Smagghe 2007), algae (Lai et al. 2002) or modified yeast cells(Lee et al. 2003)(Beck et al. 2006)(Bazin et al. 2013)(Stanford and Weinberg 2010)(Escher et al. 2005)(Colosi and Kney 2011) to suspected sources of EDC and measure the physiological response. Rather than determining the concentration of any suite of EDC, these latter assay techniques evaluate the cumulative effect of many EDC that may act in concert, without the need for identifying a specific compounds. These techniques are often considered a coarse and initial indication of environmental/public health risk.

The present study was initiated to survey the various biological assays and determine whether they can be economically and feasibly used in our area to determine the estrogenicity of wastewater percolate,

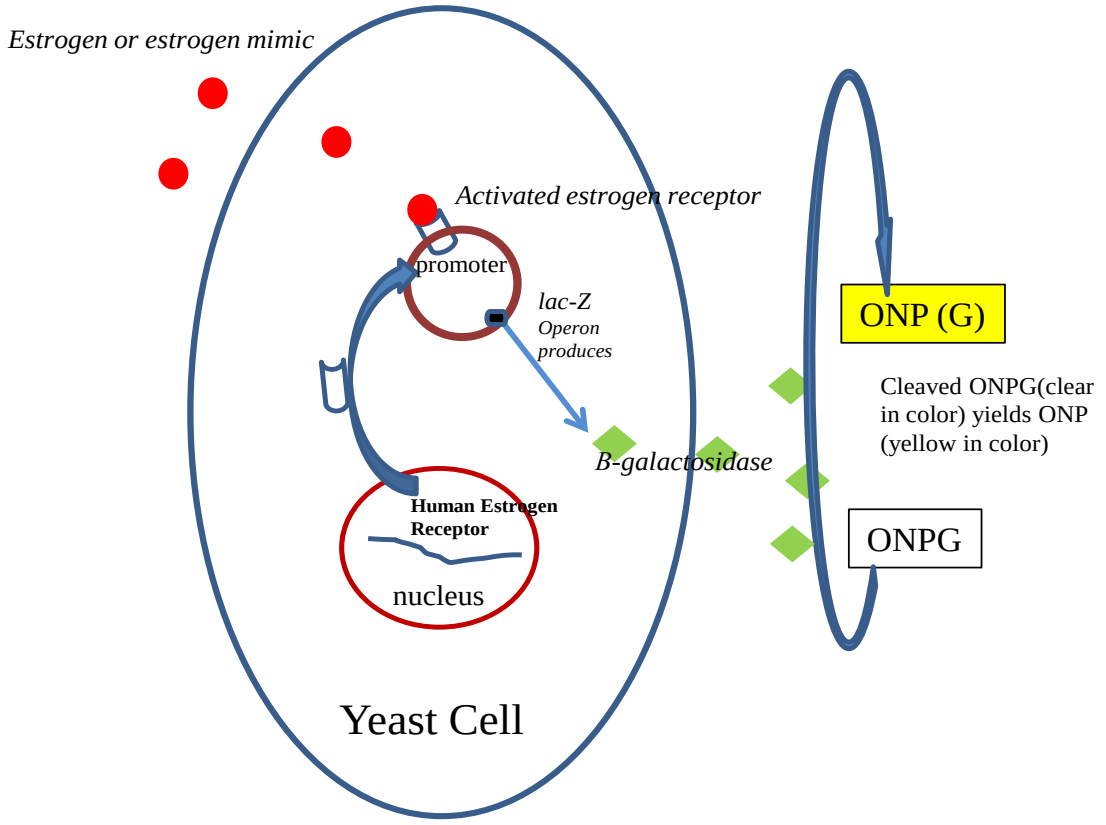
effluent or waterbodies. Estrogenicity refers to the ability of a compound to mimic the physiological actions of estrogen. It is a subset of activity of the entire endocrine systems, and is perhaps the most understood and studied regarding detrimental effects. The goal of this project was to find a rapid and inexpensive test that could further inform future more-expensive and comprehensive efforts. These latter efforts would likely involve the more expensive (cost prohibitive in our case) analytical techniques such as solid-phase extraction (SPE) followed by analyses using instruments such as Liquid Chromatography–Mass spectrometry (using two mass spectrophotometers) or LC-MS/MS. In consideration of the resources available and the need to inform any further more expensive assays, the focus of our efforts was to find a method that met the following criteria:

1. Presented results that are reproducible
2. Can be conducted following minimal training of a laboratory technician
3. Sample cost < $100/sample

Following a survey of various biological assays, a simple assay described in peer-reviewed literature was identified. Two investigators: Joseph C. Colosi, Natural Science Department, DeSales University, Center Valley, Pennsylvania, USA and Arthur Kney Department of Civil and Environmental Engineering, Lafayette College, Easton, Pennsylvania, USA published an article titled A YEAST ESTROGEN SCREEN WITHOUT EXTRACTION PROVIDES FAST, RELIABLE MEASURES OF ESTROGENIC ACTIVITY in the journal titled *Environmental Toxicology and Chemistry* (Vol. 30, No. 10, pp. 2261–2269, 2011). This study served as the basis of our efforts.

The method used in the Colosi and Kney study was a modified Yeast Estrogen Screen (YES)technique similar to many others used(Routledge and Sumpter 1996)(Beck et al. 2006, Stanford and Weinberg 2010, Bistan et al. 2012, Bazin et al. 2013)with the exception that the processing step of Solid Phase Extraction (SPE) was not required. These authors reported promising results without using this time-intensive step, which lead our investigators to consider it more efficient than other YES methods that require SPE.

This analytical technique compared favorably in a number of critical areas. Table 1 estimates the costs of the mammalian, YES (with SPE) and YES_{NE} (no extraction). The table was prepared by Joseph Colosi and modified by George Heufelder as part of a request to EPA for continued funding of the project beyond the present support period. A comparison of the relative complexity of each of these techniques is illustrated in Figure 1.As can be seen, the solid phase extraction, drying and reconstitution of each sample is a costly and more complicated step that further compelled the investigation of a Yeast Estrogen Screening (YES) test that did not require it. In addition, the initial investment for equipment in the YES_{NE}



Yeast Estrogen Screens (YES) involve the use of a yeast that has been encoded with a human estrogen receptor which causes intercellular reactions that ultimately result in a color change in a compound that is proportional to the number of estrogen receptor sites activated or the concentration of the estrogenic compounds. It is usually expressed in Estradiol equivalents. Here the ONPG changes from clear to yellow when the cell has been exposed to estrogen or an estrogen mimic.

DETAIL

Ligands (estrogen and estrogen mimics), bind with Human Estrogen Receptor which induces the operon *lac-Z* to produce a proportionate amount of β-galactosidase which cleaves a proportionate amount of ONPG (normally clear in color) to ONP (yellow in color) which is then read using a photometer at 420 nm wavelength.

did not require SPE equipment or the costs associated with the extraction cartridges, solvents and other associated consumables. The associated labor cost for the extraction steps could also be forgone. Although there are other YES techniques that do not require extraction(Balsiger et al. 2010), we considered the willingness of the principle investigators of the Colosi-Kney study to assist us through the process of method development a key influence in pursuing this method.

Characteristic	Mammalian cell assay In consultation with Dr. Thomas Wiese twiese@xula.edu	YES	YESne
Equipment cost to establish	$50,000+	$15,000	$10,000
Need for solid phase extraction (SPE)	yes	yes	no
Cost for SPE per sample	$50-100	$50-100	0
Assay material cost per sample (4 reps, 3 runs)	$100-200	$35	$35
Assay labor cost per sample ($25/hour) (4 reps, 3 runs)	$500-800	$100	$40
Total cost per sample (4 reps, 3runs)	$650-1,100	$200-300	$75
Level of sophistication required for technician	High	Moderate	Moderate
Sensitivity to matrix effects	Yes	?	Greater than for mammalian cell assay
Sensitivity to E2	0.1parts per trillion	2 parts per trillion	2 parts per trillion
Sensitive to estrogenic compounds besides E2 including E1, EE2, E3, detergents, Bisphenol A, etc.	yes	yes	yes

Table 1. Comparison of Mammalian cell assay and selected Yeast Estrogen Screen assays.

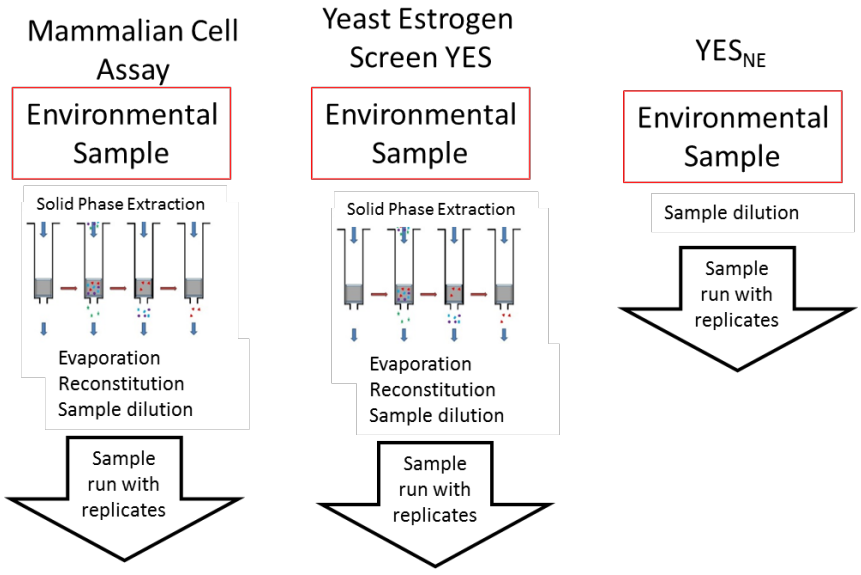


Figure 1. Comparison of selected biological assay complexity.

Materials and Methods

All methods used for our assays were identical to those of Colosi and Kney (Colosi and Kney 2011) and calculations and results were tabulated using Excel™ Spreadsheets provided by these authors. A representative copy of the spreadsheet from one run is presented in Appendix 1. During May – June 2014, both of these authors visited the Barnstable County Department of Health and Environment Laboratory on two occasions and trained a laboratory technician in the methodology. Side by side sample analyses also occurred.

Samples for analyses were obtained from a variety of sources including percolate beneath soil absorption systems and treatment units located at the Massachusetts Alternative Septic System Test Center (MASSTC), area ponds having differing degrees of development on the shoreline, and laboratory distilled and filtered water. All samples from MASSTC or ponds were first collected in pre-cleaned beakers and subsequently filtered in the field under sterile conditions through a 0.2 micron pre-sterilized syringe filter into pre-cleaned amber vials. Samples were stored at temperatures not exceeding $4^{o}C$ during transport to and storage at the laboratory.

Samples were run multiple times and many duplicate runs were conducted in an attempt to develop elements of Quality Assurance Project Plan. The laboratory technician was "blind" to the sample location on many occasions to avoid bias in the interpretation of results.

Results

Representative sample runs are presented in Appendix 2. Over 80 analytical runs were conducted, with approximately 20 performed under the observation of the authors of the method. During many analytical events, using the same standards, the standard curve could not be duplicated (see Runs # 55-58 on 8/25/14 – Appendix 2). This resulted in values from samples taken on the same day with runs using the same standards varying in excess of 30% (see Runs 59 & 60 on 8/26/14 – Appendix 2). Although on some dates the standard curves were consistent (Runs 61 & 62 on 9/3/15 – Appendix 2), this observation was not prevalent. See for instance Run 65 & 66 on 9/8/15 which exhibited highly variable standard curves using the same standards.

No consistent patterns could be observed in the estradiol equivalents between sampling runs of samples from identical locations. For instance figure 2 shows estradiol equivalents from selected percolate samples from soil columns as indicated. These data indicate generally higher attenuation (lower estradiol equivalents) in all four-foot soil columns compared with the six-inch sand depths. This contrasts with

data from some of the same locations (from the same dates) assayed at a different time (figure3) when the sandy loam and loamy sand columns exhibited generally higher estradiol equivalents compared with

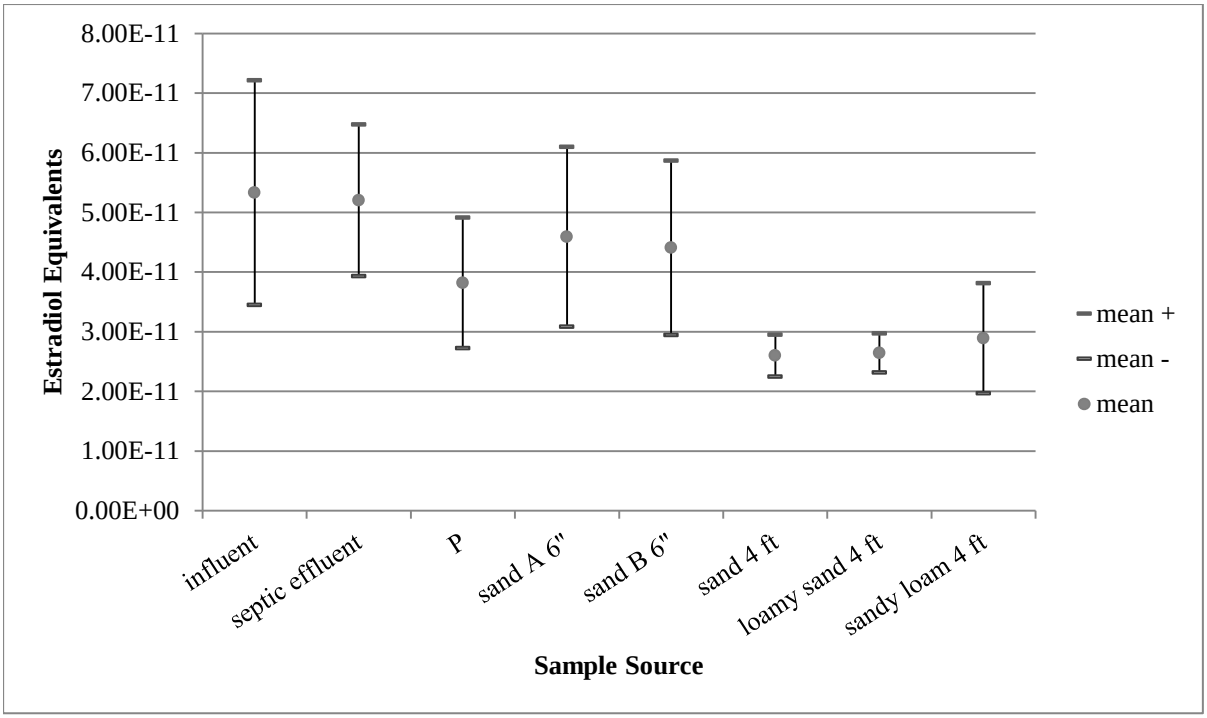


Figure 2. Estradiol equivalents in percolate from selected soil columns collected at the Massachusetts Alternative Septic System Test Center July 9, 2014. Mean and range indicated."P"= sample below 18" of loamy sand.

the six-inch sand depths. These and other inconsistencies in the data raise questions regarding our ability to duplicate the results of Colosi and Kney.

Discussion

Of the many experiments conducted and published using the recombinant yeast strain *Saccharomyces cerevisiae* (originally provided by Professor John P. Sumpter, UK), only one to this author's knowledge (Balsiger et al. 2010) has attempted to detect estrogenic activity of various compounds by exposing the yeast directly to environmental samples. The majority of experiments employ an extraction phase using various methods(Bistan et al. 2012, Lv et al. 2016, 2016)(Chen et al. 2006)(Cabana et al. 2007)(Routledge and Sumpter 1996).

The reasons for our inability to duplicate the repeatability of the Colosi and Kney study are unknown. Those authors indicated that they "introduced under- graduate non-science majors to the topic of endocrine disruptors by their successful completion of laboratory exercises using YES_{NE}(no extraction) to measure estrogenicity of influent ,effluent, and stream water"(Colosi and Kney 2011), however there is no detail given regarding how the success was measured. In the present study, the laboratory technician

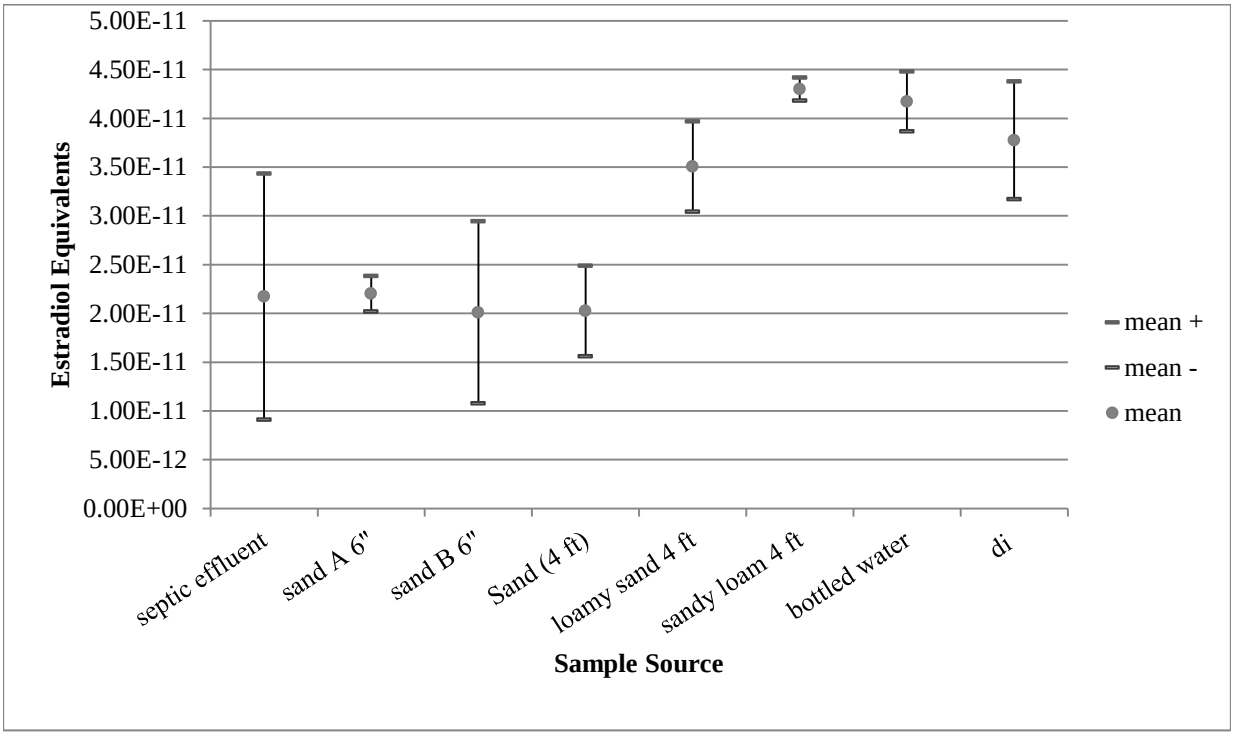


Figure 3. Estradiol equivalents in percolate from selected soil columns collected at the Massachusetts Alternative Septic System Test Center July 9, 2014. Mean and range indicated. DI = distilled water.

was a graduate of a biology program and was tutored by Colosi and Kney in the method. The conditions were controlled in the Barnstable County Department of Health and Environment Laboratory, and record keeping was rigorously performed.

The nature of the influent wastewater in the two studies would appear to be similar. They were both influent samples taken from municipal scale treatment plants. One source of variability may be, however, the wastewater effluent samples. The processes in the Colosi and Kney study were classified as different types of secondary treatment. These plants had short (24 h or less) hydraulic residence times (HRT) compared with the relatively long (generally > 3 days) residence time in the soils-based treatment systems tested. It may also be that the generally higher nitrate values and decreased pH (caused by the nitrification in the soil coincident with low alkalinity) accounted for the difference. Colosi and Kney did posit that high nitrate or phosphate may also induce an abnormal growth rate in the yeast used.

For whatever the cause, our data were not adequate for consistent quantitative comparison of onsite septic system percolate. We had hoped that it would provide a coarse quantitative took so that operational and design adjustments to onsite septic systems could be made in order to optimize the stabilization of estrogenic compounds.

Despite our inability to quantify estrogenicity using this method, the reader should understand that YES assays in general are widely used with success on both environmental and food product testing(Hermanowicz and Wozei 2002)(Beck et al. 2006)(Routledge and Sumpter 1996)(Bazin et al. 2013)(Mazur and others 2000). In the majority of studies, the solid phase extraction with subsequent evaporation and analyte reconstitution was used and produced acceptable reproducibility and adequate recoveries. Since these steps add significantly to the costs of the sample (estimates provided in table 1), our attempt here was to see if the method could produce acceptable results without using them. We can only conclude that, although the authors of the originating work and others, notably Balsiger et al.(Balsiger et al. 2010) who reference the relative ease of this type of assay still rely on a stability of conditions and expertise of analytical staff that we did not have in this project.

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Appendix 1 -

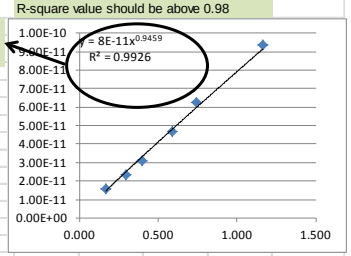
Sample spreadsheet components from a representative sample run. Note four samples assayed for each run and standards and spiked samples are conducted on each run.

Experiment	82	new standards, new yeast, old samples												
Date of collection														
Date of plate incubation		Standard curve				Samples, no spike				Samples plus spike				
Paste	Yeast measurement OD 620nm													
		1	2	3	4	5	6	7	8	9	10	11	12	
OD 620 yeast growth post z-buffer and agitation	A	0.122	0.109	0.127	0.109	0.107	0.110	0.114	0.115	0.197	0.226	0.120	0.209	
	B	0.118	0.113	0.107	0.102	0.105	0.109	0.108	0.112	0.184	0.205	0.151	0.175	
	C	0.119	0.110	0.107	0.104	0.087	0.106	0.104	0.110	0.140	0.122	0.168	0.142	
	D	0.119	0.110	0.110	0.109	0.095	0.115	0.106	0.109	0.136	0.127	0.172	0.153	
	E	0.126	0.105	0.106	0.094	0.091	0.104	0.102	0.107	0.155	0.150	0.176	0.169	
	F	0.126	0.108	0.110	0.109	0.096	0.109	0.106	0.110	0.176	0.161	0.164	0.158	
	G	0.137	0.124	0.122	0.105	0.101	0.113	0.114	0.114	0.118	0.246	0.105	0.133	
	H	0.143	0.124	0.123	0.112	0.117	0.106	0.118	0.127	0.133	0.130	0.141	0.132	
standard deviation	0.032	(The smaller the better)												
minimum	0.067													
maximum	0.246	(Min and max should be close)												
average	0.127													
		Standard curve				Samples, no spike				Samples plus spike				
Paste	OD 414 yellow measurment			No stop										
		1	2	3	4	5	6	7	8	9	10	11	12	
	A	1.539	1.585	1.589	1.549	1.536	1.477	1.496	1.553	0.582	0.544	0.391	0.686	
	B	1.200	1.117	1.134	1.133	1.117	1.177	1.145	1.113	0.596	0.569	0.526	0.577	
	C	1.056	0.956	1.007	0.958	0.965	1.005	0.984	1.008	0.444	0.387	0.514	0.479	
	D	0.783	0.764	0.828	0.829	0.825	0.834	0.770	0.769	0.421	0.411	0.546	0.486	
	E	0.696	0.688	0.726	0.687	0.670	0.690	0.703	0.710	0.501	0.473	0.637	0.616	
	F	0.573	0.575	0.562	0.572	0.511	0.544	0.502	0.471	0.524	0.492	0.683	0.590	
	G	0.387	0.398	0.403	0.408	0.405	0.409	0.398	0.400	0.930	0.824	0.912	0.912	
	H	0.404	0.398	0.405	0.411	0.421	0.410	0.456	0.408	0.921	0.934	0.956	0.922	
blank	0.405	0.405												
minimum	0.387													
maximum	1.589													
Calculated by subtracting blank from each value		Standard curve				Samples, no spike				Samples plus spike				
	OD 414 yellow measurment					minus blank								
		1	2	3	4	5	6	7	8	9	10	11	12	
	A	1.135	1.181	1.185	1.145	1.132	1.073	1.092	1.149	0.178	0.140	-0.014	0.282	
	B	0.796	0.713	0.730	0.729	0.713	0.773	0.741	0.709	0.192	0.165	0.122	0.173	
	C	0.652	0.552	0.603	0.554	0.561	0.601	0.580	0.604	0.040	-0.018	0.110	0.075	
	D	0.379	0.360	0.424	0.425	0.421	0.430	0.366	0.365	0.017	0.006	0.142	0.082	
	E	0.292	0.284	0.322	0.283	0.266	0.286	0.299	0.306	0.097	0.068	0.233	0.212	
	F	0.169	0.171	0.158	0.168	0.107	0.140	0.098	0.066	0.120	0.088	0.279	0.186	
	G	-0.018	-0.007	-0.002	0.003	0.001	0.004	-0.007	-0.005	0.526	0.420	0.508	0.508	
	H	-0.001	-0.007	0.001	0.006	0.017	0.005	0.052	0.003	0.517	0.530	0.552	0.518	
should be 0	blank	0.000												
	blank - blank	0.000												
			A	0.025			A	0.035			A	0.122		
			B	0.037			B	0.030			B	0.030		
			C	0.047			C	0.020			C	0.054		
			D	0.033			D	0.035			D	0.063		
Standard deviations Should be below 0.1			E	0.018			E	0.018			E	0.062		
(standard deviation above .1 suggests bad values)			F	0.006			F	0.030			F	0.084		
Remove bad values before analysis.			G	0.009			G	0.005			G	0.048		
			H	0.005			H	0.022			H	0.016		

Values of samples must be multiplied by multiplier because they were diluted by 1/2 (or more) with 2X medium but values of spike should not be doubled. Values not doubled in this table to estimate revcovery of spike.

Key in coefficient and exponent into I42 and copy to all cells- I68 through M83.

R-square value should be above 0.98



Standard curve

		Estradial Molar	OD Average	OD414 A	B	C	D	OD Average	Estradial Molar
	E4.5	9.38E-11	1.161	1.135	1.181	1.185	1.145	1.161	9.38E-11
	E5	6.25E-11	0.742	0.796	0.713	0.730	0.729	0.742	6.25E-11
	E5.5	4.69E-11	0.590	0.652	0.552	0.603	0.554	0.590	4.69E-11
	E6	3.13E-11	0.397	0.379	0.360	0.424	0.425	0.397	3.13E-11
	E6.5	2.34E-11	0.295	0.292	0.284	0.322	0.283	0.295	2.34E-11
Spike	E7	1.56E-11	0.166	0.169	0.171	0.158	0.168	0.166	1.56E-11
	E8	7.80E-12	-0.006	-0.018	-0.007	-0.002	0.003		7.80E-12
	DMSO	7.81E-12	0.000	-0.001	-0.007	0.001	0.006	0.000	0.00E+00

Edit as needed	Dilution factor		OD414					Estradiol equivalent					(spiked-	((spiked-spike)/
Source	0.5	Spike (E7)?	rep1	rep2	rep3	rep4	average	rep1	rep2	rep3	rep4	Average	no spike)	spike)%
#1 9/23/14	0.5		1.132	1.073	1.092	1.149	1.111	9.00E-11	8.55E-11	8.70E-11	9.13E-11	8.85E-11		
#19/23/14 spike	0.5	yes	0.178	0.140	-0.014	0.282	0.146	1.54E-11	1.22E-11	#NUM!	2.38E-11	1.28E-11	-7.57E-11	-485.25%
#1 9/23/14	0.5		0.713	0.773	0.741	0.709	0.734	5.79E-11	6.25E-11	6.00E-11	5.76E-11	5.95E-11		
#1 9/23/14 spike	0.5	yes	0.192	0.165	0.122	0.173	0.163	1.65E-11	1.43E-11	1.07E-11	1.49E-11	1.41E-11	-4.54E-11	-291.00%
#2 9/23/14	0.5		0.561	0.601	0.580	0.604	0.586	4.60E-11	4.92E-11	4.75E-11	4.94E-11	4.80E-11		
#2 9/23/14 spike	0.5	yes	0.040	-0.018	0.110	0.075	0.052	3.66E-12	#NUM!	9.68E-12	6.70E-12	4.71E-12	-4.33E-11	-277.65%
#2 9/23/14	0.5		0.421	0.430	0.366	0.365	0.395	3.50E-11	3.57E-11	3.06E-11	3.05E-11	3.30E-11		
#2 9/23/14 spike	0.5	yes	0.017	0.006	0.142	0.082	0.062	1.59E-12	6.53E-13	1.24E-11	7.30E-12	5.58E-12	-2.74E-11	-175.46%
#3 9/23/14	0.5		0.266	0.286	0.299	0.306	0.289	2.25E-11	2.42E-11	2.52E-11	2.58E-11	2.44E-11		
#3 9/23/14 spike	0.5	yes	0.097	0.068	0.233	0.212	0.152	8.58E-12	6.18E-12	1.99E-11	1.81E-11	1.33E-11	-1.12E-11	-71.62%
#3 9/23/14	0.5		0.107	0.140	0.098	0.066	0.103	9.43E-12	1.22E-11	8.66E-12	6.01E-12	9.09E-12		
#3 9/23/14 spike	0.5	yes	0.120	0.088	0.279	0.186	0.168	1.05E-11	7.81E-12	2.36E-11	1.60E-11	1.45E-11	5.46E-12	34.99%
#4 9/23/14	0.5		0.001	0.004	-0.007	-0.005	-0.002	5.64E-14	4.59E-13	#NUM!	#NUM!	#NUM!		
#4 9/23/14 spike	0.5	yes	0.526	0.420	0.508	0.508	0.490	4.33E-11	3.49E-11	4.19E-11	4.19E-11	4.05E-11	#NUM!	#NUM!
#4 9/23/14	0.5		0.017	0.005	0.052	0.003	0.019	1.59E-12	5.56E-13	4.71E-12	3.61E-13	1.84E-12		
#4 9/23/14 spike	0.5	yes	0.517	0.530	0.552	0.518	0.529	4.26E-11	4.36E-11	4.53E-11	4.26E-11	4.35E-11	4.17E-11	267.26%

Values multiplied by multiplier (inverse of dilution factor) to get true estimate of concentration

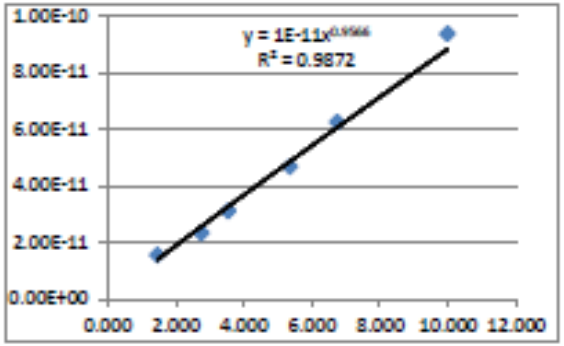
			uncorrected			Kney correction		
Experiment	82		Est. equiv. Parts per trillion			Est. equiv. Parts per trillion		
Date of collection	0		Average	% spike recovery		Average	% spike recovery	
Date of plate incubation	01/00/00							
	Multiplier							
#1 9/23/14	2		1.77E-10	48.1	-485.3%	1.80E-10	49.1	-533.7%
#2 9/23/14	2		1.19E-10	32.4	-291.0%	1.25E-10	33.9	-340.6%
#3 9/23/14	2		9.60E-11	26.1	-277.7%	1.07E-10	29.2	-321.9%
#4 9/23/14	2		6.59E-11	17.9	-175.5%	7.06E-11	19.2	-200.8%
#3 9/23/14	2		4.89E-11	13.3	-71.6%	5.47E-11	14.9	-116.5%
#REF!	2		1.82E-11	4.9	35.0%	1.96E-11	5.3	2.8%
#4 9/23/14	2		#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#REF!	2		3.68E-12	1.0	267.3%	3.55E-12	1.0	227.0%

Choose the set with % spike recoveries closest to 100%
% recoveries below 60% and above 130% are far from 100%
#NUM is negative, take it as zero

	Dilution		Yeast growth OD620				
Samples	factor	Spike 07	rep1	rep2	rep3	rep4	average
#1 9/23/14	0.5		0.107	0.110	0.114	0.115	0.112
#19/23/14 spike	0.5	yes	0.197	0.226	0.120	0.209	0.188
#2 9/23/14	0.5		0.105	0.109	0.108	0.112	0.109
#2 9/23/14 spike	0.5	yes	0.184	0.205	0.151	0.175	0.179
#3 9/23/14	0.5		0.087	0.106	0.104	0.110	0.102
#3 9/23/14 spike	0.5	yes	0.140	0.122	0.168	0.142	0.143
#4 9/23/14	0.5		0.095	0.115	0.106	0.109	0.106
#4 9/23/14 spike	0.5	yes	0.136	0.127	0.172	0.153	0.147
#3 9/23/14	0.5		0.091	0.104	0.102	0.107	0.101
#3 9/23/14 spike	0.5	yes	0.155	0.150	0.176	0.169	0.163
#REF!	0.5		0.096	0.109	0.106	0.110	0.105
#REF!	0.5	yes	0.176	0.161	0.164	0.158	0.165
#4 9/23/14	0.5		0.101	0.113	0.114	0.114	0.111
#4 9/23/14 spike	0.5	yes	0.118	0.246	0.105	0.133	0.151
#REF!	0.5		0.117	0.106	0.118	0.127	0.117
#REF!	0.5	yes	0.133	0.130	0.141	0.132	0.134
Standard Curve			rep1	rep2	rep3	rep4	average
S4.5	1		0.122	0.109	0.127	0.109	0.117
S5	1		0.118	0.113	0.107	0.102	0.110
S5.5	1		0.119	0.110	0.107	0.104	0.110
S6	1		0.119	0.110	0.110	0.109	0.112
S6.5	1		0.126	0.105	0.106	0.094	0.108
S7	1		0.126	0.108	0.110	0.109	0.113
S8	1		0.137	0.124	0.122	0.105	0.122
DMSO	1		0.143	0.124	0.123	0.112	0.126

	These should all be about the same
Average all samples	0.133
Average of all without spikes	0.108
Average of all with spikes	0.159
Average sample 1	0.150
Average sample 2	0.144
Average sample 3	0.122
Average sample 4	0.127
Average sample 5	0.132
Average sample 6	0.135
Average sample 7	0.131
Average water	0.126
Average for standard curve	0.115

Key in coefficient and exponent into Y68 and copy to all cells- Y68 through AC83.



Kney correction (adjusts for yeast growth)

Standard curve

	Estradial Molar	OD Average	OD414/OD620 A	B	C	D	OD Average	Estradial Molar
E4.5	9.38E-11	9.989	9.299	10.830	9.327	10.500	9.989	9.38E-11
E5	6.25E-11	6.752	6.742	6.305	6.818	7.142	6.752	6.25E-11
E5.5	4.69E-11	5.360	5.475	5.014	5.631	5.322	5.360	4.69E-11
E6	3.13E-11	3.548	3.181	3.268	3.850	3.894	3.548	3.13E-11
E6.5	2.34E-11	2.763	2.313	2.700	3.033	3.005	2.763	2.34E-11
Spike E7	1.56E-11	1.471	1.337	1.579	1.432	1.537	1.471	1.56E-11
E8	7.80E-12	-0.040	-0.128	-0.052	-0.012	0.033		7.80E-12
DMSO	7.81E-12	0.002	-0.003	-0.052	0.004	0.058	0.002	0.00E+00

Dilution factor	Spike(E7)?	OD420/OD620 rep1	rep2	rep3	rep4	average	Estradiol equivalent rep1	rep2	rep3		(spiked-Average	spike)/no spike)	spike)%
0.5		10.575	9.750	9.575	9.987	9.972	9.55E-11	8.83E-11	8.68E-11	9.04E-11	9.02E-11		
0.5	yes	0.901	0.617	-0.113	1.347	0.688	9.05E-12	6.30E-12	#NUM!	1.33E-11	6.99E-12	-8.32E-11	-533.65%
0.5		6.786	7.087	6.856	6.326	6.764	6.24E-11	6.51E-11	6.31E-11	5.84E-11	6.23E-11		
0.5	yes	1.041	0.802	0.805	0.986	0.908	1.04E-11	8.10E-12	8.12E-12	9.86E-12	9.12E-12	-5.31E-11	-340.59%
0.5		6.443	5.665	5.572	5.486	5.792	5.94E-11	5.25E-11	5.17E-11	5.10E-11	5.37E-11		
0.5	yes	0.282	-0.143	0.652	0.525	0.329	2.98E-12	#NUM!	6.64E-12	5.40E-12	3.45E-12	-5.02E-11	-321.89%
0.5		4.426	3.735	3.448	3.344	3.738	4.15E-11	3.53E-11	3.27E-11	3.17E-11	3.53E-11		
0.5	yes	0.121	0.051	0.823	0.533	0.382	1.33E-12	5.82E-13	8.30E-12	5.47E-12	3.98E-12	-3.13E-11	-200.78%
0.5		2.918	2.745	2.926	2.855	2.861	2.79E-11	2.63E-11	2.79E-11	2.73E-11	2.73E-11		
0.5	yes	0.623	0.457	1.321	1.251	0.913	6.36E-12	4.72E-12	1.31E-11	1.24E-11	9.17E-12	-1.82E-11	-116.47%
0.5		1.109	1.280	0.920	0.605	0.978	1.10E-11	1.27E-11	9.23E-12	6.18E-12	9.79E-12		
0.5	yes	0.679	0.543	1.698	1.174	1.024	6.90E-12	5.58E-12	1.66E-11	1.17E-11	1.02E-11	4.33E-13	2.78%
0.5		0.005	0.040	-0.057	-0.039	-0.013	6.23E-14	4.58E-13	#NUM!	#NUM!	#NUM!		
0.5	yes	4.453	1.705	4.633	3.816	3.702	4.17E-11	1.67E-11	4.51E-11	3.60E-11	3.50E-11	#NUM!	#NUM!
0.5		0.141	0.052	0.436	0.028	0.164	1.54E-12	5.90E-13	4.52E-12	3.22E-13	1.78E-12		
0.5	yes	3.883	4.073	3.911	3.920	3.947	3.66E-11	3.83E-11	3.69E-11	3.69E-11	3.72E-11	3.54E-11	227.00%

Appendix 2

Representative run data including the regression curve, correlation coefficient and comments regarding consistency.

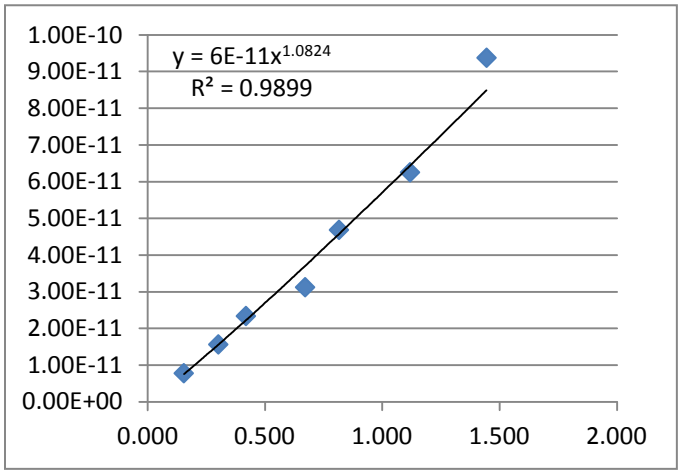
Run Number: 53

Date of Run: 08/13/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Potts 1-6 8/12/14	#NUM!	#NUM!
2	alpha 8/12/14	8.52E-11	23.2
3	beta 8/12/14	7.51E-11	20.4
4	column sand 8/12/14	#NUM!	#NUM!
5	loamy sand 8/12/14	#NUM!	#NUM!
6	sandy loam 8/12/14	2.27E-11	6.2
7	septic effluent 8/12/14	#NUM!	#NUM!
8	channel influent	1.01E-10	27.4

Standard Curve:



Comments:
#NUM! Indicates values were below blank values, essentially zero

Interpretation: In this set of samples the relative values appear in line with intuition. Influent is the highest level, with systems having six inches of sand beneath point of influent distribution having less by ~ 20%, the four-foot sand columns (sand and 70:30 mix) having non-detect. The problem area is that POTTS1-6 which is a 60:40 mix has negligible numbers with only 18 inches of this material beneath the point of influent distribution does not compare correctly with the "sandy loam" above which is four feet of the same material. It should have had less than the 18 inches, but showed a 6.2 ppt value.

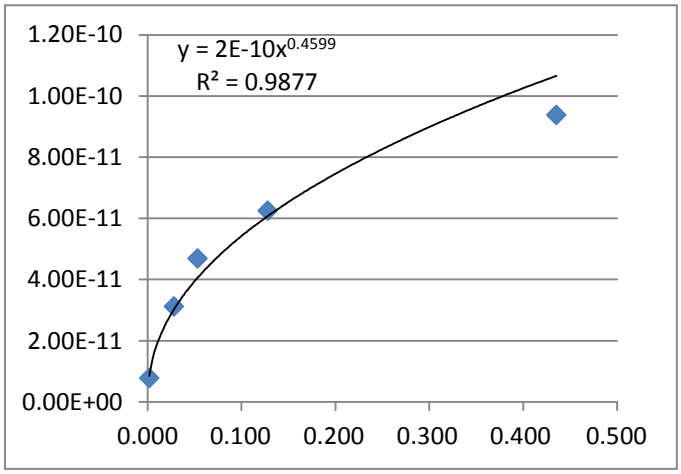
Run Number: 55

Date of Run: 08/25/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	trickling filter 8/25/14	2.31E-10	62.8
2	peat filter 8/25/14	2.56E-10	69.6
3	Potts 1-6 8/25/14	2.22E-10	60.5
4	alpha 8/25/14	2.88E-10	78.4
5	beta 8/25/14	2.83E-10	77.1
6	beta 8/25/14	2.84E-10	77.2
7	influent 8/25/14	3.05E-10	82.9
8	septic tank effluent 8/25/14	#NUM!	#NUM!

Standard Curve:



Comments:
#NUM! Indicates values were below blank values, essentially zero
Standard values E6.5 and E8 were removed from standards graph due to reported values being less than the blank

Interpretation:
In this run, again the technologies with minimal soil passage (six inches of sand) have some reductions in Estradiol Equivalents (EE), but only ~6% reduction compared to the influent. The septic tank effluent, which immediately precedes the soil absorption system, had no detectable EE, which does not compare with all the distal treatment components (trickling filter, peat, soils systems etc.) which have values of 20% less than the influent.

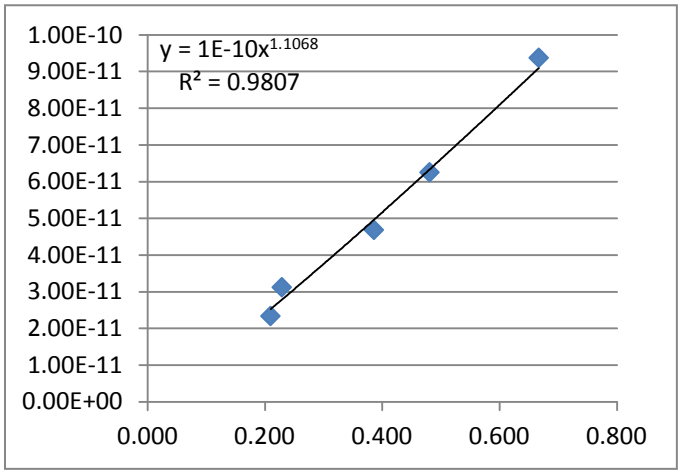
Run Number: 56

Date of Run: 08/25/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	trickling filter 8/25/14	8.73E-11	23.8 (48%)
2	peat filter 8/25/14	9.68E-11	26.3 (42%)
3	Potts 1-6 8/25/14	7.92E-11	21.6 (53%)
4	alpha 8/25/14	1.45E-10	39.5 (13%)
5	beta 8/25/14	1.42E-10	38.7 (14%)
6	beta 8/25/14	1.45E-10	39.5
7	influent 8/25/14	1.67E-10	45.5
8	septic tank effluent 8/25/14	7.99E-12	2.2

Standard Curve:



Comments:
Blank value removed from consideration due to resulting negative sample values
Standard values E7 and E8 were removed from standards graph due to outlying values

Interpretation:
These results indicate removal in six inches of sand (13-14%), with better removal in the 60:40 sand mix of Potts (53%) and that performance is better than the trickling filter and the peat filter similar to Run #55. Note that septic tank effluent is still quite low which is counter-intuitive since the reductions from influent to other treatment are not that low.

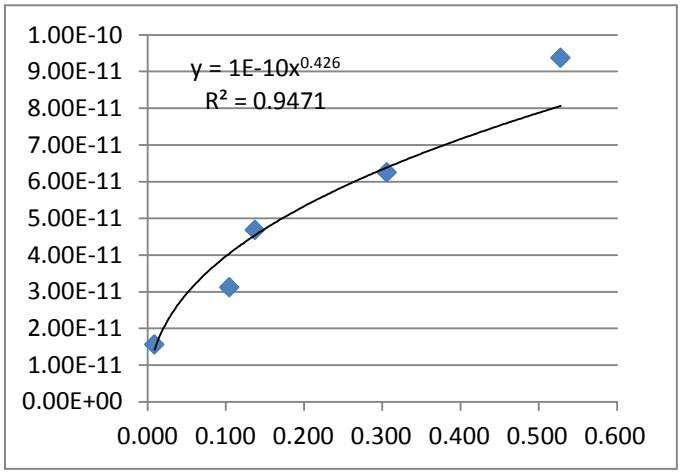
Run Number: 57

Date of Run: 08/25/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	trickling filter 8/25/14	1.28E-10	34.9 (26%)
2	peat filter 8/25/14	1.28E-10	34.7 (26%)
3	Potts 1-6 8/25/14	1.04E-10	28.4 (40%)
4	alpha 8/25/14	1.67E-10	45.3 (4%)
5	beta 8/25/14	1.74E-10	47.2
6	beta 8/25/14	1.53E-10	41.7 (11%)
7	influent 8/25/14	1.74E-10	47.2
8	septic tank effluent 8/25/14	#NUM!	#NUM!

Standard Curve:



Comments:
Standard values E7 and E8 were removed from standards graph due to outlying values, and to improve the R^2 (formerly 0.84)

#NUM! indicates values were below blank values, essentially zero

Interpretation:
This run showed less removal in the six inches of soil (alpha-beta); however relatively better removal in the trickling filter, Potts1-6, and Peat systems which are consistent with Run # 56. Again, septic tank effluent had not detected levels which is counter intuitive since treatment paths that are distal to a septic tank have readable values.

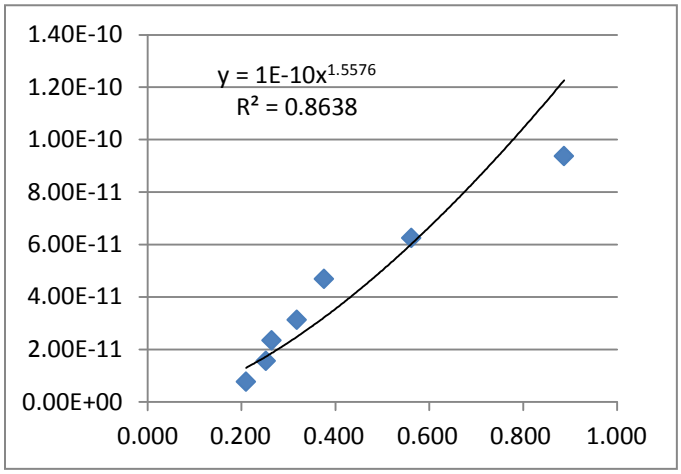
Run Number: 58 adjusted

Date of Run: 08/25/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	trickling filter 8/25/14	8.84E-11	24.0
2	peat filter 8/25/14	8.65E-11	23.5
3	Potts 1-6 8/25/14	7.14E-11	19.4
4	alpha 8/25/14	1.48E-10	40.3
5	beta 8/25/14	1.36E-10	36.9
6	beta 8/25/14	1.26E-10	34.2
7	influent 8/25/14	2.03E-10	55.1
8	septic tank effluent 8/25/14	2.26E-12	0.6

Standard Curve:



Comments:
Blank value removed from consideration due to resulting negative sample values

Interpretation:
This run is very consistent with Run #56 and show that In order of removal, the trickling filter, the peat filter and the Potts1-6 (which is 18 inches of 60:40 sand mix beneath the point of septic tank effluent distribution) remove higher levels of EE than the alpha and beta which are six inches of sand beneath the point of wastewater dispersal. Again, septic tank effluent has inconsistently low levels of EE

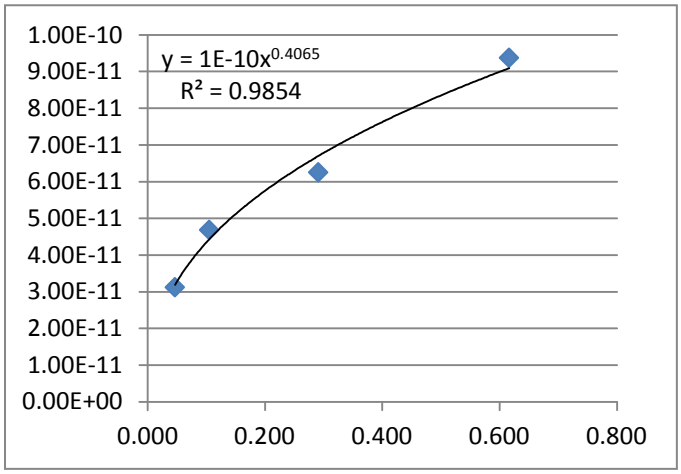
Run Number: 58

Date of Run: 08/25/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	trickling filter 8/25/14	1.26E-10	34.3
2	peat filter 8/25/14	1.25E-10	33.9
3	Potts 1-6 8/25/14	1.13E-10	30.7
4	alpha 8/25/14	1.57E-10	42.8
5	beta 8/25/14	1.52E-10	41.3
6	beta 8/25/14	1.47E-10	40.1
7	influent 8/25/14	1.77E-10	48.1
8	septic tank effluent 8/25/14	#NUM!	#NUM!

Standard Curve:



Comments:

Standard values E6.5 and E8 were removed from standards graph due to negative values, reported values being less than the blank

#NUM! indicates values were below blank values, essentially zero

Interpretation:

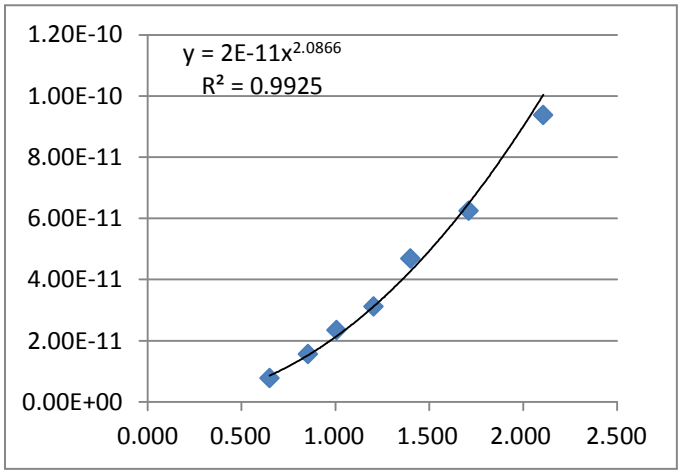
Run Number: 59

Date of Run: 08/26/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	bottled water 8/4	7.79E-11	21.2
2	alpha 7/9/14	4.72E-11	12.8
3	column sand 7/9/14	4.87E-11	13.2
4	loamy sand 7/9/14	7.80E-11	21.2
5	sandy loam 7/9/14	8.75E-11	23.8
6	septic influent 7/9/14	6.56E-11	17.8
7	beta 7/9/14	5.66E-11	15.4
8	DI	8.59E-11	23.4

Standard Curve:



Comments:

Blank value removed from consideration due to resulting negative sample values

Interpretation:

These samples show some inconsistencies. “DI” is distilled water from the laboratory and “bottled water” have higher levels than septic tank influent.

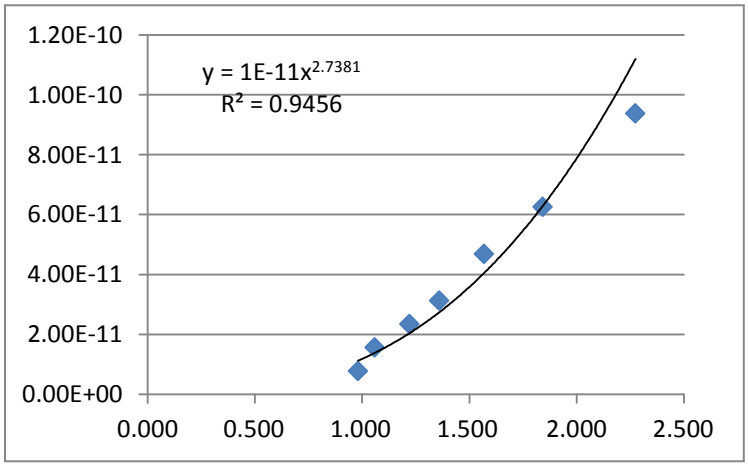
Run Number: 60

Date of Run: 08/26/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	bottled water 8/4	2.74E-11	7.5
2	alpha 7/9/14	1.65E-11	4.5
3	column sand 7/9/14	1.63E-11	4.4
4	loamy sand 7/9/14	2.52E-11	6.9
5	sandy loam 7/9/14	3.66E-11	10.0
6	septic influent 7/9/14	2.86E-11	7.8
7	beta 7/9/14	2.40E-11	6.5
8	DI	3.24E-11	8.8

Standard Curve:



Comments:

Blank value removed from consideration due to resulting negative sample and standard values

Interpretation:
This run shows many of the relative comparisons as Run #59; however the DI water still has a higher EE than septic tank influent which is counter intuitive. The absolute values range from 33-34% of the absolute values from Run #59 underscoring an issue with using the measurements.

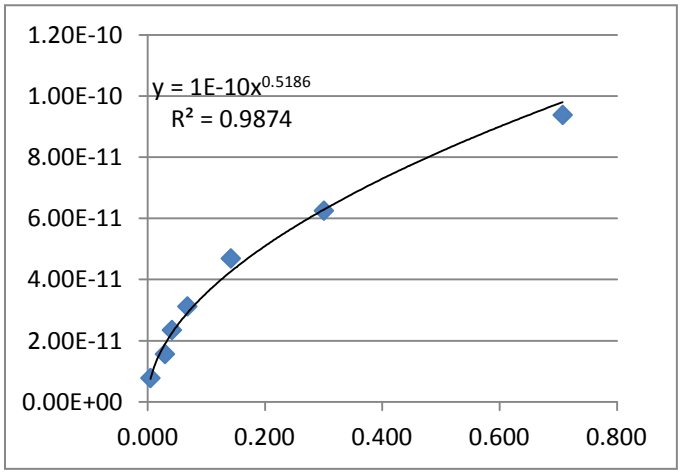
Run Number: 61

Date of Run: 09/03/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	8/25/2014 influent	1.53E-10	41.5
2	8/25/2014 influent	1.56E-10	42.5
3	5/5/2014 influent	1.47E-10	40.0
4	5/5/2014 influent	1.37E-10	37.3
5	8/12/2014 influent	1.69E-10	46.0
6	8/12/2014 influent	1.71E-10	46.4
7	6/8/2014 influent	1.32E-10	35.9
8	6/8/2014 influent	1.37E-10	37.3

Standard Curve:



Comments:

Interpretation:
This run was to determine the consistency in the method. The influent was used. The average value for EE was 40.9 ppt (38.1 – 43.6, p=.05) which appears very consistent for this single run and it appears that the influent is fairly constant over time. Paired samples were run in Run #62 from these same four sampling dates.

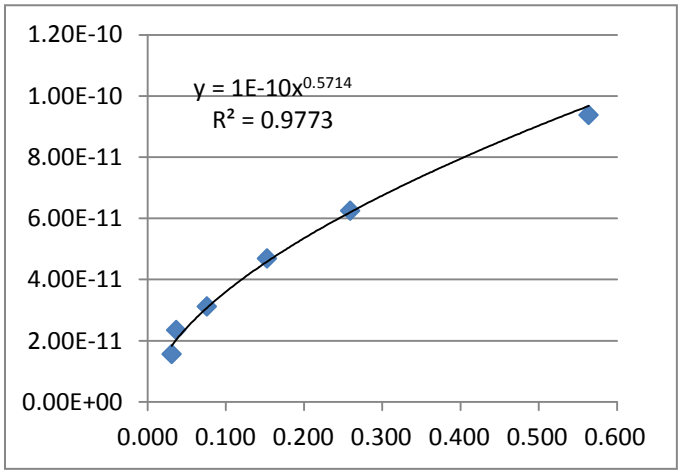
Run Number: 62

Date of Run: 09/03/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	8/25/2014 influent	1.52E-10	41.2
2	8/25/2014 influent	1.53E-10	41.6
3	5/5/2014 influent	1.18E-10	32.0
4	5/5/2014 influent	1.24E-10	33.6
5	8/12/2014 influent	1.41E-10	38.3
6	8/12/2014 influent	1.44E-10	39.2
7	6/8/2014 influent	1.05E-10	28.5
8	6/8/2014 influent	1.12E-10	30.3

Standard Curve:



Comments:
Standard value E8 removed from standards graph due to reported value being less than the blank

Interpretation:

This sampling run suggest that the freshest (collected 8/25) samples exhibit the least variability in results (standard deviation =0.6), compared with the other pairs collected on May 5, August 12 and June 8 (st deviation 3.6, 4.3 and 4.5 respectively).

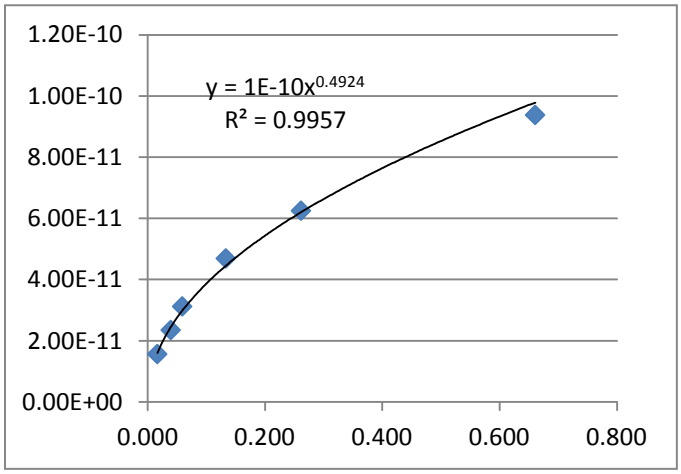
Run Number: 63

Date of Run: 09/03/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	8/25 alpha	1.88E-10	51.1
2	8/25 alpha	1.83E-10	49.7
3	7/9 alpha	1.90E-10	51.7
4	6/12 alpha	2.29E-10	62.4
5	5/5 alpha	1.81E-10	49.4
6	8/25 beta	1.75E-10	47.7
7	8/12 beta	1.68E-10	45.7
8	7/9 beta	1.70E-10	46.3

Standard Curve:



Comments:
Standard value E8 removed from standards graph due to reported value being less than the blank

Interpretation:

SEE RUN #64

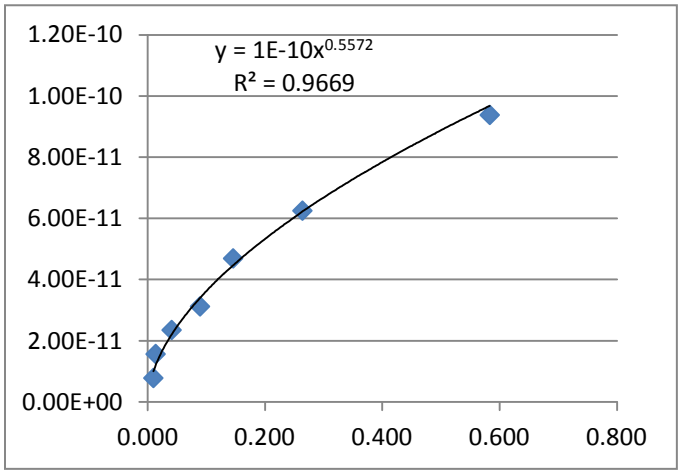
Run Number: 64

Date of Run: 09/03/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	8/25 alpha	1.62E-10	43.9
2	8/25 alpha	1.62E-10	43.9
3	7/9 alpha	1.64E-10	44.6
4	6/12 alpha	2.11E-10	57.3
5	5/5 alpha	1.70E-10	46.2
6	8/25 beta	1.63E-10	44.2
7	8/12 beta	1.56E-10	42.4
8	7/9 beta	1.63E-10	44.3

Standard Curve:



Comments:
Blank value removed from consideration due to negative sample results

Interpretation:
Runs #63 and #64 were conducted gain to determine the variability and stability of the method. The average difference between the duplicated samples was 4.7 (range 2.0 – 7.2). However the ALPHA and BETA run on 8/13 for the samples taken on 8/12 were 23.2 and 20.4 respectively (see Run # 53), and the ALPHA and BETA run on 8/25 (Run #55) indicate 77.1 and 77.2 EE respectively. This shows some inconsistency across analysis dates for the same sample. In one instance the numbers were higher when run closer to the collection date and in the other instance they were lower.

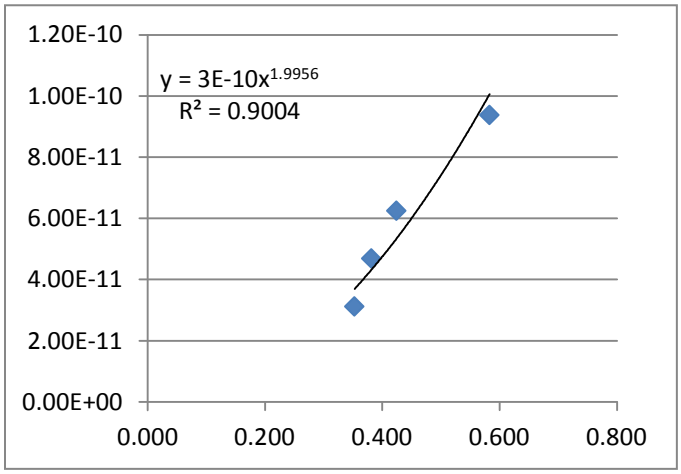
Run Number: 65

Date of Run: 09/08/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster pond 9/8/14	2	14.9
2	Round pond 9/8/14	2	14.6
3	Shiverick's pond 9/8/14	2	14.3
4	6/12 alpha	2	54.1
5	column sand 9/8/14	2	37.3
6	loamy sand 9/8/14	2	14.6
7	sandy loam 9/8/14	2	16.7
8	alpha 9/8/14	2	43.1

Standard Curve:



Comments:

Standard values E6.5, E7, and E8 were removed from standards graph due to negative values, reported values being less than the blank

Interpretation:

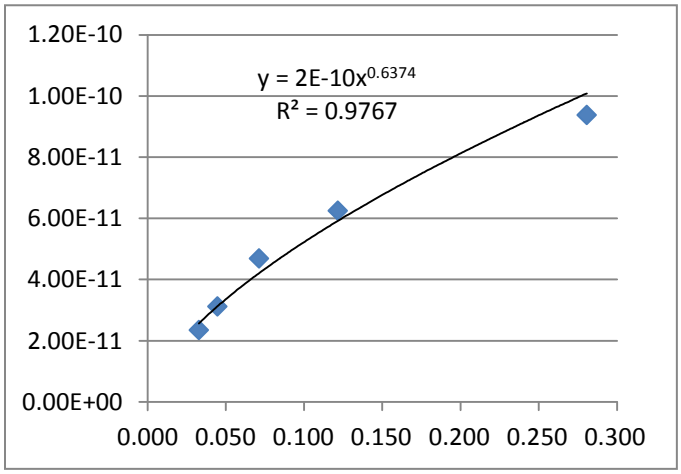
Run Number: 66

Date of Run: 09/08/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster pond 9/8/14	#NUM!	#NUM!
2	Round pond 9/8/14	1.28E-11	3.5
3	Shiverick's pond 9/8/14	2.29E-11	6.2
4	6/12 alpha	1.91E-10	52.0
5	column sand 9/8/14	1.43E-10	38.8
6	loamy sand 9/8/14	#NUM!	#NUM!
7	sandy loam 9/8/14	2.19E-11	6.0
8	alpha 9/8/14	1.71E-10	46.5

Standard Curve:



Comments:

Standard values E7 and E8 were removed from standards graph due to outlying values
#NUM! indicates values were below blank values, essentially zero

Interpretation:

These values are the first samples from ponds in the area. The values from ALHA and BETA seem consistent with previous values. The R^2 is good but the sand column is much higher than previously reported (Run #53, #59, #60), but the sandy loam (60:40 mix) value nearly matches the Run #53.

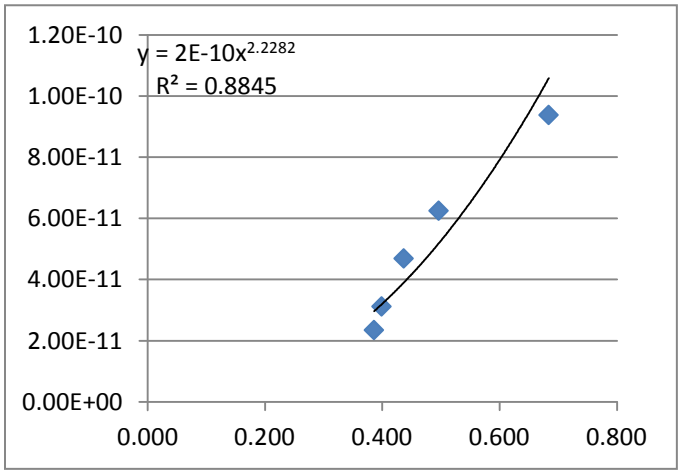
Run Number: 67 adjusted

Date of Run: 09/09/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	oyster pond 9/8/14	2.23E-11	6.1
2	round pond 9/8/14	2.63E-11	7.2
3	Shiverick's pond 9/8/14	2.46E-11	6.7
4	6/12 alpha	2.47E-10	67.2
5	column sand 9/8/14	6.28E-11	17.1
6	loamy sand 9/8/14	2.45E-11	6.7
7	sandy loam 9/8/14	2.66E-11	7.2
8	alpha 9/8/14	8.29E-11	22.6

Standard Curve:



Comments:
Standard values E7 and E8 were removed from standards graph due to outlying values and to improve R^2 (formerly 0.5603)
Blank value removed from consideration due to negative sample results

Interpretation:

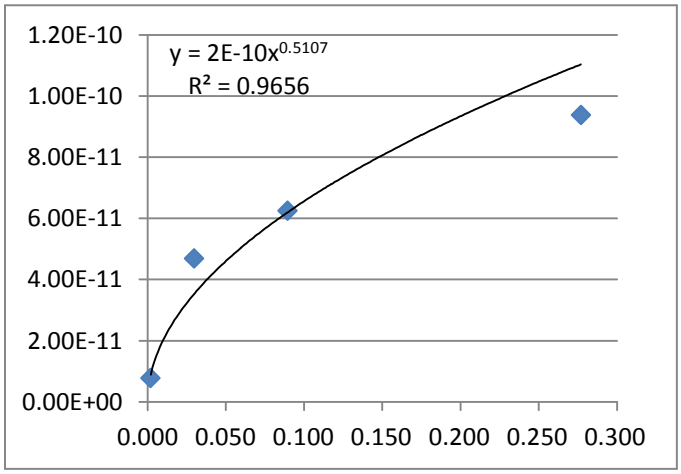
Run Number: 67

Date of Run: 09/09/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster pond 9/8/14	#NUM!	#NUM!
2	Round pond 9/8/14	#NUM!	#NUM!
3	Shiverick's pond 9/8/14	#NUM!	#NUM!
4	6/12 alpha	2.50E-10	68.1
5	column sand 9/8/14	6.56E-11	17.8
6	loamy sand 9/8/14	#NUM!	#NUM!
7	sandy loam 9/8/14	#NUM!	#NUM!
8	alpha 9/8/14	1.15E-10	31.2

Standard Curve:



Comments:
#NUM! Indicates values were below blank values, essentially zero
Standard values E6, E6.5, and E7 were removed from standards graph due to negative values, reported values being less than the blank

Interpretation:
This run was nearly identical to the previous day's run, however the numbers are lower (with the exception of 6/12 ALPHA).

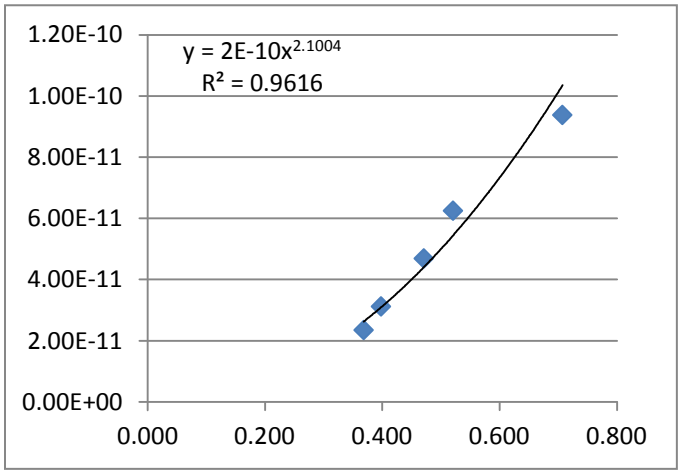
Run Number: 68 adjusted

Date of Run: 09/10/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster pond 9/8/14	2.61E-11	7.1
2	Round pond 9/8/14	2.53E-11	6.9
3	Shiverick's pond 9/8/14	2.32E-11	6.3
4	6/12 alpha	2.57E-11	7.0
5	column sand 9/8/14	6.65E-11	18.1
6	loamy sand 9/8/14	2.67E-11	7.3
7	sandy loam 9/8/14	2.99E-11	8.1
8	alpha 9/8/14	7.91E-11	21.5

Standard Curve:



Comments:

Blank value removed from consideration due to negative sample results
Standard values E7 and E8 were removed from standards graph due to outlying values

Interpretation:

With this run, the values return to levels comparable with Run #66, however the numbers for ALPHA 9/8/14, ALPHA 6/12/14 and column sand (9/8/14) are less than half of that observed in Run #66.

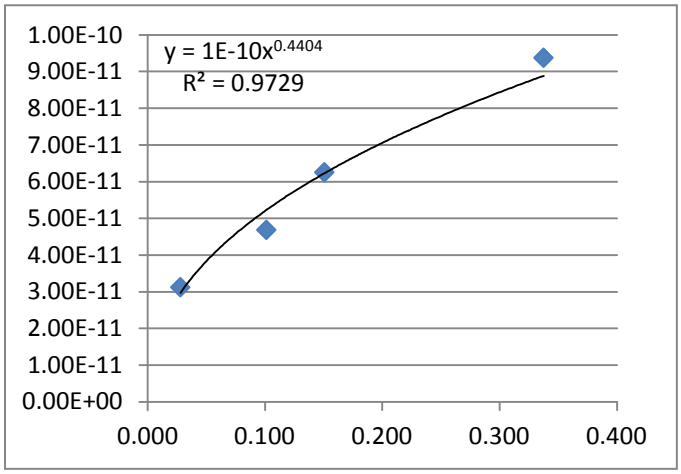
Run Number: 68

Date of Run: 09/10/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster pond 9/8/14	#NUM!	#NUM!
2	Round pond 9/8/14	#NUM!	#NUM!
3	Shiverick's pond 9/8/14	#NUM!	#NUM!
4	6/12 alpha	#NUM!	#NUM!
5	column sand 9/8/14	5.61E-11	15.3
6	loamy sand 9/8/14	#NUM!	#NUM!
7	sandy loam 9/8/14	#NUM!	#NUM!
8	alpha 9/8/14	7.01E-11	19.1

Standard Curve:



Comments:
#NUM! Indicates values were below blank values, essentially zero
Standard values E6.5, E7, and E8 were removed from standards graph due to negative values, reported values being less than the blank

Interpretation:

If we questionably eliminate three of the standards from the curve, the curve looks more like the "typical" curve and the values are all lower than Run #66.

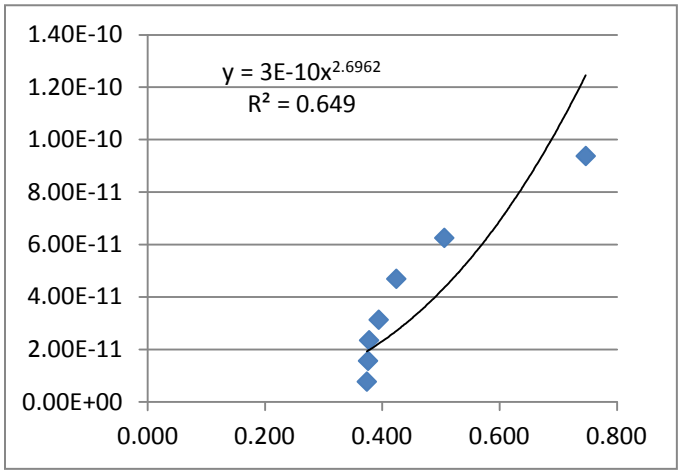
Run Number: 69

Date of Run: 09/10/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	oyster pond 9/8/14	1.21E-11	3.3
2	round pond 9/8/14	2.29E-11	6.2
3	Shiverick's pond 9/8/14	1.76E-11	4.8
4	6/12 alpha	1.73E-11	4.7
5	column sand 9/8/14	6.60E-11	17.9
6	loamy sand 9/8/14	1.46E-11	4.0
7	sandy loam 9/8/14	2.15E-11	5.8
8	alpha 9/8/14	8.10E-11	22.0

Standard Curve:



Comments:
Blank value removed from consideration due to negative sample results

Interpretation:

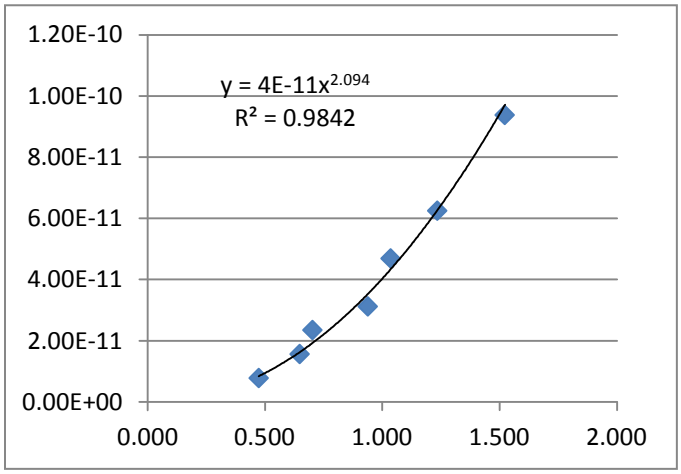
Run Number: 70

Date of Run: 09/12/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster pond 9/8/14	1.08E-11	2.9
2	Round pond 9/8/14	9.07E-12	2.5
3	column sand 9/7/14	3.24E-11	8.8
4	Jenkins pond 9/8/14	6.38E-12	1.7
5	Shiverick's pond 9/8/14	5.89E-12	1.6
6	alpha 9/8/14	5.13E-11	14.0
7	sterile Di	1.03E-11	2.8
8	Sterile DI	1.11E-11	3.0

Standard Curve:



Comments:
Blank value was removed from consideration due to resulting negative sample values.

Interpretation:

The Standard Curve looks good. No standards are excluded. The ALPHA value is one half of Run #67 which is perplexing.

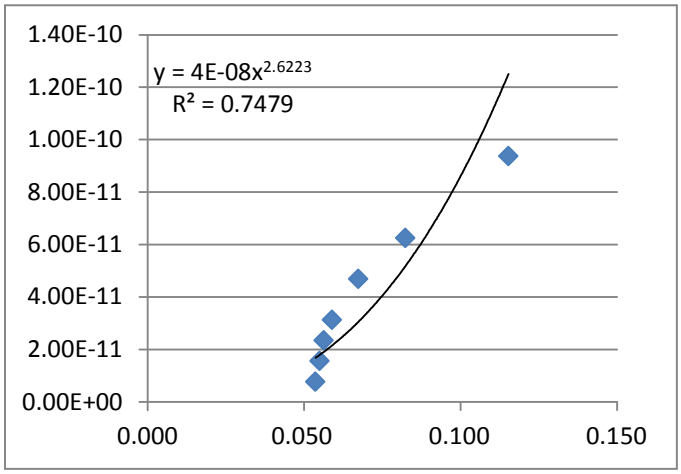
Run Number: 71

Date of Run: 09/16/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	alpha9/16/14	1.03E-10	28.0
2	septic effluent 9/16/14	7.03E-11	19.1
3	influent 9/16/14	6.59E-11	17.9
4	column sand 9/16/14	6.25E-11	17.0
5	loamy sand 9/16/14	6.09E-11	16.6
6	sandy loam 9/16/14	7.87E-11	21.4
7	Potts 1-6 9/16/14	6.00E-11	16.3
8	influent 9/16/14	6.51E-11	17.7

Standard Curve:



Comments:
Experiment was run using the micro-centrifuge tube protocol
Blank value was removed from consideration due to resulting negative sample values.

Some kind of contamination noted in the yeast activity: Yeast growth was very good, yellow color did not develop. Incubation continued until yellow developed and a pattern was discerned

Interpretation:

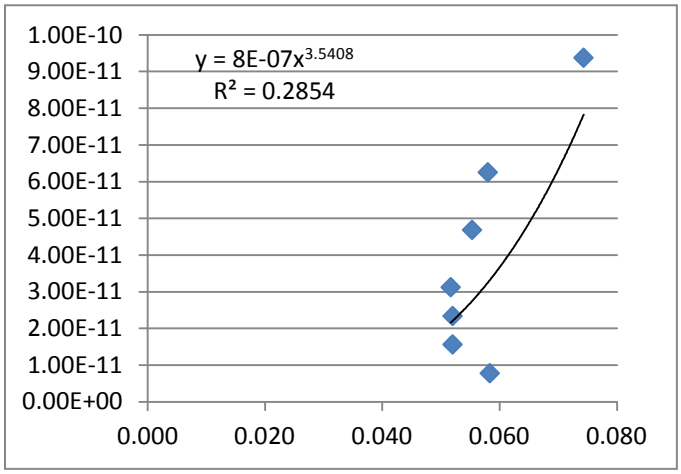
Run Number: 72

Date of Run: 09/17/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	oyster pond 9/8/14	7.85E-11	21.3
2	round pond 9/8/14	7.70E-11	20.9
3	Shiverick's pond 9/8/14	7.11E-11	19.3
4	Jenkins pond 9/8/14	4.76E-11	12.9
5	column sand 9/8/14	1.32E-10	36.0
6	loamy sand 9/8/14	6.16E-11	16.8
7	sandy loam 9/8/14	4.25E-11	11.5
8	alpha 9/8/14	5.79E-11	15.7

Standard Curve:



Comments:
Experiment was run using the micro-centrifuge tube protocol
Blank value was removed from consideration due to resulting negative values.

Some kind of contamination noted in the yeast activity: yeast growth was very good, yellow color did not develop.

Interpretation:

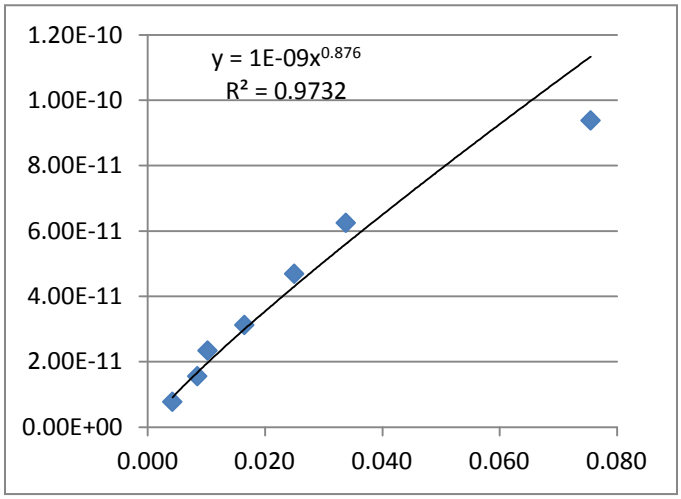
Run Number: 73

Date of Run: 09/22/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	alpha9/16/14	2.43E-11	6.6
2	septic effluent 9/16/14	4.53E-11	12.3
3	influent 9/16/14	4.68E-11	12.7
4	column sand 9/16/14	1.93E-11	5.2
5	loamy sand 9/16/14	1.77E-10	48.1
6	sandy loam 9/16/14	4.54E-10	123.5
7	Potts 1-6 9/16/14	2.63E-10	71.4
8	personal sample 9/22/14	1.25E-10	34.0

Standard Curve:



Comments:
Strong yeast growth, but the yellow color was very weak.
The pattern in the yellow color developed after hours in the incubator at 35°C

Interpretation:

Standard curve not bad (with exception of one value), values not comparable with previous runs; however this was the first time samples taken on these dates were run. "Personal sample" was saliva from the analyst.

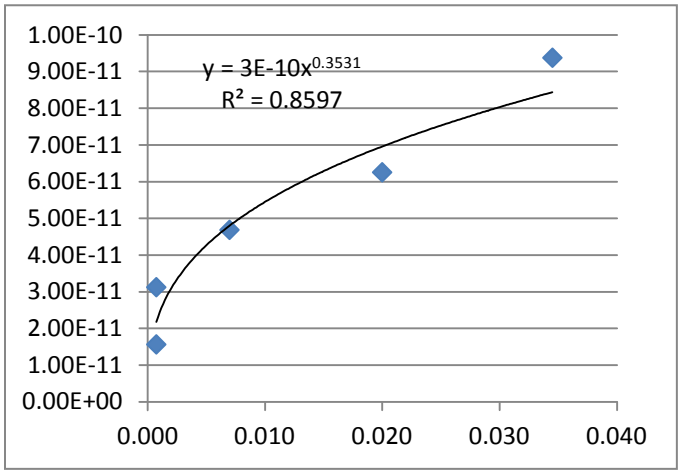
Run Number: 74

Date of Run: 09/22/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster pond 9/8/14	2.15E-10	58.5
2	Round pond 9/8/14	1.78E-10	48.4
3	Shiverick's pond 9/8/14	2.56E-10	69.7
4	Jenkins pond 9/8/14	2.35E-10	63.9
5	column sand 9/8/14	2.26E-10	61.6
6	loamy sand 9/8/14	2.52E-10	68.4
7	sandy loam 9/8/14	2.30E-10	62.5
8	alpha 9/8/14	4.84E-10	131.5

Standard Curve:



Comments:
Standard values E6.5 and E8 were removed from standards graph due to negative values, reported values being less than the blank
The yellow color was very weak, but a pattern was evident with an additional incubation at 35°C before stop solution was added.

Interpretation:

These results are much higher for the ponds than Runs #67, #68 (and adjusted)and #70 (Which had a very good R^2). Soil columns and the ALPHA sample were also higher than other runs.

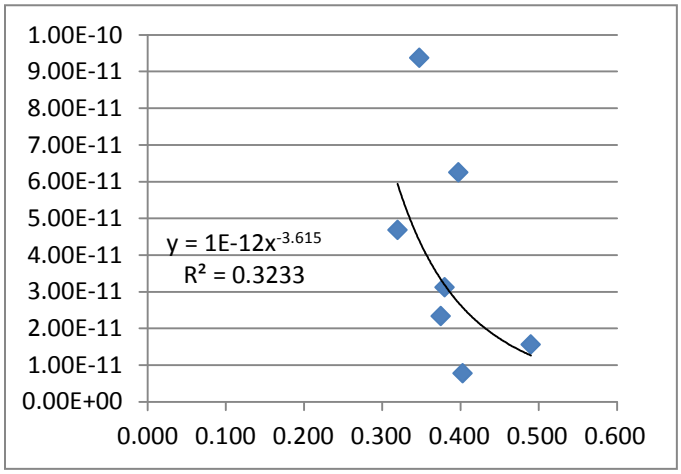
Run Number: 76

Date of Run: 09/24/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	column sand 9/23/14	4.32E-11	11.7
2	column sand 9/23/14	5.64E-11	15.3
3	loamy sand 9/23/14	7.30E-11	19.8
4	loamy sand 9/23/14	2.45E-10	66.6
5	sandy loam 9/23/14	2.42E-11	6.6
6	sandy loam 9/23/14	9.15E-11	24.9
7	alpha 9/23/14	3.23E-11	8.8
8	alpha 9/23/14	2.28E-11	6.2

Standard Curve:



Comments:
Samples tested in duplicate
Blank value eliminated because most sample and spike values were below
Some kind of contamination noted in the yeast activity. The yellow color that developed did so only after hours in the incubator at 35°C, suggesting degradation of ONPG naturally, and not because of enzyme activity.

Interpretation:

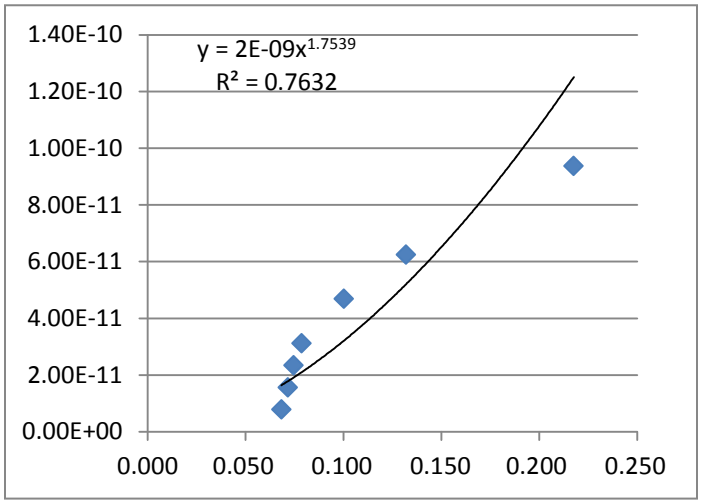
Run Number: 77

Date of Run: 09/30/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	column sand 9/23/14	5.58E-11	15.2
2	loamy sand 9/23/14	3.70E-11	10.1
3	sandy loam 9/23/14	3.68E-11	10.0
4	alpha 9/23/14	1.89E-10	51.4
5	DI water	3.96E-11	10.8
6			
7			
8			

Standard Curve:



Comments:
Microfuge tube protocol
Three repetitions of standards run with four reps of each of the five samples.
The yellow color was very weak, but a pattern was evident with an additional incubation at 35°C before stop solution was added.

Interpretation:

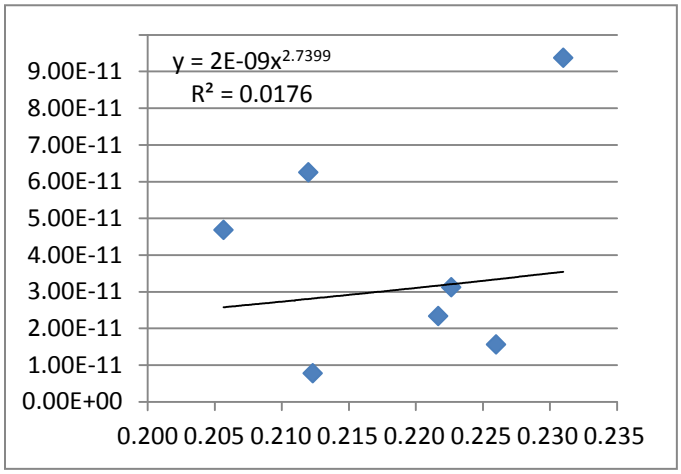
Run Number: 78

Date of Run: 09/30/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	column sand 9/23/14	1.66E-10	45.2
2	loamy sand 9/23/14	6.90E-11	18.8
3	sandy loam 9/23/14	9.93E-11	27.0
4	alpha 9/23/14	8.15E-11	22.2
5	DI water	6.65E-11	18.1
6			
7			
8			

Standard Curve:



Comments:
Three repetitions of standards run with four reps of each of the five samples.

Some kind of contamination noted in the yeast activity. The yellow color that developed did so only after hours in the incubator at 35°C, suggesting degradation of ONPG naturally, and not because of enzyme activity.

Interpretation:

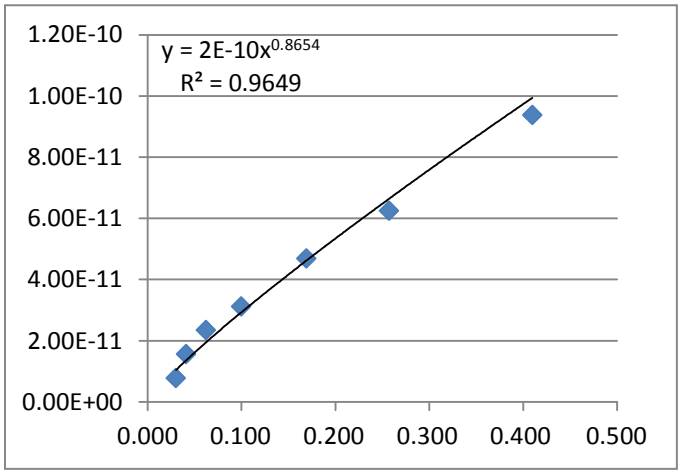
Run Number: 79

Date of Run: 10/06/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster pond 9/7/14	7.56E-11	20.6
2	Round pond 9/7/14	4.74E-11	12.9
3	Shiverick's pond 9/7/14	2.16E-11	5.9
4	Jenkins pond 9/7/14	1.99E-11	5.4
5	column sand 9/8/14	#NUM!	#NUM!
6	loamy sand 9/8/14	6.58E-12	1.8
7	sandy loam 9/8/14	5.91E-11	16.1
8	alpha 9/8/14	2.75E-10	74.9

Standard Curve:



Comments:
#NUM! Indicates values below the blank; essentially zero
Blank value adjusted to eliminate high value in one of the reps.

Interpretation:

Values for soil columns do not compare with data from Run #74 or Run #68. Calibration curve looks good. Pond values do not compare favorably with Run #70 or Run #74 which show a dominance of values below and above these data respectively.

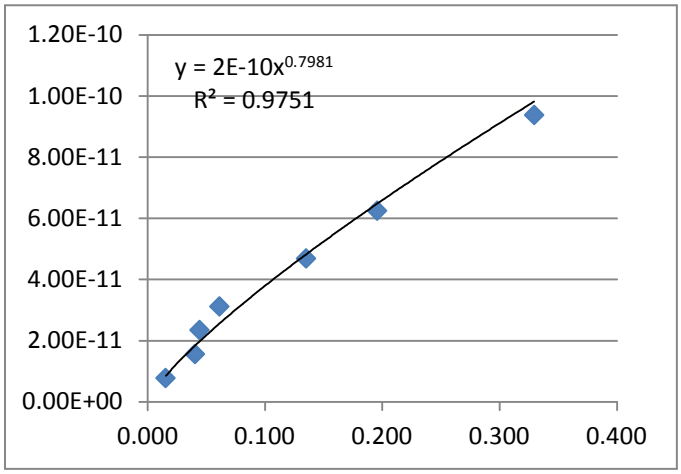
Run Number: 80

Date of Run: 10/06/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	alpha9/16/14	7.60E-11	20.7 (6.6)
2	septic effluent 9/16/14	5.01E-11	13.6 (12.3)
3	influent 9/16/14	3.65E-11	9.9 (12.7)
4	column sand 9/16/14	2.77E-11	7.5 (5.2)
5	loamy sand 9/16/14	7.77E-11	21.1 (48.1)
6	sandy loam 9/16/14	5.72E-11	15.6 (123.5)
7	Potts 1-6 9/16/14	2.04E-11	5.5 (71.4)
8	personal sample 9/22/14	3.41E-11	9.3 (34.0)

Standard Curve:



Comments:

Interpretation:

Compare this Run with Run #73 ALPHA 6.6(Run #73) – 20.7, VALUES IN PARENS ABOVE COME FROM RUN #73. Not very comparable.

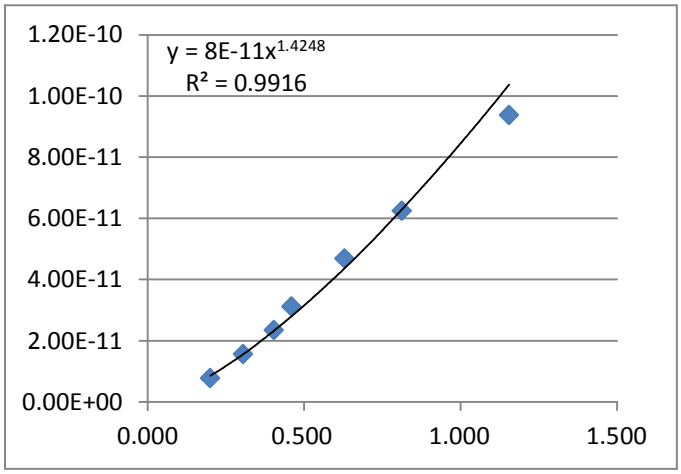
Run Number: 81 adjusted

Date of Run: 10/07/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster Pond 9/7/14	1.05E-11	2.9
2	Round Pond 9/7/14	1.15E-11	3.1
3	Shiverick's Pond 9/7/14	7.43E-12	2.0
4	Jenkins Pond 9/7/14	7.16E-12	1.9
5	column sand 9/8/14	9.81E-11	26.7
6	loamy sand 9/8/14	1.68E-10	45.8
7	sandy loam 9/8/14	8.71E-12	2.4
8	alpha 9/8/14	7.68E-11	20.9

Standard Curve:



Comments:
Blank adjusted to 0 because most sample values were below the blank value.
Microfuge tube run, only three repetitions of each series

Interpretation:
Pond values most comparable to Run #70, but soil columns still inconsistent.

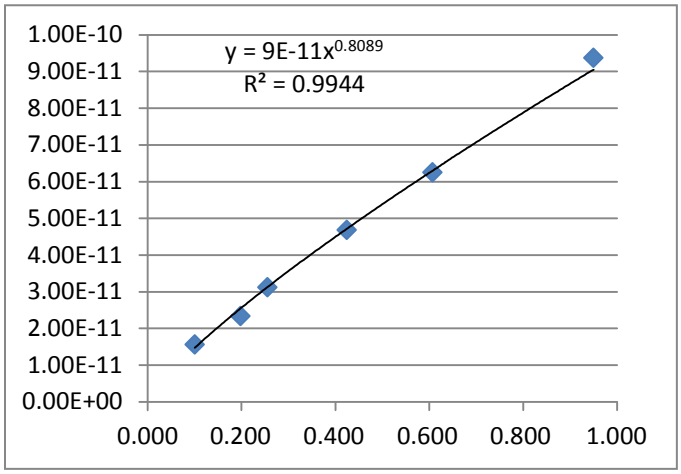
Run Number: 81

Date of Run: 10/07/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Oyster Pond 9/7/14	#NUM!	#NUM!
2	Round Pond 9/7/14	#NUM!	#NUM!
3	Shiverick's Pond 9/7/14	#NUM!	#NUM!
4	Jenkins Pond 9/7/14	#NUM!	#NUM!
5	column sand 9/8/14	1.03E-10	28.1
6	loamy sand 9/8/14	1.55E-10	42.2
7	sandy loam 9/8/14	#NUM!	#NUM!
8	alpha 9/8/14	8.44E-11	23.0

Standard Curve:



Comments:
Blank value higher than most sample values
#NUM! Indicates values below the blank; essentially zero
Microfuge tube run, only three repetitions of each series

Interpretation:

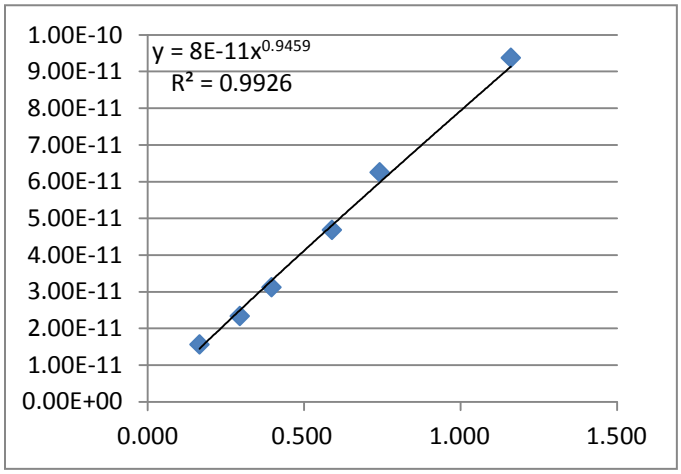
Run Number: 82a

Date of Run: 10/08/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	column sand 9/23/14	2.80E-11	7.6
2	column sand 9/23/14	3.13E-11	8.5
3	loamy sand 9/23/14	2.25E-12	0.6
4	loamy sand 9/23/14	2.34E-12	0.6
5	sandy loam 9/23/14	1.51E-11	4.1
6	sandy loam 9/23/14	1.87E-11	5.1
7	alpha 9/23/14	7.87E-11	21.4
8	alpha 9/23/14	8.67E-11	23.6

Standard Curve:



Comments:
Standard E8 removed from graph due to negative value; original value below blank.
Tests 82a and 82b conducted on the same plate, only 2 reps of samples and samples + spikes, with two full sets of standards tested (standards sets a and b)

Interpretation:

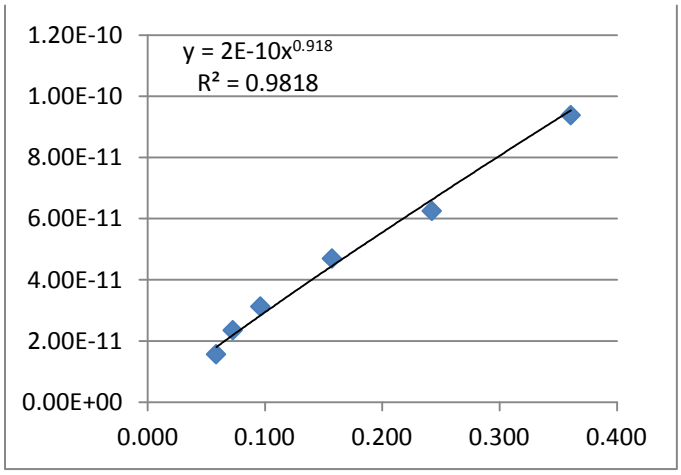
Run Number: 83

Date of Run: 10/15/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Septic tank effluent 10/15/14	9.61E-11	26.1
2	alpha 10/15/14	9.93E-11	27.0
3	standard septic10/15/14	9.12E-11	24.8
4	column sand low load 10/15/14	9.16E-11	24.9
5	loamy sand low load 10/15/14	3.65E-12	1.0
6	sandy loam low load 10/15/14	6.63E-12	1.8
7	column sand high load 10/15/14	6.43E-11	17.5
8	loamy sand high load 10/15/14	9.11E-12	2.5

Standard Curve:



Comments:
Standard E8 removed to improve R^2 (formerly 0.889)

Interpretation:

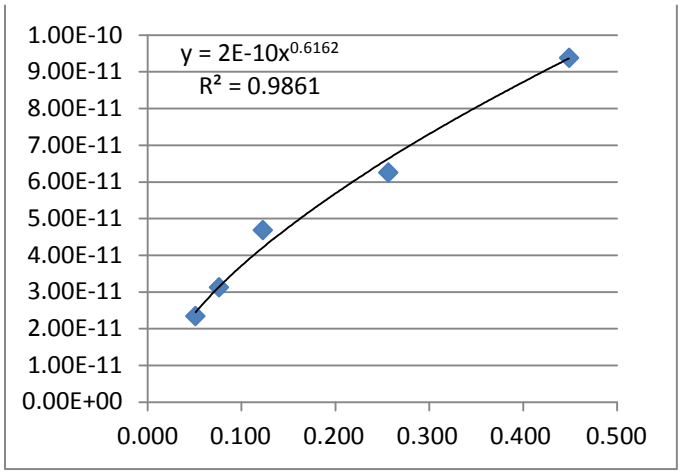
Run Number: 84

Date of Run: 10/15/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Septic tank effluent 10/15/14	2.01E-10	54.8
2	alpha 10/15/14	1.70E-10	46.3
3	standard septic10/15/14	1.50E-10	40.9
4	column sand low load 10/15/14	1.56E-10	42.5
5	loamy sand low load 10/15/14	2.95E-11	8.0
6	sandy loam low load 10/15/14	2.88E-11	7.8
7	column sand high load 10/15/14	1.30E-10	35.5
8	sandy loam high load 10/15/14	8.95E-11	24.3

Standard Curve:



Comments:
Standards values E7 and E8 were removed to improve the R^2 (formerly 0.9119).

Interpretation:

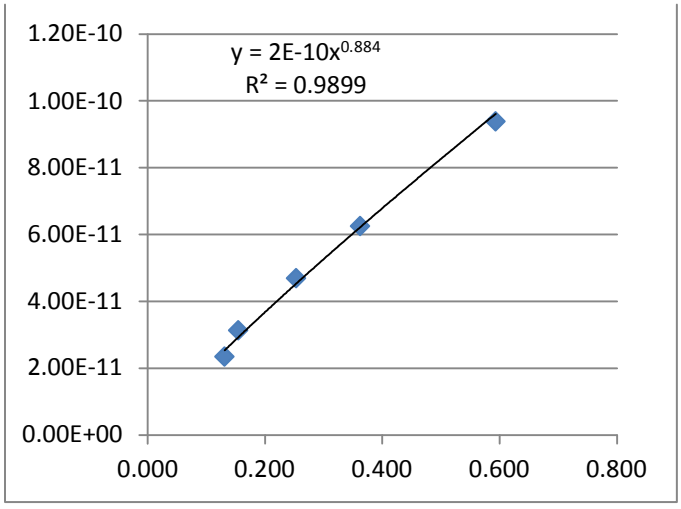
Run Number: 85 adjusted

Date of Run: 10/20/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Septic Effluent	2.40E-10	65.3
2	alpha	1.40E-10	38.0
3	standard septic	3.67E-11	10.0
4	loamy sand low	3.33E-11	9.0
5	column sand low	3.70E-11	10.1
6	sandy loam low	3.24E-11	8.8
7	column sand high	3.73E-11	10.1
8	loamy sand high	6.12E-11	16.7
9	sandy loam high	3.01E-11	8.2

Standard Curve:



Comments:

Experiment run using the micro-centrifuge tube protocol
Blank value removed from consideration due to negative sample results
Standard values E7 and E8 were removed from standards graph due to outlying values
3 repetitions only were run of each standard, and sample

Interpretation:

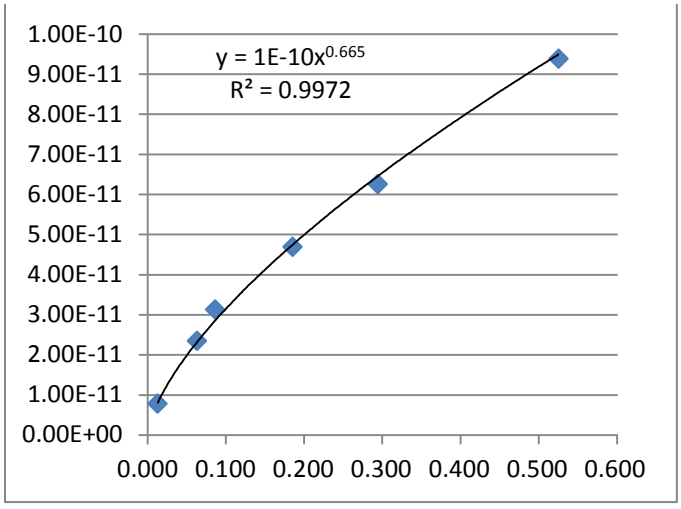
Run Number: 85

Date of Run: 10/20/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Septic Effluent	1.25E-10	34.0
2	alpha	7.66E-11	20.8
3	standard septic	#NUM!	#NUM!
4	loamy sand low	#NUM!	#NUM!
5	column sand low	#NUM!	#NUM!
6	sandy loam low	#NUM!	#NUM!
7	column sand high	9.74E-13	0.3
8	loamy sand high	2.79E-11	7.6
9	sandy loam high	#NUM!	#NUM!

Standard Curve:



Comments:

Experiment run using the micro-centrifuge tube protocol
Standard value E7 was removed from standards graph due to outlying values
3 repetitions only were run of each standard, and sample
#NUM! indicates negative results in the equation, essentially zero

Interpretation:

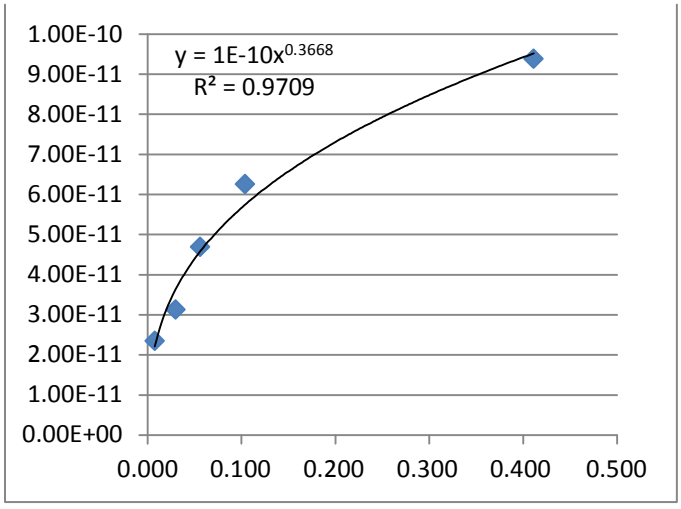
Run Number: 86

Date of Run: 10/20/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	Septic Effluent	1.65E-10	45.0
2	alpha	1.29E-10	35.1
3	standard septic	1.27E-10	34.6
4	loamy sand low	5.09E-11	13.9
5	column sand low	1.25E-10	34.1
6	sandy loam low	0.00E+00	0.0
7	column sand high	1.25E-10	34.1
8	loamy sand high	1.10E-10	29.8
9	sandy loam high	6.75E-11	18.3

Standard Curve:



Comment:

Standard values E6.5 and E7 were removed from standards graph due to negative values
3 repetitions only were run of each standard and sample

Interpretation:

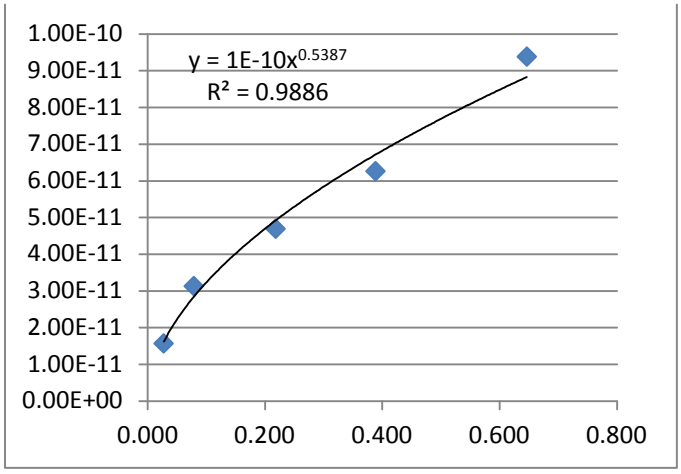
Run Number: 87

Date of Run: 10/21/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	#1	2.18E-10	59.4
2	#2	2.82E-11	7.7
3	#3	3.99E-11	10.9
4	#4	1.12E-11	3.0
5	#5	#NUM!	#NUM!
6	#6	3.33E-12	0.9
7	Di water	#NUM!	#NUM!
8	buffer	#NUM!	#NUM!

Standard Curve:



Comment:

#NUM! indicates values were below blank values, essentially zero

Interpretation:

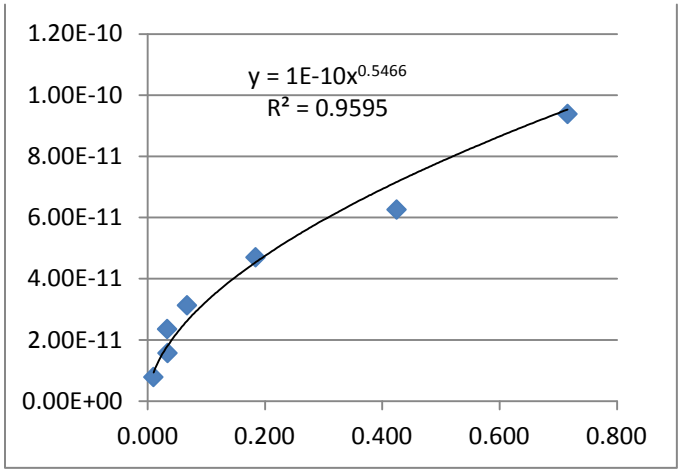
Run Number: 88

Date of Run: 10/21/14

Samples:

Sample number	Sample Source	Estradiol Equivalent	Parts per trillion
1	#1	1.68E-10	45.6
2	#2	9.29E-11	25.3
3	#3	1.31E-10	35.6
4	#4	9.89E-11	26.9
5	#5	7.97E-12	2.2
6	#6	5.26E-11	14.3
7	Di water	#NUM!	#NUM!
8	buffer	#NUM!	#NUM!

Standard Curve:



Comment:

#NUM! indicates values were below blank values, essentially zero

Interpretation: