REDUCTION OF ESTROGENIC BIOACTIVITY IN EFFLUENT THROUGH ELECTRON BEAM IRRADIATION

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Reduction of Estrogenic Bioactivity in Wastewater Through Electron Beam Irradiation.
(May 2015)

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Clean water availability has been made scarce by droughts in Texas caused by longer and hotter summers. Therefore, adopting new water reuse strategies has become important. A concern with recycled water has been the increase of emerging contaminants such as pharmaceuticals and chemicals, specifically estrogenic compounds, due to industrialization. Over exposure to exogenous estrogenic compounds has been shown to lead to cancers and diseases such as reproductive disorders. These adverse health effects can occur in humans and animals when estrogens are not effectively removed from wastewater effluent during water reclamation processes. Electron beam (eBeam) irradiation is an environmentally friendly technology that utilizes high energy electrons to yield rapid reduction and oxidation reactions, and has been shown to break down estrogens in wastewater biosolids. Our hypothesis is that estrogenic compounds, estrone (E1), 17-β-estradiol (E2), and bisphenol A (BPA), can be effectively broken down in wastewater effluent using eBeam irradiation. Should the estrogens be broken down, they will no longer be bioactive and capable of causing adverse health effects. The reduction in bioactivity of the estrogen was quantified using a yeast estrogen screening assay (YES assay) after eBeam irradiation of wastewater effluent at doses between 2 kGy and 15 kGy. The

detection limits of the YES Assay were 25pM for E1 and E2 and 0.25pM for BPA. Reduction was seen in E2 at an absorbed dose of 15kGy. However, reproducible reduction of estrogenic bioactivity for E1 and BPA could not be determined from irradiation trials. The YES Assay was originally developed with E2 and therefore it is possible that it is not an optimal assay to detect reduction in bioactivity for E1 and BPA. Overall we are not able to conclude that eBeam irradiation is an effective reduction method for the estrogens in wastewater effluent, and further studies are needed to confirm our hypothesis.

ACKNOWLEDGMENTS

This research was supported by the State of Texas Initiative, "Creation and Deployment of Water-Use Efficient Technology Platforms" and the USDA-NIFA project H87808. We would also like to thank and acknowledge Dr. Suresh D. Pillai, Jessica McKelvey, Charlotte Hieke, and Ralf Singh for their guidance and support.

NOMENCLATURE

BPA	Bisphenol A
CPRG	Chlorophenol red-β-D-galactopyranoside; chromogenic substrate
CSTX	Carter's Creek Wastewater Treatment Facility
DMSO	Dimethyl sulfoxide
E1	Estrone
E2	17-β-Estradiol
eBeam	Electron beam
EDC	Endocrine disrupting chemical
TAMU	Texas A&M University Wastewater Treatment Facility
UV	Ultra violet

CHAPTER I

INTRODUCTION

Importance of Effective Wastewater Reuse

Water is a vital resource that is a basic human necessity; however, it is becoming scarce. A leading cause for this scarcity is the persistence of droughts across the United States. Between March 2014 and March 2015 the percentage of D4, the most extreme drought classification, has nearly doubled within the continental United States [1]. In particular, within the same timeframe, 99% of California was in a state of drought. Currently, all across the United States, water restrictions are in effect. These restrictions prohibit the excessive use of clean water for the purposes of watering lawns, dishwashing in restaurants, and multiple other uses [2]. For example, in many cities, watering lawns is only allowed once a week between 7am – 11am and 7pm – 11pm [2]. These city-wide restrictions are in effect due to the low amounts of water available in local aquifers, which are the main source utilized for city water supplies.

Water supplies could potentially be supplemented with treated water from wastewater treatment facilities. Instead, this water is released directly into the environment, which is then termed effluent. Before the effluent could be considered safe for the public and used to supplement water supplies, it would need a secondary treatment process prior to entering a conventional drinking water treatment facility. Secondary treatment processes are needed due to the speculation that unsafe compounds remain in the effluent, demonstrated by negative environmental impacts such as those observed in fish and alligator populations [3]. The introduction of these compounds, called emerging contaminants, has increased with

industrialization. Emerging contaminants of concern in effluent include pharmaceuticals and chemicals, specifically estrogenic compounds [4]. Due to this increase in contaminants, it has become of great importance to discover new ways to enhance current water reclamation technologies [5].

Current water reclamation technologies utilized in a water reclamation facility include membrane filtration, ultra violet (UV) light radiation, and oxidative chemicals. Membrane filtration utilizes various pore-size membranes, ranging from 0.001µm to 1.0µm. These filters are capable of removing pesticides, metal ions, salt, viruses, pathogenic bacteria, and other harmful contaminants [6]. UV light employs radiation to attack cell walls, making them unable to reproduce. In Japan, these methods have been used to reclaim water such that biodiversity and fish populations were restored within rivers [6]. Oxidative chemicals, such as chlorine dioxide and ferrate, are employed to control microbiological growth. This technology is most commonly used and is inexpensive; however, its effectiveness is limited by the turbidity of the water [6]. Therefore, it is necessary to use this method in conjunction with other reuse technologies such as electron beam (eBeam). The implementation of such technologies in a water reclamation facility could help to improve the reclamation process and increase water availability.

eBeam as an Enhancement for Current Decontamination Methods

eBeam irradiation utilizes high energy electrons, which travel at 99.99999% the speed of light [7]. These electrons have the capability to produce free radicals from water, which attack contaminants and can render them harmless through rapid reduction and oxidation reactions [8]. Currently, eBeam technology is used for the sterilization of medical devices, phytosanitation,

and food pasteurization [9]. The United States Environmental Protection Agency has approved the use of eBeam as a wastewater treatment technology for biosolids [10].

In previous studies, eBeam irradiation has been shown to decrease the amount of estrogenic bioactivity and pathogenic contaminants in biosolids [11, 12]. Additionally, in our lab, it has been proven that eBeam irradiation had an increased reduction of estrogenic compounds when paired with other reducing technologies such as chlorine dioxide and ferrate. This synergistic effect allows decreased use of current treatments to receive the same or greater reduction in contaminants as compared to the use of only one treatment [11].

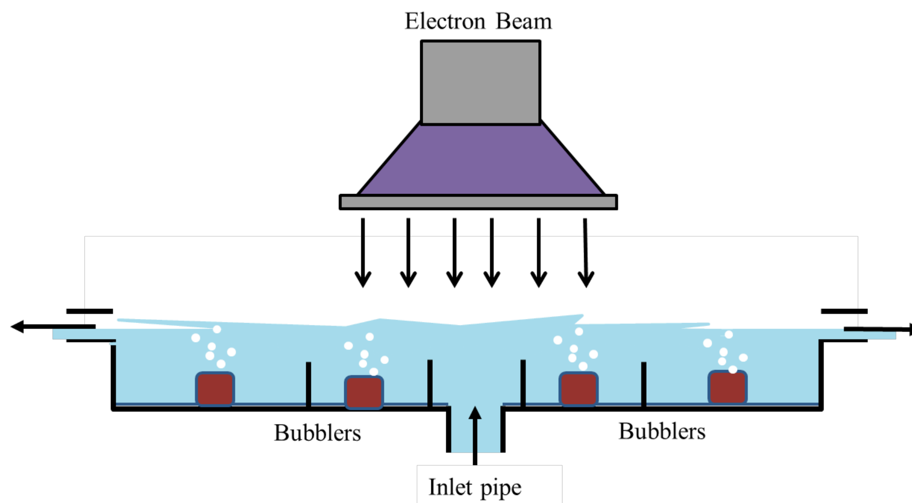


Figure 1: Diagram describing a potential modality of electron beam integration into a water reclamation facility. Adapted from an image in the article "Treat Water Right" in the *Daily Star* [13].

Therefore, less chemical supplementation and lower doses of irradiation would be required to decontaminant wastewater effectively. Furthermore, this would prevent the addition of excess decontamination chemicals such as chlorine to effluent which has been proven to lead to various

diseases such as stomach discomfort, anemia, eye irritation, and nervous system effects [13]. Figure 1 shows a potential modality to incorporate eBeam technology into a water reclamation facility. With the addition of eBeam technology in a water reclamation facility, a greater reduction of contaminants can be achieved using lower amounts of treatment. Therefore, eBeam irradiation could increase the efficiency of water reuse while safeguarding against emerging contaminants; thus proving to be a solution to the scarcity of water caused by droughts and industrialization.

Issues with Estrogenic Compounds in Effluent

Of many possible contaminants, estrogenic compounds can be noted as especially harmful in excessive amounts. Estrogens naturally produced by the human body include estrone (E1) and 17-β-estradiol (E2). In the environment, estrogenic bioactivity can also be found in a variety of chemical structures that usually aren't predicted to display such activity, predominantly known as xenoestrogens [14]. Xenoestrogens have the capability of binding to the human estrogen receptor without having the characteristic estrogenic structure. Bisphenol A (BPA) is a xenoestrogen found most commonly in plastics as well as in pesticides [3]. Estrogenic compounds have been proven to act as endocrine disrupting chemicals (EDCs). EDCs are defined as chemicals that affect the developmental, reproductive, neurological, and immune functions in humans and wildlife [15]. EDCs have the capability to bind to multiple receptors found in the human body. These receptors include, but are not limited to, nuclear receptors, nonnuclear steroid hormone receptors, nonsteroidal receptors, orphan receptors, and the estrogen receptor [16]. When an EDC binds to a receptor within the human body it blocks the endogenous hormone from binding. By preventing the native hormone from binding, cell signals are blocked

and the body fails to respond properly [15]. EDCs have been linked to reproductive disorders such as early puberty, male infertility, abnormalities in male reproductive organs, and female reproductive diseases; it has also been suggested that EDCs cause breast cancer, uterine cancer, testicular cancer, and thyroid cancer [17].

Yeast Estrogen Screening Assay

A method to measure estrogenic bioactivity is the yeast estrogen screening assay (YES Assay) developed by Routledge and Sumpter [14]. The YES Assay incorporates a recombinant yeast, *Saccharomyces cerevisiae* (*S. cerevisiae)*, which was modified to have a human estrogen receptor (hER) encoded into its chromosome. With this hER, the yeast is capable of expressing the lac-Z gene in the presence of estrogenic compounds. The lac-Z-gene produces β-galactosidase that metabolizes chlorophenol red-β-D-galactopyranoside (CPRG), a chromogenic substrate, resulting in a color change from yellow to red [14]. The amount of color change is directly related to the concentration of bioactive estrogenic compounds present in the solution. Therefore, using spectrophotometry, the concentration of bioactive estrogenic compounds can be quantified. Figure 2 demonstrates the reaction of the human estrogen receptor (hER) integrated in the genome of *S. cerevisiae* while in the presence of estrogenic compounds.

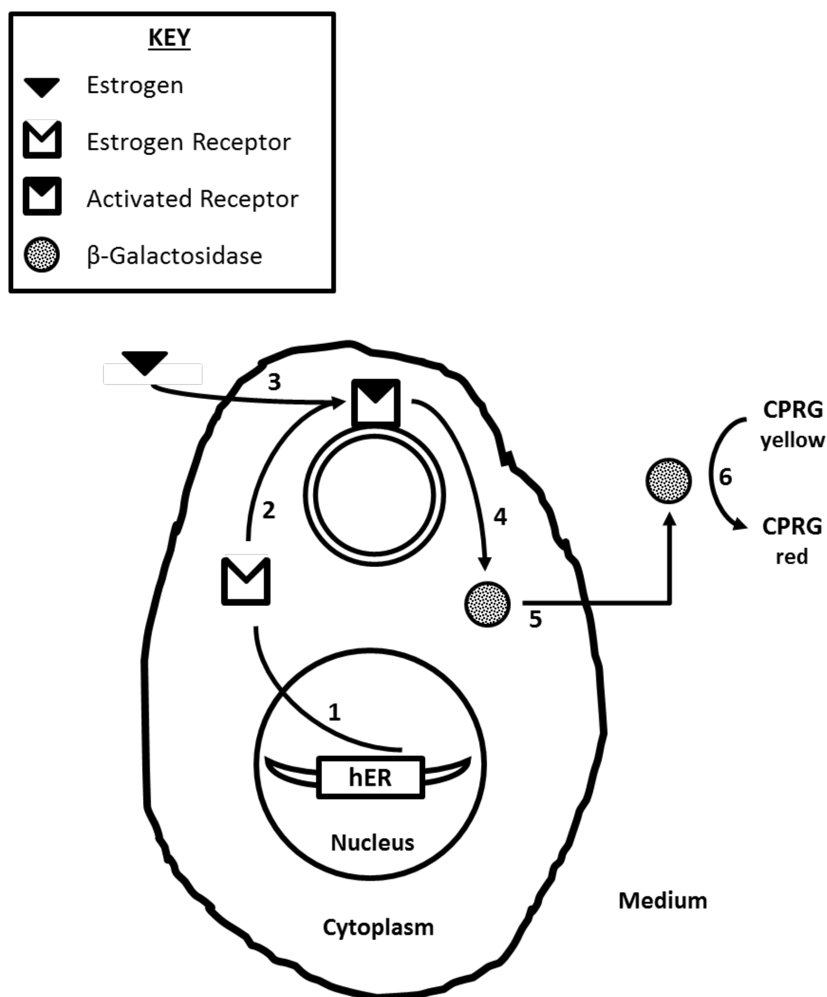


Figure 2. Yeast Cell with Integrated Human Estrogen Receptor. 1: Human estrogen receptor (hER) integrated in the nucleus of *S. cerevisiae*. 2: Unbound hER. 3: Activation of hER through binding of estrogen. 4: Expression of Lac-Z gene. 5: Production of β-galactosidase. 6: CPRG color change from yellow to the red in the presence of β-galactosidase. Adapted from an image in the journal article "Estrogenic Activity of Surfactants and Some of Their Degradation Products Assessed Using a Recombinant Yeast Screen" by Routledge and Sumpter [14].

CHAPTER II

METHODS

Preparation of Media and Reagents

All glassware to be used was cleaned to remove estrogenic compounds. Initially, glassware was placed a 1% Alconox (Alconox Inc., White Plains, New York) bath consisting of 10g Alconox in 1L deionized water for at least four hours. The glassware was then rinsed with deionized water and placed in a secondary DriContrad (Decon Laboratories Inc., King of Prussia, Pennsylvania) bath consisting of 7.4g DriContrad in 1L deionized water for four hours. The glassware was then rinsed and placed in an oven (Fischer Scientific Isotemp 500 Series, Waltham, Massachusetts) for four hours at 150 °C and then autoclaved (Steris Amsco Century SG – 120 ScientificGravity Sterilizer, Mentor, Ohio) at 121°C for 15 minutes.

Any materials, unless specified otherwise, were purchased from Sigma, St. Louis, Missouri. Minimal media was prepared by adding 13.61g KH_2PO_4, 1.98g $(NH_4)_2SO_4$, 4.2g KOH pellets, 0.2g MgSO4, 1mL $Fe_2(SO_4)_3$ solution (40mg $Fe_2(SO_4)_3$ /50mL H_2O), 50mg L-leucine, 50mg L-histidine, 50mg adenine, 20mg L-arginine-HCl, 20mg L-methionine, 30mg L-tyrosine, 30mg L-isoleucine, 30mg L-lysine-HCl, 25mg L-phenylalanine, 100mg L-glutamic acid, 150mg L-valine, and 375mg L-serine to 1L of double-distilled water. The media was then filtered through a 0.22µm bottle filter (VWR, Radnor, Pennsylvania) and stored at room temperature.

A 20% (w/v) glucose solution was prepared by adding 24g glucose to a final volume of 120mL deionized water and dispensed into 20mL aliquots. An L-aspartic acid solution was prepared by

adding 240mg L-aspartic acid into 60mL deionized water and dispensed into 20mL aliquots. Both the glucose solution and L-aspartic acid test tubes were then autoclaved at 121°C for 15 minutes, then stored at room temperature. An L-threonine solution was prepared by adding 720mg L-threonine into 30mL deionized water and dispensed into 10mL aliquots. The test tubes were then autoclaved at 121°C for 15 minutes, then stored at 4°C. A copper (II) sulfate solution was prepared by adding 49.94mg copper (II) sulfate into 10mL deionized water. The solution was then sterile filtered through a 0.2µm sterile syringe filter (VWR, Radnor, Pennsylvania) into two 5mL aliquots. A CPRG solution was prepared, as needed, by adding 20mg CPRG to 2mL deionized water and then sterile filtered through a 0.2µm sterile syringe filter.

The vitamin solution was prepared by adding 8mg thiamine, 8mg pyridoxine, 8mg pantothenic acid, 40mg inositol, and 20mL of biotin solution (2mg biotin/100mL deionized H_2O) to 180mL double-distilled water. The solution was then filter sterilized through 0.22µm pore size filters (VWR, Radnor, Pennsylvania) and 10mL aliquots were stored at 4°C in sterile glass bottles.

S. cerevisiae growth plates were made by adding 1.5% bacto agar (BD, Sparks, Maryland) to minimal media (1.5g bacto agar/90mL minimal media) and then autoclaved at 121°C for 15 minutes, and cooled in a water bath to approximately 50°C. Once cooled, 10mL glucose solution, 2.5mL L-aspartic acid solution, 1mL vitamin solution, 0.8mL L-threonine solution, and 250µL copper (II) sulfate solution was added and mixed, then poured into the agar. Growth plates were immediately poured into petri dishes and stored at 4°C in the dark.

Growth medium was prepared as needed with 5mL glucose solution, 1.24mL L-aspartic acid solution, 0.5mL vitamin solution, 0.4mL L-threonine solution, 125µL Copper (II) sulfate solution and 45mL minimal medium.

Estrogen 1mM stock solutions were made in dimethyl sulfoxide (DMSO) and stored away from light. Stock solutions were made of the natural estrogens estrone (0.0135g/50mL DMSO), 17-β-estradiol (0.0136g/50mL DMSO), and the xenoestrogen bisphenol A (0.0114g/50mL DMSO).

S. cerevisiae Growth

To obtain a culture of the *S. cerevisiae*, a -80°F freezer stock vial was quickly thawed and streaked onto growth plates. These plates were then incubated for 48 hours at 32°C until colonies developed, and then stored at 4°C for up to one month.

S. cerevisiae Growth Curves

Growth curves were created by preparing fresh growth medium in a 100mL flask and inoculating with one colony forming unit (CFU) of yeast from the stored growth plates. The inoculated medium was then incubated in an orbital shaker set to 225rpm 32°C. The optical density at 600nm (OD600) was taken at 2, 4, 6, 8, 10, 12, 24, 33, 49, and 58 hours after inoculation by pipetting 100µL of the inoculated medium into a sterile cuvette. This cuvette was then placed into the spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany) that had been calibrated with the blank cuvette made with sterile, fresh growth media.

Determination of *S. cerevisiae* Concentration

The CFU concentration of yeast was determined from serial dilutions after 24 hours of growth in fresh growth media inoculated with one CFU incubated at 32°C and 225rpm. After 24 hours of growth, the OD600 value was obtained spectrophotometrically. Then 10-fold serial dilutions were made in phosphate buffered saline (PBS) in a biological safety cabinet (Labconco Pulifier Class II Biosafety Cabinet, Kansas City, Missouri). Then 100µL of the dilutions from 10^{-4} to 10^{-8} were plated onto growth plates. The plates were then incubated at 32°C for 48 hours or until colonies formed. After colonies formed, they were enumerated to determine the CFU/mL.

YES Assay

The YES assay began by inoculating fresh growth medium with one CFU of yeast and allowing it to incubate in the orbital shaker (Lab-Line Incubator Shaker, Tripunithura, India) at 32°C and 225rpm until it reached log phase of growth at approximately 24 hours; which was confirmed by taking the OD600 and recorded. Then seeded assay medium was prepared by adding 0.5mL stock CPRG solution to fresh growth media and inoculating it with 2mL of the log phase growth medium. Ten-fold dilutions for E1, E2 and BPA or irradiated and extracted estrogens were prepared in a 96 well dilution plate (VWR, Radnor, Pennsylvania) in DMSO. Using a multichannel pipette (VWR, Radnor, Pennsylvania) 5µL of the appropriate dilutions were transferred into triplicate wells of another 96 well assay plate (Costar, Corning, New York) containing 195µL of the seeded assay medium. At least three wells containing only seeded assay medium and DMSO were used as negative controls. The plate was then mixed thoroughly with a multichannel pipette (Eppendorf, Hamburg, Germany). The lid of the well plate was carefully taped down such that there was no air flow or potential evaporation of the solutions. The assay

plate was then placed in an incubator (Fischer Scientific, Waltham, Massachusetts) at 32°C in the dark. Every 24 hours the assay plate was mixed to prevent settling of the yeast. Once the DMSO negative controls began to change color from yellow to red, the assay plate was read immediately on a spectrophotometer at 535nm and the data was plotted absorbance versus estrogen concentration.

Water Samples

The test water samples were effluent samples collected from the Carter's Creek Wastewater Treatment Facility (CSTX) and Texas A&M Wastewater Treatment Facility (TAMU) effluent. Samples were collected and stored at 4°C for a maximum of 48 hours.

eBeam Irradiation

The experiment was carried out in a Biosafety Level 2 lab with access to the National Center for Electron Beam Research (NCEBR) on the Texas A&M University campus. The effluent samples were spiked to make concentrations of 10^{-5}M E1, 10^{-5}M E2 and 10^{-4}M BPA, and aliquoted to make individual pouches (sealed air-tight). One pouch of each effluent sample was left un-spiked and was used as the effluent control. Each pouch was then triple-packed according to biosafety level two (BSL2) procedures and transported to NCEBR to be irradiated. Spiked samples were irradiated using a 10MeV electron beam at target doses corresponding to 0, 2, 4, 8, 10, and 15kGy. The un-spiked and un-irradiated (0kGy) effluent control samples were both transported to the NCEBR as well, but not irradiated.

eBeam irradiation doses were measured using internationally validated Alanine pellet dosimeters (Harwell Dosimeters, Oxfordshire, United Kingdom). The dosimeters (in heat-sealed pouches) were placed within the sample to insure correct absorbance of the eBeam irradiation dose and measured using the Bruker E-scan spectrometer (Bruker, Billerica, Massachusetts). Previous studies validated the pouch system used to insure effective irradiation with a dose-uniformity ratio (DUR) of approximately 1.0 [12].

Estrogen Extraction

After eBeam irradiation, the pouches were aseptically cut open and poured into individual 15mL glass tubes, and the estrogens were extracted using 99% w/v ether (VWR, Radnor, Pennsylvania) in a fume hood (Kewaunee Scientific Engineering, Adrian, Michigan). To extract the estrogens, 5mL of ether was added to each 1mL sample and vortexed (Fischer Scientific Vortex Mixer, Waltham, Massachusetts) to mix thoroughly. The tubes were placed in a dry ice bath for one minute to allow the aqueous phase to solidify. Once solidified, the organic phase (supernatant) was removed and transferred into new sterile, glass tubes. The tubes were left open in the fume hood, in the dark, until the ether evaporated. Estrogens were resuspended in 100µL of DMSO, and then vortexed. Samples were transferred into 1.5mL labeled sterile tubes and stored in the dark until processed in the YES Assay.

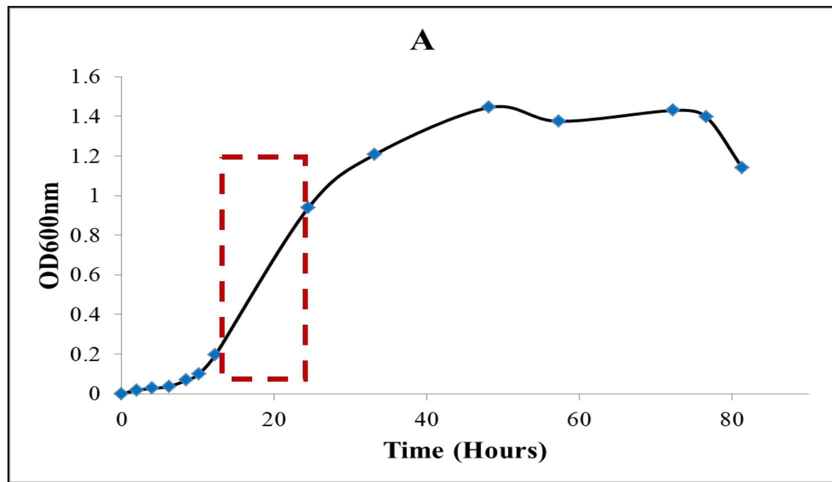
CHAPTER III

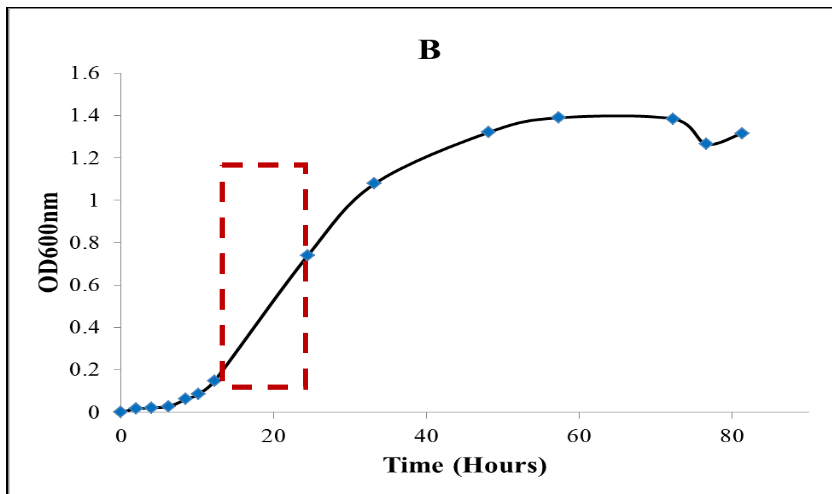
RESULTS

Growth Curve

The results from each OD600 reading were graphed versus time (hours). The log phase was then determined from the graphs shown in Figure 3 from when the yeast was in the middle of exponential growth; between 15 and 25 hours. The log phase of growth had to be determined to know when to inoculate the seeded assay medium for the YES Assay. The growth curve was repeated once to insure accuracy in determining the log phase of yeast growth.

To determine the concentration of yeast cells during the log phase, an inoculated growth media was grown to log phase and the OD600 was taken. Two sets of a 10-fold serial dilution of the log phase growth media were made and the dilutions between 10^{-4} and 10^{-7} were plated on growth plates and counted. This indicated the CFU concentration corresponding to the OD600 recorded earlier. The concentration of yeast during log phase was calculated to be $7.18 \times 10^{6} \pm 0.78$ CFU/mL.





Log Phase

Figure 3. ***S. cerevisiae*** **Growth Curves** A: Trial one of determining the log phase of *S. cerevisiae.* B: Trial two of determining log phase of *S. cerevisiae.*

Detection Limit Determination

The sensitivity of *S. cerevisiae*'s capability to bind to the estrogenic compounds and produce a color change was determined with the YES Assay. A range of dilutions from 10^{-5}M to 10^{-12}M for E1and E2, and from 10^{-3}M to 10^{-10}M for BPA, were plated following the YES Assay procedure and the resulting plate is shown in Figure 4.

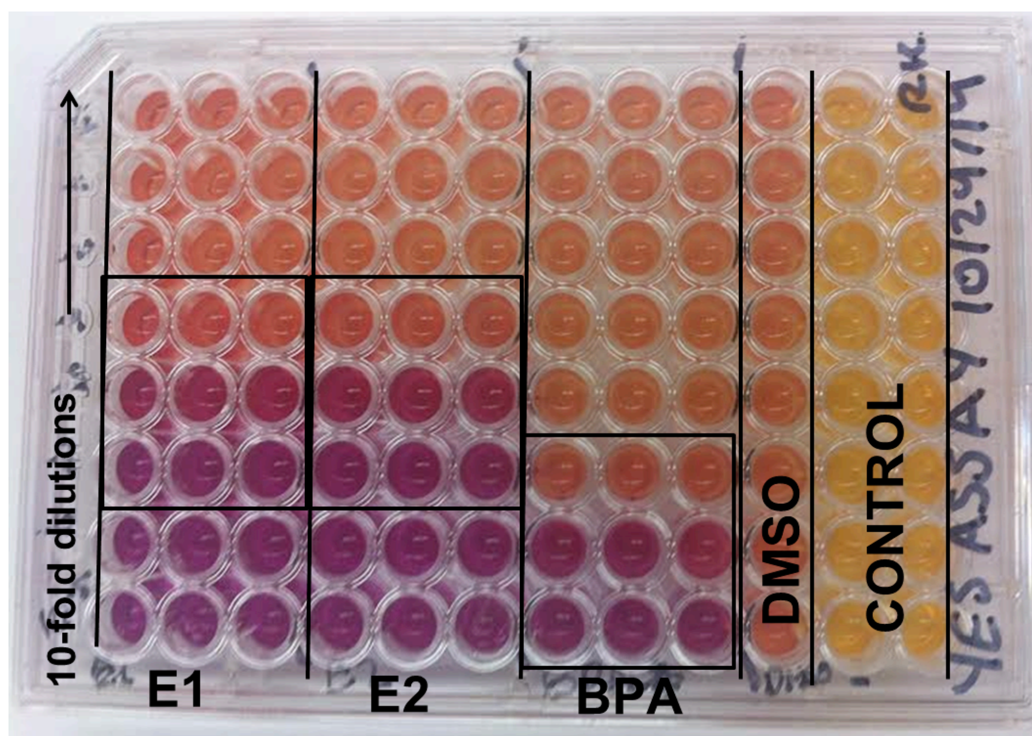
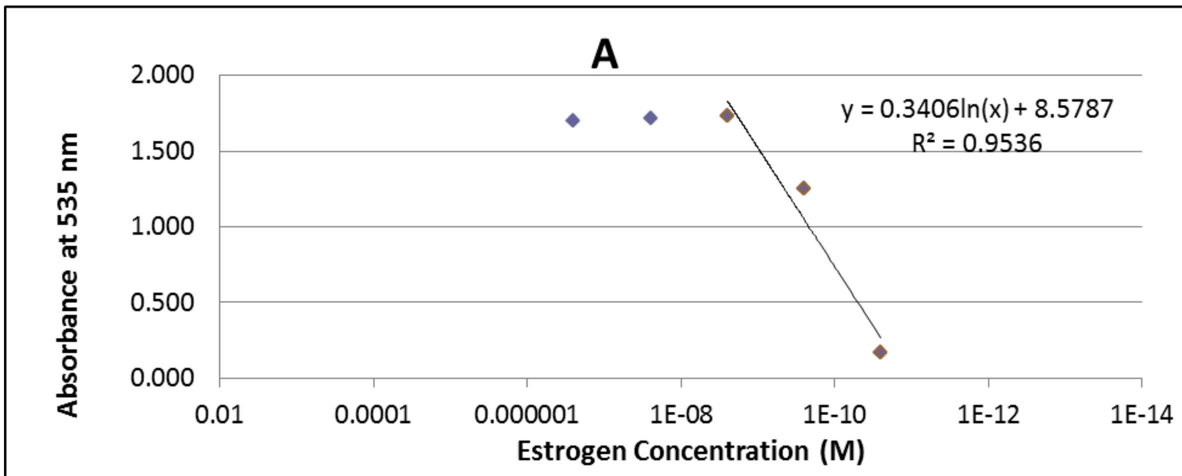
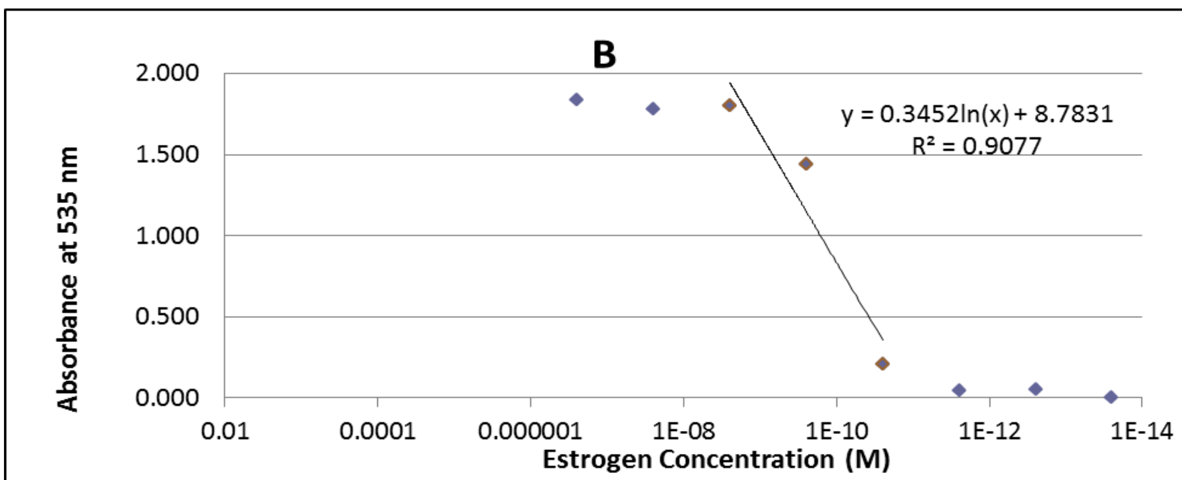


Figure 4. YES Assay Detection Limit Plate Black boxes indicate the detection limit range for each estrogen in the YES Assay. E1and E2 have detection limit ranges of 25pM – 2500pM. BPA has a detection limit range of 0.25pM – 25pM.





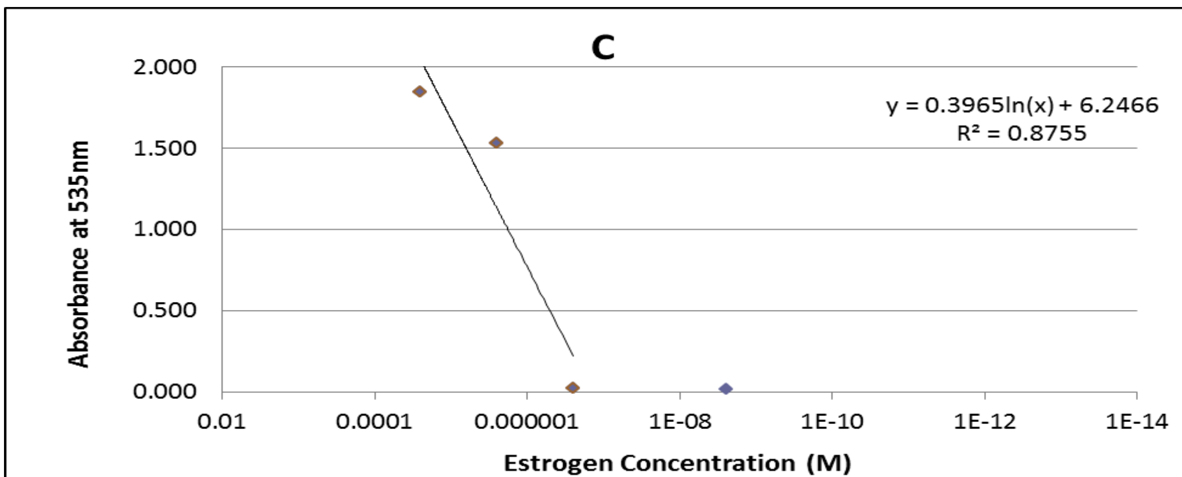


Figure 5. Detection Limit Range of *S. cerevisiae* in YES Assay. A: Detection limit of E1 demonstrating detection limit range of 25pM – 2500pM. B: Detection limit of E2 demonstrating detection limit range of 25pM – 2500pM. C: Detection limit of BPA demonstrating detection limit range of 0.25pM – 25pM. Linear lines depict the detection limit range for the YES Assay for each estrogen tested.

Figure 5 demonstrates the detection limit of the yeast, *S. cerevisiae*, for each of the estrogens tested. The detection limit for E1 and E2 was 25pM, and for BPA the detection limit was 0.25μM. These values represent the minimum concentration of estrogen required for the yeast to detect the presence of the estrogen in solution, as detected by a change in its absorbance at 535nm. E1and E2 have detection limit ranges of 25pM – 2500pM. BPA has a detection limit range of 0.25pM – 25pM as shown with lines.

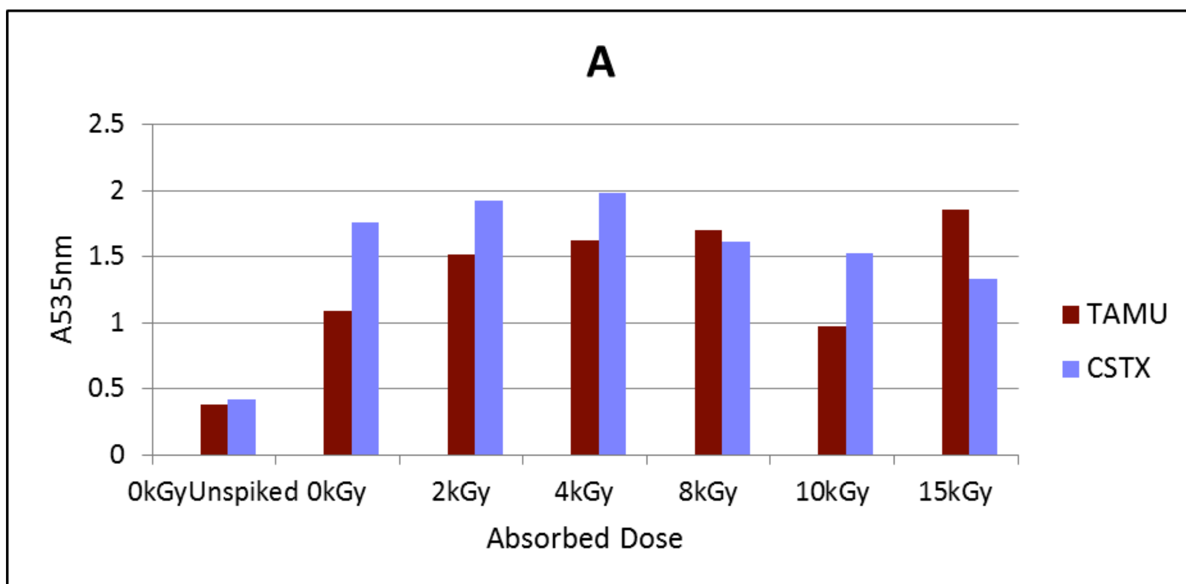
Yeast Estrogen Screening Assay of Irradiated Samples

Effluent from TAMU and CSTX were collected and spiked with estrogens. The variation of effluents ensured that a variety of wastewater treatment methods were represented in the experiment; this variation included the aerobic digestion method, which is utilized at TAMU, and the anaerobic digestion method, which is used at the CSTX. Samples were spiked with respective estrogens and irradiated at NCEBR with doses between 0kGy and 15kGy. The samples were processed for estrogen extraction according to methods, serially diluted in DMSO, and then plated in the 96-well assay plate according to the detection limits predetermined such that a reduction could be quantified in the YES Assay detection range. After incubation, the absorbance at 535nm versus irradiation dose was plotted.

Trial 1 samples were incubated for 72 hours which was the amount of time required for DMSO control samples to display activity through color change. However, at this time point many of the estrogens were on the upper limits of the detection limit ranges. Also, the recovery of the samples from Trial 1 was variable due to the packaging method; the recovery ranged from 50% to 100% for each irradiated effluent sample. Trial 2 data was collected at 48 hours to ensure

samples were detected in the middle of the ranges for each estrogen tested. Also, in Trial 2 the packaging method was optimized such that recovery was 100% for each sample.

E1, shown in Figure 6, displayed no observable reduction in Trial 1 or Trial 2 for either effluent sample at any absorbed dose. E2, shown in Figure 7, displayed reduction at 15kGy in Trial 1 for both effluent samples. The reduction in the CSTX effluent was comparable to the unspiked effluent whereas in the TAMU effluent it was not. In Trial 1, the sample at 8kGy for TAMU effluent is not shown due to 100% loss in the extraction process. E2 in Trial 2 displayed reduction at absorbed doses between 2kGy and 15kGy for TAMU effluent samples, and between 10kGy and 15kGy for CSTX effluent samples. However, the level of reduction was not comparable to the unspiked effluent. BPA, shown in Figure 8, in Trial 1 displayed reduction at absorbed doses between 2kGy and 15kGy for TAMU effluent samples, but the level of reduction was not comparable to the unspiked effluent. There was no observable reduction for BPA in CSTX effluent samples at any absorbed dose. BPA in Trial 2 displayed no significant reduction for either effluent sample at any absorbed dose.



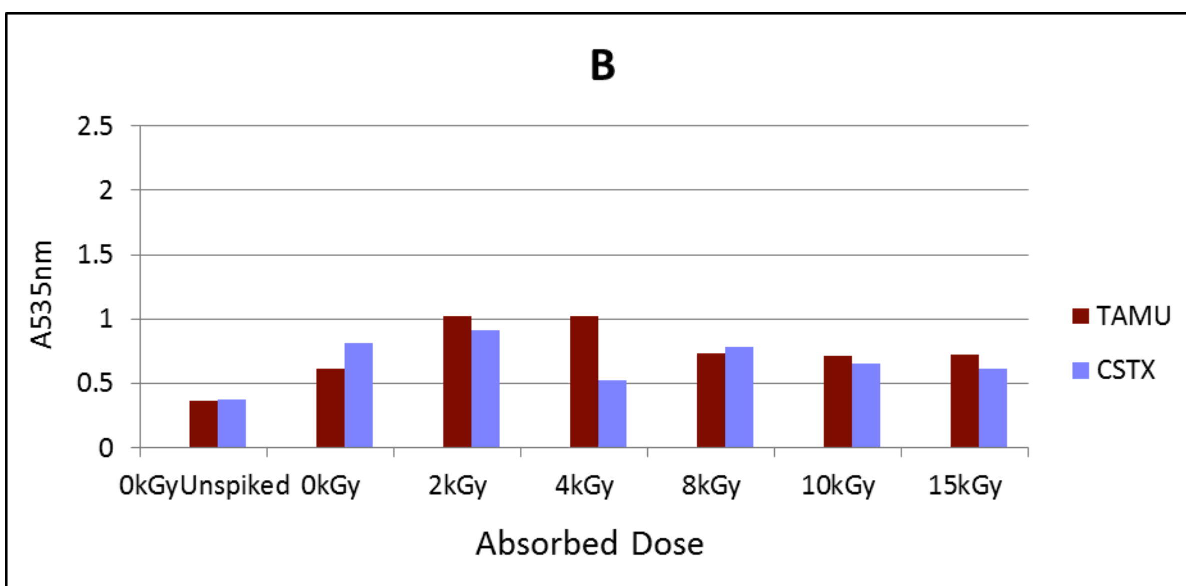
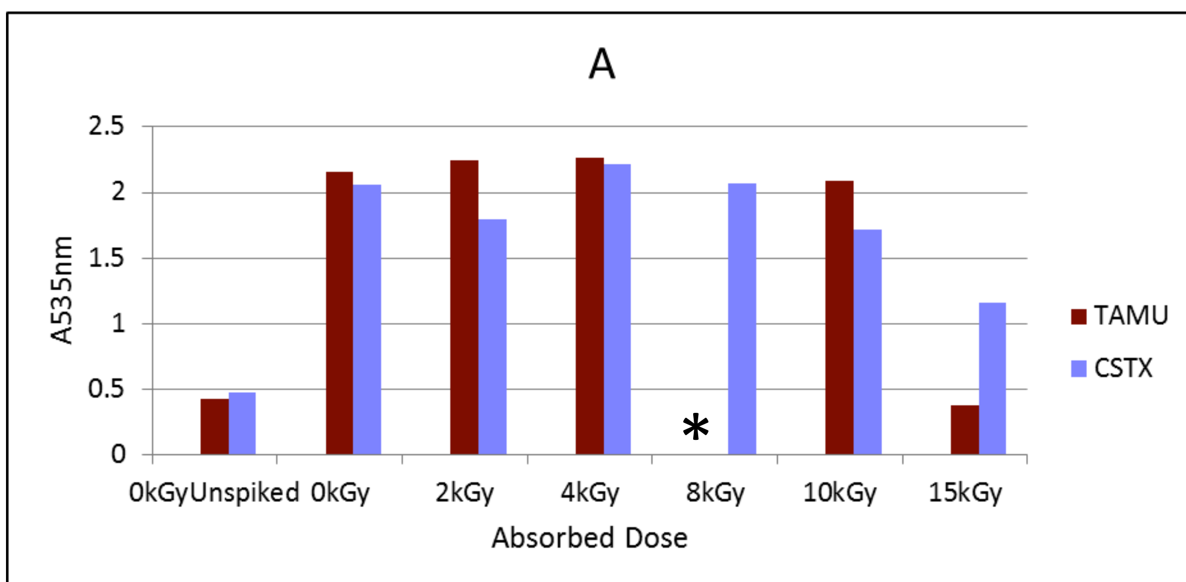


Figure 6. E1 Spiked Effluent Samples (TAMU and CSTX) Irradiated at Various Absorbed Doses. A: Trial 1 incubated for 72 hours at 32°C. B: Trial 2 incubated for 48 hours at 32°C.



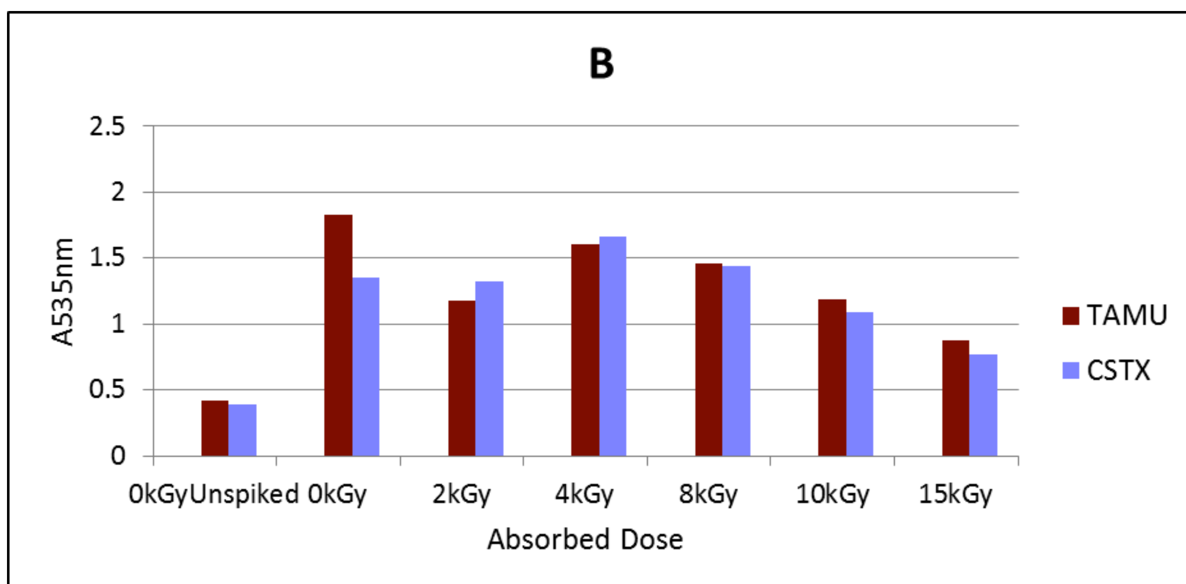
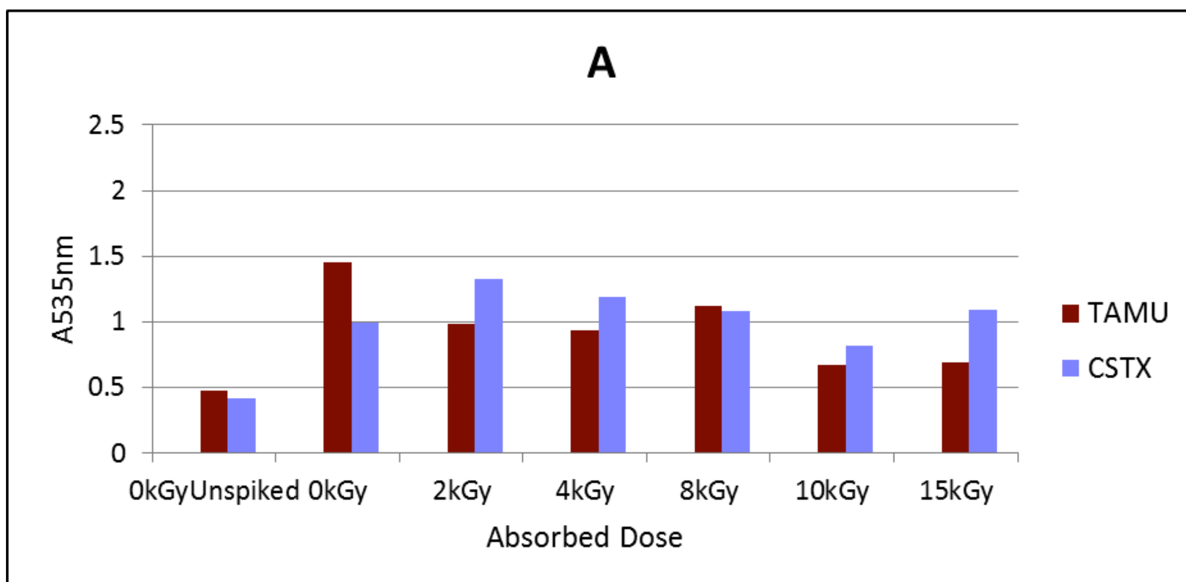


Figure 7. E2 Spiked Effluent Samples (TAMU and CSTX) Irradiated at Various Absorbed Doses.. A: Trial 1 incubated for 72 hours at 32°C. Sample at 8kGy for TAMU effluent was lost in the extraction process. B: Trial 2 incubated for 48 hours at 32°C.



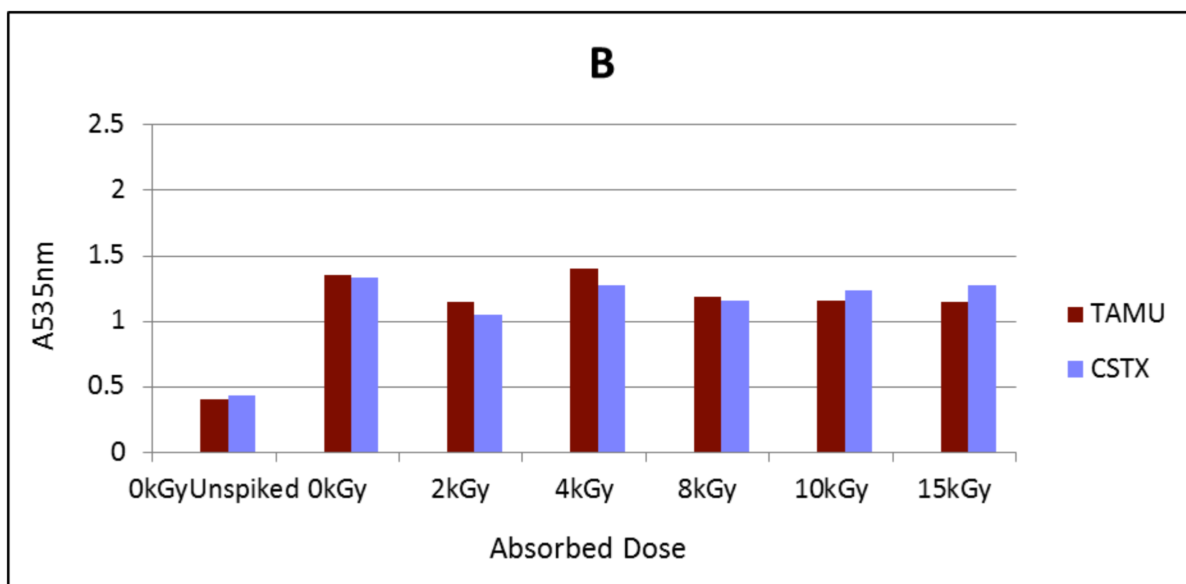


Figure 8. BPA Spiked Effluent Samples (TAMU and CSTX) Irradiated at Various Absorbed Doses. A: Trial 1 incubated for 72 hours at 32°C. B: Trial 2 incubated for 48 hours at 32°C.

CHAPTER IV

CONCLUSION

A significant reduction in Trial 1 was seen in E2 at 15kGy for both TAMU and CSTX effluents. Specifically, there was an 82.3% reduction in TAMU effluent from 0kGy to 15kGy, and a 43.8% reduction in CSTX effluent from 0kGy to 15kGy. However, a majority of errors in the assay occurred in Trial 1 and these errors can be attributed to the efficiency of the extraction method. In Trial 1, sample recovery ranged from 50% to 100% across all samples. The inconsistent loss of samples in Trial 1 created results that demonstrated no logical trend, including the control sample for E1 spiked TAMU effluent at 0kGy which contained less estrogenic bioactivity than that of the sample irradiated at 15kGy. Therefore, indicating unreliability of the results in Trial 1.

In Trial 2, after extraction process optimization, recovery was close to 100% for all samples, and the results were determined to be more reliable. There was not a reduction observed for E1 spiked TAMU effluent, but instead a 16.7% increase in bioactivity between 0kGy spiked TAMU effluent and 15kGy spiked TAMU effluent. However, E1 spiked CSTX effluent demonstrated a 24.0% reduction in bioactivity within the same parameters. E2 demonstrated the greatest reduction in bioactivity between 0kGy spiked effluents and 15kGy spiked effluents. The percent reduction between these samples included 52.3% for TAMU and 43.0% for CSTX. BPA also demonstrated an overall reduction between 0kGy spiked effluents and 15kGy spiked effluents; however, this percentage only included a 15.5% reduction for TAMU and 4.37% for CSTX.

While overall decreases were observed between 0kGy spiked effluents and 15kGy spiked effluents in Trial 2, a bell curve trend occurred in most all trials. This bell curve demonstrates an increase in estrogenic bioactivity between 2kGy spiked effluents and 8kGy spiked effluents. Due to the occurrence of this trend, it is possible to infer that lower doses (2kGy-8kGy) of eBeam irradiation, when in contact with estrogenic compounds, creates by-products that are more bioactive; thus showing an increase in estrogenic bioactivity.

When compared to the reduction found in E2, the lower amount of reduction in E1 and BPA can be attributed to the basis that the YES Assay was originally designed for E2 detection [14]. Additionally, E2 has been shown to have up to five times more binding capability than E1 [18]. Also, from results shown in detection limit trials for BPA, it required significantly higher concentrations to display similar bioactivity when compared to the natural estrogens E1 and E2. Therefore, the YES Assay may not be an effective method to quantify the change in bioactivity for E1 and BPA.

To prove our hypothesis and quantify reduction of estrogenic bioactivity after eBeam irradiation in effluent, an alternative method of estrogenic bioactivity detection is required. In our lab, the use of the breast cancer cell line, ZR-75, has effectively quantified a reduction in estrogenic bioactivity in biosolids. Therefore, the ZR-75 is a potentially more effective assay to detect reduction of estrogenic bioactivity than the YES Assay.

Although eBeam as a single technology was not definitively proven to be efficient in reducing bioactivity of all estrogenic compounds, synergy with other reclamation technologies is still a

possibility. Our lab has shown that eBeam irradiation has participated in synergistic reactions with oxidative chemicals, effectively reducing bioactivity of estrogenic compounds in biosolids [11]. Therefore, trials with eBeam irradiation coupled with oxidative chemicals may be able to reduce the bioactivity of estrogenic compounds to undetectable amounts in effluent.

At this time, more trials are required with the YES Assay to definitively prove the effectiveness of eBeam irradiation as a method for reclaiming effluent water for reuse. If the reduction between 0kGy and 15kGy can be more precisely quantified, eBeam irradiation could increase the efficiency of water reuse while safeguarding against emerging contaminants; thus proving to be a solution to the scarcity of water caused by droughts and industrialization.

REFERENCES

1. Rosencrans, M. United States Drought Monitor. (accessed 03/12/2015).

2. Drought Restrictions: Stage 2. www.saws.org (accessed 4/10/2015).

3. Toze, S. Reuse of Effluent Water - Benefits and Risks. *Agricultural Water Management* 2006*, 80*, 4/10-147-159.

4. Spangenberg, N. Water Resources Impact: Contaminants of Emerging Concern. *3* 2007*, 9*, 3/12/2015.

5. Kjelgren, R.; Rupp, L.; Kilgren, D. Water Conservation in Urban Landscapes. Thesis. Department of Plants, Soils, and Biometeorology, Utah State University, 2000. HortScience, 2000. Print.

6. GEC; UNEP. Water and Wastewater Reuse. United Nations Environment Programme 2007, 1, 9-12.

7. The E-Beam Facility. http://ebeam.tamu.edu (accessed 3/7/2015).

8. Gehringer, S. Advances in Radiation Processing of Wastewater – Basics of the Process 2003, 7-8.

9. Hamm, R..; Hamm, M. (Eds). 2012. Industrial accelerators and their applications. World Scientific pp. 421

10. U. S. EPA, 1993. The standards for the use or disposal of sewage sludge. Final 40 CFR Part 503 Rules. EPA 822/Z–93/001.

11. Pillai, S. D.; Reimers, R. Disinfecting and Stabilizing Biosolids Using E-Beam and Chemical Oxidants. 2010, U4R06, ES-1-5-11.

12. Praveen, C.; Jesudhasan, P.R.; Reimers, R.S.; Pillai, S.D. "Electron Beam Inactivation of Selected Microbial Pathogens and Indicator Organisms in Aerobically and Anaerobically Digested Sewage Sludge." Bioresource Technology 144 (2013): 652-57. Web.

13. Khan, M. A. Treat the Water Right. *The Daily Star* 2009*, 3*.

14. Routledge, E.; Sumpter, J. Estrogenic Activity of Surfactants and Some of Their Degradation Products Assessed Using a Recombinant Yeast Screen. Thesis. Department of Biology and Biochemistry, Brunel University, 1995. N.p.: Setac, 1996. Print.

15. Endocrine Disruptors. www.niehs.nih.gov (accessed 4/10/2015).

16. Holbrook, R.; Novak, J.; Grizzard, T.; Love, N. Estrogen Receptor Agonist Fate During Wastewater and Biosolids Treatment Processes: A Mass Balance Analysis. Environ Sci Technol. 2002, 36, 4/10/15-4533-4539.

17. Endocrine Disruptors. www.greenfacts.org (accessed 4/10/2015).

18. Wise, A.; O'Brien, K.; Woodruff, K. Are Oral Contraceptive a Significant Contributor to the Estrogenicity of Drinking Water? Environ. Sci. Technol. 2011, 45, 4/10-51-60.