NM WRRI Student Water Research Final Report

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2. **Project title - Evaluating Endocrine Disrupting Effects of Treated Produced Water on Human Cell Lines**

3. **Description of research problem and research objectives –**

According to the U.S. Energy Information Administration in 2022, the United States witnessed a significant contribution of unconventional hydrocarbon resources to its energy landscape. Approximately 2.84 billion barrels of crude oil were extracted directly from tight oil reservoirs, comprising 66% of the total U.S. crude oil production for that year (EIA, 2024a). Concurrently, the production of 28.6 trillion cubic feet of dry natural gas from shale formations represented 79% of the total U.S. dry natural gas production in 2022 (EIA, 2024b). The U.S. crude oil production had grown 5.6% or 0.6 million barrels per day (b/d) in 2022 compared with 2021, according to the Monthly Crude Oil and Natural Gas Production report. Texas and New Mexico made up the largest portion of the expansion in U.S. crude oil production in 2022. The Permian Basin, a productive oil basin situated on the border of West Texas and eastern New Mexico, holds the top position in oil production for these two states. In 2022, New Mexico achieved a remarkable milestone by experiencing its third consecutive year of significant growth in crude oil production, outpacing all other U.S. states. Specifically, New Mexico's crude oil production increased by 0.3 million barrels per day (b/d), reaching a record high of 1.6 million b/d for the state (Roman, 2023). This significantly impacts both the global energy landscape and regional economies, including the state of New Mexico.

Despite the upsides, there are several downsides to the extraction of shale oil and gas. The recovery of shale oil and gas generates the byproduct produced water (PW) in large quantities. PW is water generated from subterranean formations that are brought to the surface during the extraction of oil & gas. PW is also referred to as brine, saltwater, and formation water. Due to its exposure to hydrocarbon-rich formations, this water possesses certain chemical traits from both the formations and the hydrocarbons. It encompasses water originating from the reservoir, water previously introduced into the formation, and any chemicals introduced during production procedures (Clark & Veil, 2009). PW is a combination of organic and

inorganic materials and the physical and chemical properties of PW depend on factors such as the geological location of the field, its geological formation, the lifetime of its reservoirs, and the type of hydrocarbon product being produced affect its characteristics (Fakhru'l-Razi et al., 2009). PW contains high levels of total dissolved solids (TDS), oil and grease, suspended solids, major elements, transition metals, naturally occurring radioactive material (NORM), organic compounds, microbes, and chemicals in hydraulic fracturing (HF) fluids such as biocides and surfactants. The chemicals in HF fluids reported by Fracfocus (fracfocus.org) may also be included in PW (Scanlon et al., 2020). Thus, the disposal and management of PW have gained significant public concern as it may pose a threat to human health and the environment. Water scarcity in several areas requires us to treat PW for beneficial reuse. PW may still include certain levels of organic pollutants even after being treated with the most sophisticated water treatment systems. Conventional water quality parameters alone cannot ensure the safe discharge and reuse of the treated water due to the complexity of PW composition. PW has been found to include small percentages of organic compounds that could disrupt the endocrine system while interfering with hormone action, as it contains mixtures of petrogenic alkylphenols and naphthenic acids (Scarlett et al., 2012). Limited information exists about the impact of PW disposal and reuse on the endocrine-disrupting effects. Therefore, this study evaluates the endocrine-disrupting effects of treated PW from the Permian basin using a human cell line. The objectives of this study are to utilize in vitro bioassays to assess the cytotoxicity of PW on human cell lines and assess the endocrine-disrupting behaviors of treated and untreated PW on human cell lines.

4. Description of methodology employed

4.1 Produced Water Treatment Technologies Utilized

This study used PW generated from the Permian Basin. Two samples were collected from two different desalination treatment systems from Orla, Texas.

4.1.1 Desalination Treatment Technology Used for Permian Basin PW

The PW samples were collected from a pilot-scale low-temperature thermal desalination unit that treats raw PW from the Permian Basin in Orla, Texas. Initially, the raw PW is pretreated with 30% hydrogen peroxide (H_2O_2) and filtered using a basket strainer (1/16" mesh screen) to decrease the amount of hydrogen sulfide (H_2S) formed and the main solids of the raw water before treating it in the main system.

The TDS of raw PW was found to be between 100,000-170,000 mg/L after 500 barrels/day of raw PW was fed into the reactor. The distillation unit used to treat these samples was a modular pilot-scale water treatment system powered by low-grade exhaust waste heat from a gas compressor. The unit contained four process loops: heating, evaporator, condenser, and cooling, operating at low temperatures and in vacuum conditions. The waste exhaust heat generated from the compressor station was recovered. It provided an average temperature supply of 85 ± 5°C. The unit was operated continuously in these conditions for 18 days, and the distillate was collected and stored at 4°C and transported for post-treatment and analysis.

4.1.2. Post-Treatment Technology Used for the Distillate from the Permian Basin PW

The Permian Basin has reported high ammonium and TOC (total organic carbon) concentrations of approximately 450-880 mg/L and 50-140 mg/L. Some of the ammonia and volatile organic compounds could stay in the distillate due to their low boiling points. Therefore, even after thermal distillation, further treatment is sometimes required to remove ammonia and residual TOC before safe disposal or reusing the treated PW. After thermal desalination, the distillate contained around 44.93 mg/L of ammonia and 42.32 mg/L of TOC. Zeolite is a porous aluminosilicate mineral with unique adsorption and cation exchange properties that enable it to be used as an adsorbent for ammonia removal. In this study, the clinoptilolite (Double Eagle, Casper WYO), the most abundant natural zeolite type, was packed in a cylindrical column to remove ammonia. The media in the zeolite column had a particle size distribution between 280-860 μm and a pore volume of 7%. Granular Activated Carbon (GAC) has been widely used for the removal of organic compounds from water due to its high surface area and strong adsorption capacity. GAC was also packed in the column in this study to remove the residual organics in the distillate, and finally, GAC followed by zeolite (GAC+ zeolite) as a unified treatment train was set up to remove organics, metals, and ammonia. The media in the GAC column (Aqua-Tech, Spectrum Brand, Inc.) has a particle size range of 425-710 μm and a pore volume of 11%. The height of the column was 30 cm, and it had an internal diameter of 2.5 cm. More operational conditions for the two columns with zeolite and GAC and GAC+zeolite are shown in Table 1 below.

Table 1: Operation conditions maintained for post-treatment

	1	2	3	
Parameters	**Zeolite**	**GAC**	**GAC + Zeolite**	
Media height (cm)	28	28	28	28
Total Bed Volume (L)	0.147	0.147	0.147	0.147
Flow rate Q (L/h)	0.12	0.12	0.12	0.12
Empty Bed Contact Time (EBCT) (h)	1.23	1.23	1.23	1.23

However, the GAC+Zeolite treatment train was the best treatment option as its removal percentages were high in potential stressors, including volatile organics, ammonia, TOC, cadmium, chromium, zinc, and manganese. After post-treatment, the ammonia was reduced to less than 0.015 mg/L, and TOC was decreased to 5.4 mg/L.

4.2 Cell Culture and Sample Preparation for Cytotoxicity Assays

4.2.1 Cell Culture and Treatments

The effect on different samples of produced water (PW) was tested on human intestinal epithelial (Caco-2) cells, human breast cancer cells (MCF-7), and a specific immortalized cell line derived from an aborted fetus or human embryonic kidney cells (HEK293). These cells were originally obtained from the American Type Culture Collection (ATCC). The cells were cultured in 75 cm^2 sterile tissue culture flasks (Thermo Fisher Scientific, MA, USA) containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. They were incubated in an atmosphere of 5% CO_2 at 37 °C. When the confluency of cells surpassed 80%, the cells were washed twice with Phosphate Buffer saline (PBS) and then detached using 0.25% Trypin-EDTA solution. The subculturing process was done once a week to perform the following assays. To assess the cytotoxic effects of PW, each of the three cell types underwent exposure to various dilutions, spanning from 6.25% to 50% PW. Treated produced water (PW) samples were utilized for the proposed study following desalination

processes from the Permian Basin. These PW samples were obtained as effluent from pilot or laboratory-scale desalination treatment units. Post-treatments, such as zeolite and granular-activated adsorption, were conducted since the concentrations of ammonia and total organic carbon (TOC) remained high. The feed PW before treatment was also used in this study for comparison purposes. Concurrently, untreated cells (used as a control) and blanks containing only the culture media were included in each experiment during the cytotoxicity assays. All treatments, controls, and blanks were performed in replicates.

4.2.2 MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) Assay

The MTT assay will measure the cellular metabolic activity during exposure to PW dilutions. The MTT assay is a colorimetric assay for the non-radioactive quantification of cell proliferation and viability, which is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. In this study, the CyQUANT MTT cell viability Assay Kit (Thermo Fisher Scientific, USA) was used to evaluate the cell viability after PW exposures according to the manufacturer's guidelines with minor adjustments. The cells were detached from the tissue culture flask and resuspended in fresh DMEM. A hemocytometer was used for cell counting. After a concentration of 1 x 10^5 cells/well was reached the cells were seeded (100 µL) in a 96-well plate and left to recover and adhere at 37 °C for 24 h. Next, the medium was removed and replaced with 100 µL of PW dilutions ranging from 6.25% to 50% (involves diluting different volumes of cell culture medium and deionized water to achieve 6.25% to 50% PW dilutions). The cells were incubated at 37 °C for 24 h. Afterward, 10 µL of MTT labeling reagent was added to each well and incubated for 4 h at 37 °C. After 4 h, 100 µL of solubilization solution (SDS-HCl) was added to each well to dissolve any purple crystals formed and incubated for another 4 h at 37 °C. Ultimately, the optical density (OD) was measured at 570 nm and 690 nm utilizing either the Epoch™ Microplate Spectrophotometer (BioTek, VT, USA) or the Synergy HTX Multimode Reader (BioTek, USA). The OD for each sample was determined by subtracting the 690 nm measurement from the 570 nm measurement (Hu et al., 2022) (Aziz et al., 2014). The percentage of cell viability was computed using the following formula:

$$\text{Cell Viability (\%)} = \frac{Mean\ OD\ of\ experimental\ samples - mean\ OD\ of\ blank}{Mean\ OD\ of\ controls - mean\ OD\ of\ blanks} \text{ x } 100$$

4.2.3 LDH (Lactate Dehydrogenase) Assay

LDH is a cytosolic enzyme in various cell types released into the cell culture medium upon damage to the plasma membrane which is a typical characteristic of cells undergoing apoptosis, necrosis, and forms of

cellular damage (Kamiloglu et al., 2020). LDH levels are related to cell membrane disruption and are a reliable indicator of cytotoxicity (Gökhan, 2022). The CyQuantTM LDH cytotoxicity assay kit (Thermo Fisher Scientific, USA) was used to measure cell lysis after exposure to PW according to the manufacturer's guidelines with minor adjustments. The optimum cell density for the LDH assay was determined by empirically performing a "total lysis" using different numbers of cells and plotting the resulting absorbance reading against the cell number. The optimum cell number was selected as 10,000 cells/100 µL (1 x 10^5 cells/ml). All cell types were seeded at a density of 1 x 10^5 cells/ml in a 96-well plate and allowed to attach by incubating at 37 °C for 24 h before treatment with different dilutions of PW. Then, the culture medium was removed, and the 96-well plate was divided into three sections; spontaneous LDH activity (treated with medium and DI water), maximum LDH activity (treated with medium, DI water), and compound-treated LDH activity where it was replaced with PW dilutions (ranging from 6.25% to 50% PW and diluting with medium and DI water) and incubated for 24 h at 37 °C. This induces membrane damage which causes cytotoxicity and LDH release. Next 10 µL of 10 x lysis buffer was added into the maximum LDH activity wells and incubated for 45 min at 37 °C. Afterward, 50 µL of the supernatant from each well was transferred to a new 96-well plate with the addition of 50 µL of the reaction mixture. It was incubated in the dark for 30 min and the reaction was stopped by the addition of 50 µL of stop solution. Finally, the optical density (OD) was measured at 490 nm and 680 nm utilizing either the Epoch™ Microplate Spectrophotometer (BioTek, VT, USA) or the Synergy HTX Multimode Reader (BioTek, USA). The OD for each sample was determined by subtracting the 680 nm measurement from the 490 nm measurement. The percentage of cytotoxicity was determined using the following formula:

$$\text{Cytotoxicity (\%)} = \left[\frac{\text{Compound-treated LDH activity} - \text{Spontanous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}}\right] \text{x } 100$$

4.2.4 CellTiter-Glo® 2.0 Cell Viability Assay

The CellTiter-Glo® 2.0 assay (Promega, Madison, USA) assesses viable cell count in culture by measuring the Adenosine triphosphate (ATP) levels, an indicator of metabolic activity. The cellular ATP reacts with luciferase, producing luminescent light proportional to the ATP concentration. The quantity of ATP directly correlates with the number of live cells in the culture (Bertoletti et al., 2022). Cells were all seeded in 96-well white opaque-walled plates at a density of 1 × 10^5 cells/well (1 × 10^6 cells/ml) and left to adhere at 37 °C for 24 h. Next, the medium was removed and replaced with 100 µL of PW dilutions ranging from

6.25% to 50% (involves diluting different volumes of cell culture medium and deionized water to achieve 6.25% to 50% PW dilutions). The cells were incubated at 37 °C for 24 h. Afterward, 100 µL of CellTiter-Glo® 2.0 reagent was added to each well. The contents were shaken on an orbital shaker for 2 min to induce cell lysis. The plate was incubated at room temperature for 10 min to stabilize the luminescent signal. Finally, the luminescence was recorded using a microplate reader Synergy HTX Multimode Reader (BioTek, USA) (Nowak et al., 2018). The study was performed in triplicates, and the cell viability was presented as a percentage of luminescence from control cells (Malinowski et al., 2022).

4.2.5 Caspase-Glo® 3/7 Apoptosis Assay

Caspase-3 and caspase-7 are both activated universally during apoptosis. Caspase-3/7 Activity was measured for cell apoptosis. The Caspase-Glo® 3/7 Assay (Promega, Madison, USA) kit is a homogeneous, luminescent assay for measuring caspase-3 and -7 activities. The purpose of caspase-3 is to control DNA fragmentation and changes in morphology due to apoptosis. In contrast, caspase-7 serves a small role in this process, however, it is mainly important to the loss of cellular viability. Caspases 3 and 7 play a major role in this area. It utilizes a luminogenic caspase-3/7 substrate, with the DEVD tetrapeptide sequence, in a reagent optimized for caspase and luciferase activities and cell lysis. The "add-mix-measure" format involves adding a single Caspase-Glo® 3/7 Reagent, leading to cell lysis, caspase cleavage of the substrate, and generating a luminescent signal produced by luciferase. The luminescence produced is proportional to the amount of caspase activity present. This assay was performed using MCF-7 cell lines. A cell density of 10^4 cells/well (1×10^5 cells/ml) was selected according to the manufacturer's guidelines (Gokhan, 2022). Cells were seeded in a white opaque 96-well plate and allowed to attach for 24 h at 37 °C. Next, the medium was removed and replaced with 100 µL of 50% PW (dilutions were made with 50 µL, 20 µL concentrated medium and 30 µL DI water). PW after thermal distillation (Distillate) and distillate after the posttreatment of GAC and zeolite were used to expose MCF-7 cell lines. The cells were incubated at 37 °C for 24 h. Afterward, the Caspase-Glo® 3/7 reagent was prepared by equilibrating the Caspase-Glo® 3/7 buffer and lyophilized Caspase-Glo® 3/7 substrate to room temperature and transferring the contents of the Caspase-Glo® 3/7 buffer bottle into the Caspase-Glo® 3/7 substrate bottle. The content was mixed to thoroughly dissolve the Caspase-Glo® 3/7 reagent. Next, the 96-well plate was removed from the incubator and allowed to equilibrate to room temperature. 100 µL of Caspase-Glo® 3/7 reagent was added to each well containing 100 µL of blank, negative control cells and treated cells in culture media. The plate was gently mixed using a plate shaker at 500 rpm for 30 seconds. The plate was incubated at room temperature for 30 min to 2 hr. The luminescence of each sample in the plate was

recorded every 30 min using a microplate reader Synergy HTX Multimode Reader (BioTek, USA) (Miret et al., 2006). A blank to measure background luminescence and a negative control to determine the basal caspase activity of the cell culture system were also carried out for comparison. The experiment was performed in replicates. The data was represented as a percentage of the negative control (Mfotie Njoya et al., 2018).

4.2.6 Griess Reagent Kit for Nitrite Determination

Nitric oxide (NO) serves as a molecular mediator in various physiological processes, including vasodilation, inflammation, thrombosis, immunity, and neurotransmission. Upon addition of the PW to the human cell lines it causes an activation of the immune cells. The immune cells release various inflammatory mediators such as NO which is detected by the Griess assay, with a detection limit of 1.0 μM nitrite. The Griess Reagent Kit (G-7921, Invitrogen, Thermo Fisher Scientific) provides all necessary components for nitrite quantitation in biological samples, where sulfanilic acid reacts with nitrite to form a diazonium salt, subsequently coupling with N-(1-naphthyl)ethylenediamine to create an azo dye measurable at 548 nm. This nitrite assay was performed by using HEK293 and MCF-7 cell lines after exposure to PW samples (feed, distillate, GAC+ zeolite post-treated PW). A calibration curve was created initially to convert absorbance readings to nitrite concentrations. Sodium nitrite solutions (1-100 μM) were prepared by diluting the standard solution with DI water (Privat et al., 1997). The Griess reagent, consisting of 10 μL N-(1-naphthyl) ethylenediamine and 10 μL sulfanilic acid, was prepared (20 μL per well). The assay took place in a 96-well plate, with each well totaling 300 μL (20 μL Griess reagent, 150 μL nitrite-containing sample, 130 μL DI water). The nitrite-containing sample was prepared by collecting the supernatant from each well containing MCF-7 and HEK-293 cell lines after 24 h exposure to the Permian basin PW samples with medium and DI water (50% and 80% PW concentrations were used for the nitrite determination assay). After adding 20 μL Griess reagent and 130 μL DI water to 150 μL sample, the 96-well plate was incubated at room temperature for 30 min. A photometric reference was also prepared by mixing 20 μL of Griess reagent with 280 μL of DI water. The absorbance in each well was measured using the optimum measurement wavelength of 548 nm. Next, the absorbance readings were converted to nitrite concentration using the calibration curve (Guevara et al., 1998) (Berisha et al., 2020).

4.3 Gene Expression After Exposure to Treated PW

4.3.1 Total RNA Extraction

Initially, MCF-7 cell lines were seeded in a 24-well plate at a cell density of 6.0 x 10^5 cells/ml and incubated in an atmosphere of 5% CO_2 at 37°C for 24 h to let it adhere. After 24 h, the media was removed carefully, exposed to culture media with 50% and 80% PW, and incubated at the same conditions for another 24 h. RNA extraction was conducted by using the PureLink® RNA Mini Kit (Thermo Fisher Scientific, United States) following the manufacturer's protocol. After 24 h exposure to PW, PW samples were removed from the 24-well plate before adding the prepared Lysis Buffer. Homogenization was carried out by transferring the lysate to a 1.5 mL RNase-free tube and passing it 5-10 times through an 18-gauge needle attached to an RNase-free syringe. The concentration of the RNA was measured by using the BioTek Epoch Microplate Spectrophotometer with a Take3 microvolume plate. The purity of RNA was analyzed by using the ratio of the absorbance value measured at 260 and 280 nm. Then, the remaining RNA was stored at -80°C for future use (Zhao et al., 2023).

4.3.2 Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

The expression of the following genes listed in Table 2 was assessed using RT-qPCR. The GAPDH gene is used as a housekeeping gene to normalize gene expression. The primers were validated based on previous literature (Van Houghton et al., 2024).

Table 2: Genes assessed using RT-qPCR

Gene	Primer	Function
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	GAPDH forward – 5'-GGCCTCCAAGGAGTAAGACC-3' GAPDH reverse – 5'-AGGGGTCTACATGGCAACTG-3'	GAPDH is a key regulatory enzyme falling into the category of a housekeeping gene that catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate during glycolysis. These genes are used as reference genes for normalizing gene expression data derived from different cell types or after exposure to various experimental treatments using RT-qPCR (NIH, 2024e).
CYP19A1 (cytochrome P450 family 19	GAPDH forward 5'-CTCCTCATCAAACCAGACATC-3' GAPDH reverse 5'-TAAAATCAACTCAGTGGCAAAG-3'	Member of the cytochrome P450 superfamily of enzymes. Responsible for encoding aromatase. This enzyme also

subfamily A member 1)		converts androgens, male sexual development hormones, to a female sex hormone, estrogen (NIH, 2024c).

Before beginning the cDNA preparation, all RNA samples were treated with DNase I, Amplification Grade (Thermo Fisher Scientific), to eliminate DNA via hydrolysis before cDNA synthesis. 8 µL of each RNA sample was mixed with 1 µL of 10× DNase I Reaction Buffer, 1 µL of DNase I, AMpt Grade, 1 U/µL in an RNase-free, 0.5 ml microcentrifuge tube and incubated at room temperature for 15 min. Afterward, 1 µL of 25 mM EDTA solution was added to the reaction mixture to inactivate the DNase I. Next, the RNA sample was heated at 65°C for 10 min and was ready for the reverse transcription.

Afterward, reverse transcription was done to form cDNA using the iScriptTM cDNA Synthesis Kit made by Bio-Rad. To make up a total volume of 20 µL per reaction, 4 µL of 5× iScript Reaction Mix, 1 µL iScript Reverse Transcriptase, and 11 µL of Nuclease-free water were added to PCR tubes. 4 µL of relevant pure RNA was added to each PCR tube, and the complete reaction mix was incubated in a thermal cycler at the following temperatures to synthesize the cDNA. The temperature for priming was set for 5 min at 20°C, reverse transcription was for 20 min at 46°C, RT inactivation was for 1 min at 95°C and the PCR tubes were held at 4°C until the next qPCR step was prepared. qPCR reactions were performed in 96-well plates with a system volume of 20 µL, containing 10 µL of 2× SsoAdanved Universal SYBR Green Supermix (Bio-Rad), 1 µL of each forward and reverse primer (10 µM), 2 µL of diluted DNA sample and 6 µL of DNA/RNA free water. Amplification and detection were carried out in a CFX Connect Real-Time system (Bio-Rad, Hercules, CA) after an initial denaturation of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, primer annealing and extension at 60 °C for 60 s. The gene expression was determined using the ddCT (Delta-Delta-CT) method. The fold change was calculated and normalized relative to the control values for cells grown in the absence of endocrine disruptors.

4.4 E-Screen Assay for the Determination of Estrogenicity

The cells cultured in 75 cm^2 sterile tissue culture flasks containing DMEM supplemented with 10% FBS, 1% penicillin-streptomycin solution, and 2 mM L-Glutamine were trypsinized, detached and cultured in a 96-well plate at a cell density of 10,000 cells per well and left to adhere for 24 h. Afterward, the cells were exposed to PW concentrations of 50% and 80% in phenol-red free DMEM containing 10% estrogen-free charcoal-stripped bovine serum, 1% penicillin-streptomycin solution, and 2 mM L-Glutamine. Each

concentration had 4 replicates per assay. The negative control had 6 replicates without hormones, and the internal positive control had 17β-Estradiol (E2) in five concentrations between 10^{-12} M and 10^{-9} M. The assay was terminated on day 6, and the cell proliferation was assessed by counting the cell number in each well using the colorimetric Sulforhodamine B (SRB) Cell Cytotoxicity Assay Kit. The SRB assay was performed without removing the culture medium and adding ¼ volume (e.g., 50 µL in 200 µL of culture medium) of Fixation Solution II to each well and incubating the plate for 1 hour at 4°C. The solution was removed and washed with 200 µL of DI water 3 times by pipetting without damaging the cell monolayer and allowed to dry at room temperature. The cells were stained by adding 45 µL of SRB Solution to each well and leaving it for 15 minutes at room temperature in the dark. The SRB Solution was removed, and each well was washed 4 times using 200 µL of 1× Wash Buffer. The wash buffers were removed by pipetting, and the plate was air-dried. Next, solubilization was done by adding 200 µL 1× Solubilization Solution to each well and shaking the plate on a plate shaker for 10 min at room temperature. The absorbance was measured using the Synergy HTX Multimode Reader (BioTek, USA) at 565 nm. The replicates were averaged for each concentration, positive and negative controls, and the proliferative effect (PE) and relative proliferative effect (RPE%) were calculated using the equations below (Schilirò et al., 2011),

$$PE = \frac{cell\ number\ max.(sample)}{cell\ number\ (negative\ control)}$$

$$RPE\ \% = \frac{(PE-1)sample}{(PE-1)E2} \text{ x } 100$$

4.5 Statistical Analysis

The results of endocrine-disrupting activity were expressed as a percentage of control and were reported as the mean ± SE. One-way ANOVA using Excel was used to determine the statistically significant differences compared to the positive control. An alpha value of 0.05 was used for all statistical tests and data with $p \leq 0.05$ was significantly different (*). Visualization of the results was done using GraphPad Prism 10.

5 Description of results; include findings, conclusions, and recommendations for further research.

5.1 Chemical Characteristics of the Treated PW

Table 3 shows the feed, distillate, and GAC+zeolite characteristics of the PW collected from Orla and Big Springs, Texas.

Table 3: Feed, Distillate, and GAC+zeolite characteristics for PW collected from Orla, Texas

Parameter	Units	Feed	Distillate	Post-treated (GAC+Zeolite)
NH_3	mg N-NH_3/L	611	44.93	<0.015
TDS	mg/L	103552	287	
TOC	mg/L	74.39	42.32	5.40
TPH	mg/L	28.0	11.33	N/A
EC	µS/cm	119056	326	171
pH	unitless	6.58	8.54	7.4
ρ	g/cm^3	1.068	1.001	
Fe^+	mg/L	4.44	0.265	0
Mn^{2+}	mg/L	0.457	0.006	0
Na^+	mg/L	24795	72.5	59.67
Ca^{2+}	mg/L	3232	6.512	4.683
Mg^{2+}	mg/L	509	1.442	0.790
K^+	mg/L	405	0.948	1.437
Ba^{2+}	mg/L	3.654	0.060	0.0062
Sr^{2+}	mg/L	838	1.588	0.008
Al^{3+}	mg/L	0.268	0.004	0
Li^+	mg/L	21.92	0.004	0
Zn^{2+}	mg/L	0.038	0.015	0
Pb^{2+}	mg/L	0.006	0.000	0
HCO_3^-	mg/L	164	179.6	
SO_4^{2-}	mg/L	345	0.664	1.624
Cl^-	mg/L	46672	84.0	1.999
H_3BO_3	mg/L	216	1.24	
SiO_2	mg/L	19.75	0.097	

PO_4^{+}	mg/L	2.33	2.54	0.0198
Si^{4+}	mg/L	9.22	0.045	8.675
Benzene	mg/L		0.501	N/A
Toluene	mg/L		0.548	N/A
Phenanthrene	mg/L		0.0014	N/A
Phenol	mg/L		0.026	0.000693
Cd	mg/L		0.0011	0.0014
Cu	mg/L		0.0095	0.0173
Cr	mg/L		0.0003	0.0000
Zn	mg/L		0.0031	0.0000
Mn	mg/L		0.0115	0.0000
Hardness	mg $CaCO_3$/L		0.52	15.82

5.2 Toxicity Assay Results of PW samples

5.2.1 Cell Viability Based on MTT Assay

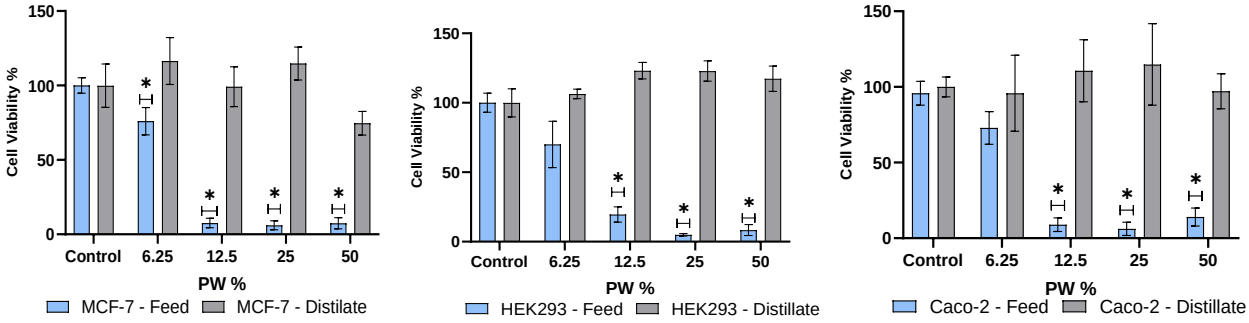

Figure 1: Cell viability for feed and distillate on MCF-7, HEK293 & Caco-2 cell lines obtained from MTT assay. The * represents the statistical difference (P_value < 0.05) in contrast to the control.

MTT assay evaluates the cellular metabolic activity by measuring the conversion of the MTT tetrazolium dye to the amount of purple formazan formed with the help of the enzyme oxidoreductase. The amount of purple formazan formed is directly proportional to the cell viability in each well (Berehu et al., 2021). Figure 1 shows a similar trend in the cell viability for the feed and distillate as the percentage of PW

increases. A concentration of 6.25 % feed PW still caused a certain amount of significant toxicity in the MCF-7 cell line (P_value < 0.05); however, not in HEK293 and Caco-2 cell lines (P_value > 0.05). The mean cell viability at 6.25 % feed PW in the MCF-7, HEK293, and Caco-2 cell lines were 79.5%, 83.7%, and 88.5% respectively. The distillate has no significant cell viability when in contrast to the control. However, the cell viability of MCF-7 cell lines was quite lower than that of the HEK293 and Caco-2 cell lines at a 50% distillate concentration. The reduction in cell viability of the MCF-7 cell lines in the distillate at 50% distillate was insignificant (P_value > 0.05). The feed contained about 611 mg/L NH_3-N (43.6 mM) and 73.49 mg/L TOC. The distillate contained 46.3 mg/L NH_3-N (3.31 mM) and 42.3 mg/L organic concentration (TOC). The effect of ammonia with ammonium chloride concentrations ranging from 0, 0.05, 0.5, 5, 25, and 50 mM on bovine mammary epithelial cells showed that the cell viability reduced significantly when the concentration was equal to or greater than 5 mM ($P < 0.05$) (Wang et al., 2018). The highest concentration of ammonia used in this study was 21.8 mM NH_3-N at 50% in the feed PW. Since the concentration of ammonia is very high in the feed and is greater than 5 mM we can state it is likely to cause the cell viability to be reduced significantly. However, in our study, the highest concentration of ammonia in the distillate used for the assay only contains 1.65 mM of ammonia at 50%. This has no significant cell viability on the human cell lines, indicating that the ammonia in the distillate does not cause any significant cell viability. We conducted a control with an ammonia concentration of 3.3 mM and obtained a cell viability of 100%, indicating no toxicity.

5.2.2 Cell Viability based on CellTiter-Glo® 2.0 Assay

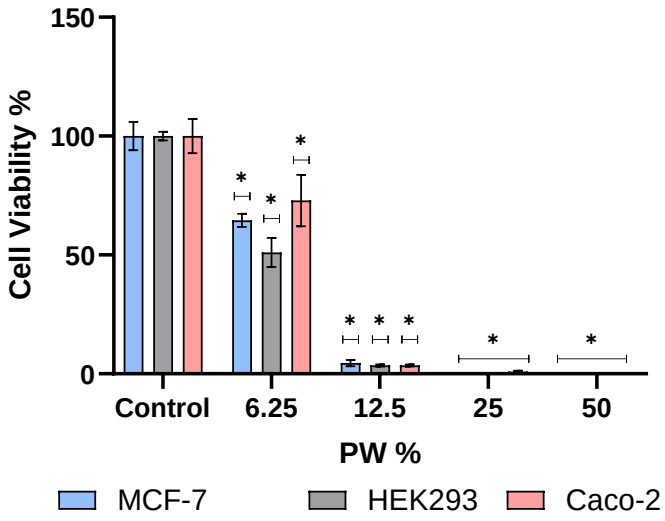

Figure 2: Cell viability for feed on MCF-7, HEK293 & Caco-2 cell line obtained from CellTiter-Glo® 2.0 Assay. The * represents the statistical difference (P_value < 0.05) in contrast to the control.

The CellTiter-Glo® 2.0 assay is an alternative to the tetrazolium salt reduction assay. It was carried out due to the limitations of the MTT assay, which has shown inconsistent results caused by chemical interference. Nowadays, there is an increase in the number of assays based on intracellular ATP measurements. The CellTiter-Glo® 2.0 assay is more accurate and provides more reliable cytotoxicity results compared to the MTT assay (Malinowski et al., 2022) (Nowak et al., 2018).

Figure 2 shows how the cell viability has reduced with increasing concentration of feed PW. Similar trends were observed in the MTT assay as well. However, this assay shows total cell death at 25% and 50% feed PW, indicating no background absorbance in the CellTiter-Glo® 2.0 assay. Different from the MTT assay at 6.25 % feed PW, there was a significant decrease in cell viability (P_value < 0.05) in all three cell lines.

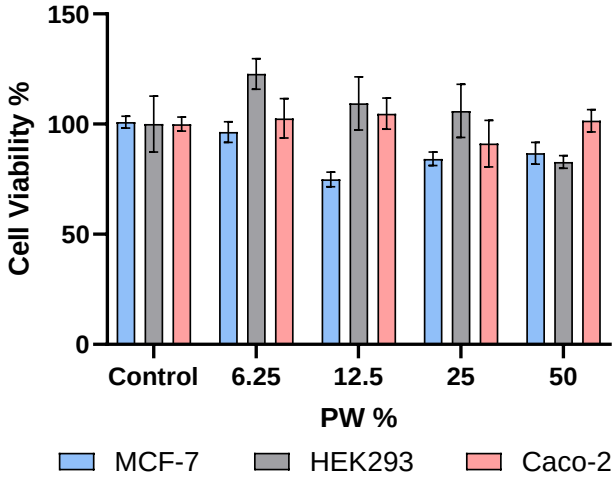

Figure 3: Cell viability for distillate on MCF-7, HEK293 & Caco-2 cell line obtained from CellTiter-Glo®2.0 Assay

Similar to the MTT assay, no significant impact on cell viability was observed with increasing concentration of distillate PW. Cell viability seems to be affected when the MCF-7 cell lines are exposed to 12.5% and 25%; however, the P_value of > 0.05 suggests the effect is insignificant in contrast to the control.

5.2.3 Cytotoxicity Based on LDH Assays

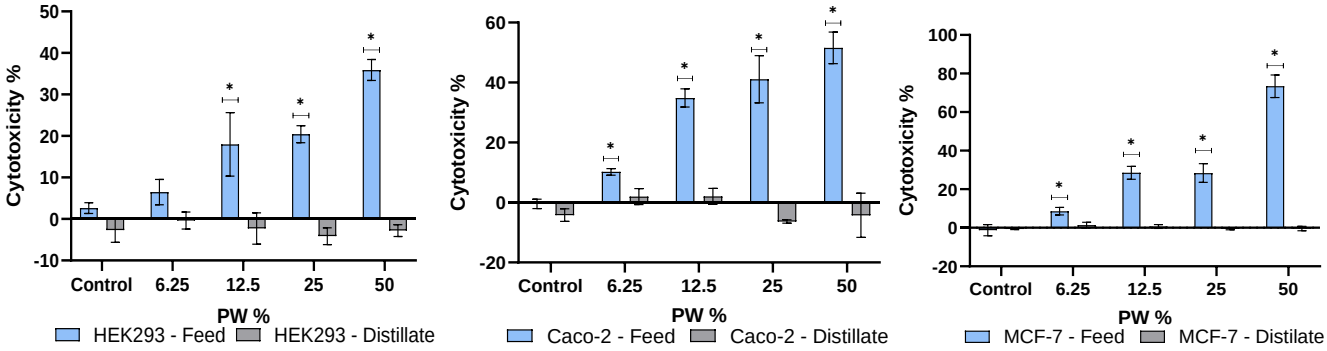

Figure 4: Cytotoxicity for feed and distillate on MCF-7 cell line obtained from LDH assay. The * represents the statistical difference (P_value < 0.05) in contrast to the control.

The LDH assay is based on releasing and losing intracellular LDH into the culture media. It is a reliable and quick test compared to the MTT assay. LDH test indicates irreversible cell death due to cell membrane disruption and necrosis (Fotakis & Timbrell, 2006). The extracellular LDH produced by the cells to the media can be evaluated by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH. NADH is oxidized by diaphorase, which reduces a tetrazolium salt (INT) to a red formazan product measured at 490 nm (ThermoFisher-Scientific, 2019). According to Figure 4, a similar trend in cytotoxicity is observed in all three cells where the cell membrane disruption or necrosis increases with increasing PW percentage. At 6.25% feed PW significant cytotoxicity was observed in Caco-2 and MCF-7 cell lines (P_value < 0.05). Compared with the Caco-2 and HEK293 cell lines, the MCF-7 cell line at a feed PW 50% showed a cytotoxicity greater than 65%, indicating the MCF-7 is more sensitive than the Caco-2 and HEK293 cell lines. The distillate PW did not result in significant cell membrane disruption with increasing concentrations of PW when in contrast to the control. This suggests that the distillate has no cytotoxicity on the human cell lines.

5.2.4 Cell Apoptosis based on Caspase-Glo® 3/7 Assays

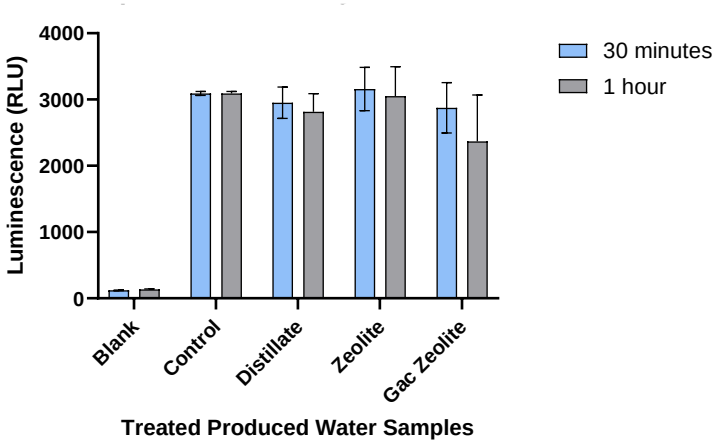

Figure 5: The production of caspase 3 and caspase 7 from MCF-7 cells after exposure to 50% distillate, distillate with zeolite post-treatment, and distillate with GAC followed by zeolite post-treatment

Cell apoptosis, also called cell-programmed death, happens when the cells are active participants in their cell death under normal physiological conditions. Factors such as lack of growth factors and changes in the hormonal environment are a few physiological signals that influence cell apoptosis. When cell

apoptosis occurs, changes in morphology are observed, such as caspase activation, separation of the cytoplasm and nucleus into membrane-bound vesicles containing ribosomes, and mitochondria that are intact morphologically (Miret et al., 2006). After the cells are exposed to PW, apoptosis occurs because the Z-DEVD substrate is cleaved by active caspase 3/7 releasing aminoluciferin. In the presence of ATP and oxygen, aminoluciferin reacts with the enzyme Luciferase to produce luminecence. The amount of luminescence produced is proportional to the caspase 3/7 activity undergone in the human cells. Cleaving these proteins may lead to cell-programmed death (Manzano et al., 2024). The maximum luminescence was recorded at 30 minutes. According to Figure 5, at PW 50%, no significant apoptosis difference between distillate and post-treated PW was observed in comparison with the control. A previous study tested the exposure to ammonium chloride. It was observed that at an ammonia concentration of 5 mM, the rate of apoptosis increased significantly in MAC-T cells in contrast to the control (Wang et al., 2018). Since 50% of distillate PW only contains around 1.65 mM ammonia, it does not seem to impact the cell apoptosis of the MCF-7 cell lines.

5.2.5 Cell Oxidative Stress Based on Nitrate Assays

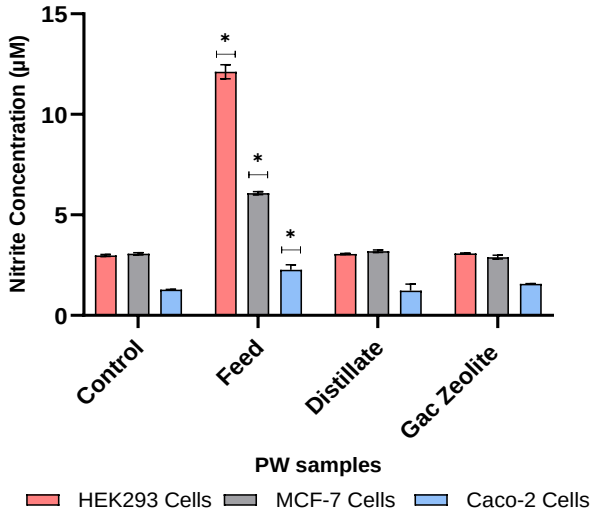

Figure 6: Nitrite concentration evaluated after exposure to 50% feed, distillate, distillate with GAC followed by zeolite post-treatment PW. The * represents the statistical difference (P_value < 0.05) in contrast to the control.

Nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems (Tuteja et al., 2004). NO has been demonstrated to be a crucial molecule in the regulation of acute and chronic inflammation and host defense mechanisms (Tripathi et al., 2007). In this study, nitrite was measured to determine the cell response to PW since it is a primary breakdown product of nitric oxide. The nitrite concentration was evaluated to determine the cell NO response to untreated and treated PW. In contrast to the control, the treated PW (distillate and distillate followed by post-treatment) did not have high nitrite concentrations. However, exposure to feed PW caused HEK293, Caco-2, and MCF-7 cell lines to undergo significant (One-way analysis of variance (ANOVA) showed a $P < 0.05$) cell inflammation or cell oxidative stress. Treated PW did not undergo cell inflammation compared to the feed PW exposed cell lines. With increasing concentrations of ammonia, it has been found that the percentage of cell apoptosis and ROS level increases significantly, indicating ammonia induces cell oxidative stress while inhibiting cell growth if the ammonia concentration is higher than 5mM. Since we have around 305.5 mg/L NH_3-N (21.8 mM) in feed PW, we can conclude that it has caused significant cell oxidative stress. The distillate contains only around 1.65 mM ammonia, so it does not affect the oxidative stress. Each cell is affected differently as the diffusion rate of ammonium ions through the cell membrane varies. Also, the rate of diffusion is low due to the charge of other ions (Wang et al., 2018). An increase in oxidative stress in the three types of human cells can cause damage to the cellular DNA and affect proteins and lipid molecules, which will induce a genotoxic stress response (Phillips, 2019).

5.3 Endocrine Disrupting Effects of Treated PW

5.3.1 The expression of genes related to estrogen production

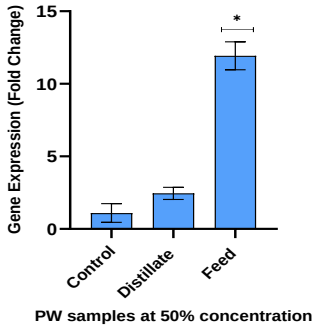

Figure 7: Gene Expression of CYP19A1 using MCF-7 cell line after 24 h exposure to 50% PW. The * represents the statistical difference (P_value < 0.05) in contrast to the control.

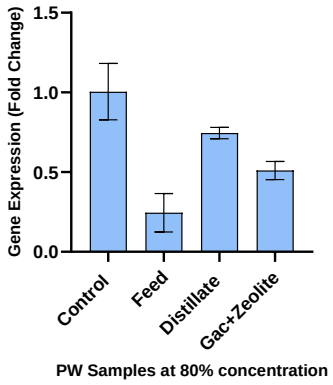

Figure 8: Gene Expression of CYP19A1 using MCF-7 cell line after 24 h exposure to 80% PW

Figures 7 and 8 show the estrogenic activity when the MCF-7 cell lines were exposed to 50% and 80% PW for the gene CYP19A1. The CYP19A1 gene encodes aromatase, an enzyme that transforms androgens into estrogens. The enzyme aromatase is important in biological processes such as cell proliferation, regulation of fat metabolism, and hormone signaling. CYP19A1 has been significantly up-regulated when adding 50% feed PW in contrast to the control (Castro-Piedras et al., 2018). The distillate also showed slight estrogenic activity compared to the control; however, it was not as significant as the estrogenic activity in the feed PW. One-way ANOVA showed a P-value < 0.05 in the feed PW indicating high endocrine-disrupting activity compared to the distillate (P-value > 0.05). Compared with 50% PW, the estrogenic activity in 80% feed PW is lower. This is because cell growth is inhibited, and the percentage of dead cells has increased since the 80% feed PW is more toxic to the MCF-7 cell lines at higher concentrations. The 80% feed PW contains around 488.8 mg/L of ammonia, 59.51 mg/L TOC, 22.4 mg/L TPH, and other chemicals that damage the cell line by causing oxidative stress due to the presence of high concentrations of ammonia, etc. Thus, the estrogenic activity in feed PW is less than the distillate and GAC zeolite post-treated PW.

5.3.2 Estrogenic Activity Observed from E-Screen Assay

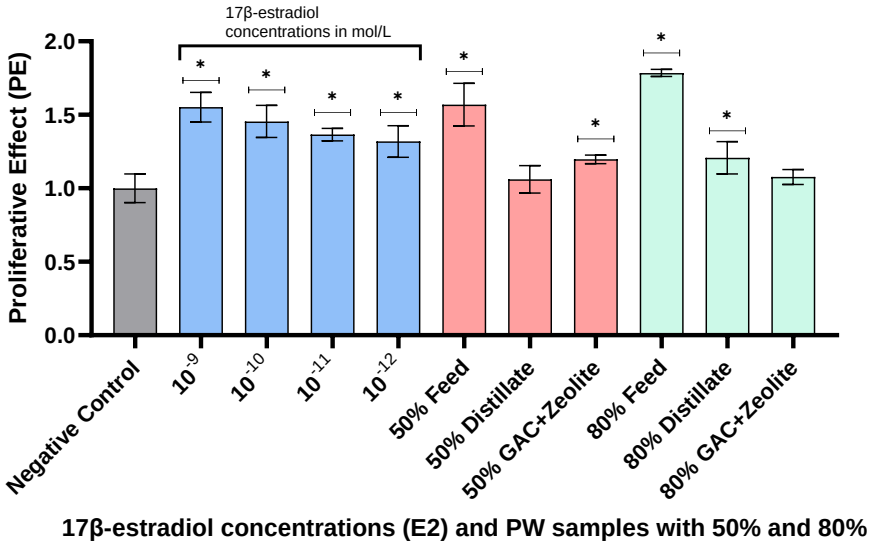

Figure 9: Proliferative effect induced in MCF-7 cell lines by 17β-estradiol (E2), 50% and 80% feed, distillate, and GAC+Zeolite Orla, Texas PW. The * represents the statistical difference (P_value < 0.05) compared with the negative control

Table 4: The RPE% (relative proliferation effect) of 50% and 80% feed, distillate, and GAC+Zeolite treated PW on MCF-7 cell lines in samples from Orla, Texas

Sample	10^{-9}M E2	10^{-10}M E2	10^{-11}M E2	10^{-12}M E2
17β-estradiol (E2) Positive Control	100.00	100.00	100.00	100.00
50% Feed	103.23	125.30	156.12	178.74
50% Distillate	10.98	13.32	16.60	19.01
50% GAC+Zeolite	35.66	43.28	53.93	61.74
80% Feed	142.15	172.53	214.97	246.12
80% Distillate	37.54	45.57	56.78	65.01
80% GAC+Zeolite	13.96	16.95	21.12	24.18

PW is known to cause endocrine-disrupting effects due to the presence of EDCs (He et al., 2010). These EDCs interact with hormone receptors, mimicking hormone activity and altering the functions of the endocrine system. Such interactions can adversely affect human and animal health (Schilirò et al., 2011). Therefore, we employed the E-Screen assay to assess the estrogenicity after exposure to treated and untreated PW. E-Screen assay uses a cell proliferation mechanism with estrogen receptors in MCF-7 cell lines to determine the estrogenic activity in the PW samples. Figure 9 shows the PE of the negative control as 1, the positive control with 17β-estradiol (E2) from 10^{-9} to 10^{-12} M between 1.55 and 1.31 respectively. The PE effect at 50% and 80% feed is higher than the treated PW and is greater than the positive control with E2. It can be concluded that 50% and 80% feed PW containing estrogen analog after treatment promotes E2-induced cell proliferation in MCF-7 cell lines compared to the control since PE > PE of E2. The cell proliferation was significantly high in the 80% feed PW ($P < 0.05$ – One-way ANOVA) relative to the negative control with no estrogen treatment. The treated PW (distillate and GAC+Zeolite PW) induced less proliferation compared to the positive control since the PE < PE of E2. However, the distillate at 80% has a significant increase in cell proliferation ($P\_value < 0.05$) which means distillate also has a considerable amount of EDC which caused the regulation of the estrogen receptors in the MCF-7 cell line. The estrogenic activity due to exposure to PW was evaluated by calculating the relative efficacy called the RPE% (relative proliferation effect). Table 4 shows the RPE for the samples mentioned above. An RPE > 100% represents full agonistic activity and an RPE < 100% represents partial agonistic activity (Schilirò et al., 2011).

6 Provide a paragraph on who will benefit from your research results. Include any water agency that could use your results.

Through this study, we can evaluate the potential risks of PW on the environment and human health. We expect that, in comparison to raw PW, the treated PW will exhibit minimal cytotoxicity and low endocrine-disrupting nature. Understanding the endocrine-disrupting nature of treated PW provides essential data for regulatory compliance. Regulators can use this information to set appropriate standards and guidelines for safe disposal or reuse. By studying the effects of different water treatment processes on endocrine disruption, researchers can identify which treatment methods are most effective in removing endocrine-disrupting constituents. The citizens of New Mexico need to understand the risks associated as NM was the second largest state for crude oil production and top 10 for natural gas production in 2022. Most areas

of NM face water scarcity challenges and treating and reusing PW can reduce some scarcity. Understanding the endocrine-disrupting potential ensures that reused PW water meets safety standards and aligns with federal guidelines, thus contributing to sustainable water resource management. In summary, assessing the endocrine-disrupting nature of treated PW in human cells can benefit NM citizens by promoting public health, environmental protection, sustainable resource management, and informed decision-making in the oil and gas industry.

7 Describe how you have spent your grant funds. Also, provide your budget balance and how you will use any remaining funds.

The NM WRRI grant helped my research to go forward because it gave me the resources and support I needed. I was able to collaborate with experts and attend conferences to share my findings while gathering more knowledge. All the budget has been used for the stipend (0.25 FTE, Spring 2024), supplies, and conference travel.

8 List presentations you have made related to the project.

Senuri Wijekoon, Evaluating Endocrine Disrupting Effects of Treated Produced Water on Human Cell Lines, NM WRRI 68th Annual New Mexico Water Conference, Albuquerque, New Mexico, Nov 2023. (poster presentation)

Senuri Wijekoon, Evaluating Endocrine Disrupting Effects of Treated Produced Water on Human Cell Lines, NMSU Biosymposium 2024, Las Cruces, New Mexico, April 2024 (poster presentation)

Senuri Wijekoon, Evaluating Endocrine Disrupting Effects of Treated Produced Water on Human Cell Lines, American Water Works Association (AWWA) 2024, Las Cruces, New Mexico, May 2024 (oral presentation)

Senuri Wijekoon, Evaluating Endocrine Disrupting Effects of Treated Produced Water on Human Cell Lines, State of the Science to Advance Diversified Water Treatment and Reuse, Las Cruces, New Mexico, June 2024 (poster presentation)

9 List publications or reports, if any, that you are preparing.

Senuri Wijekoon, Yeinner Tarazona, Pei Xu and Yanyan Zhang, Evaluating Endocrine Disrupting Effects of Treated Produced Water on Human Cell Lines (manuscript in preparation for journal submission)

10 List any other students or faculty members who have assisted you with your project.

This research was supported by my faculty advisor Dr. Yanyan Zhang, Dr. Pei Xu and Mauricio Tarazona at New Mexico State University.

11 Provide special recognition awards or notable achievements as a result of the research including any publicity such as newspaper articles, or similar.

No applicable

12 Provide information on degree completion and future career plans. Funding for student grants comes from the New Mexico Legislature and legislators are interested in whether recipients of these grants go on to complete academic degrees and work in a water-related field in New Mexico or elsewhere.

Degree completion – 06th August 2024

Future career plans – To do a PhD in Environmental Engineering

Upon completion of my studies, my ultimate objective is to contribute to both industry and academia, leveraging my expertise in environmental engineering to foster a sustainable world.

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