NM WRRI Student Water Research Grant Final Report

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2. Project title: Comprehensive Toxicological Assessment of Raw and Treated Oilfield Produced Water Using the Zebrafish Model

3. Description of research problem and research objectives

Produced water (PW), generated at a rate of approximately 25 billion barrels annually in the U.S., is a complex byproduct of oil and gas extraction. To date, multiple organic and inorganic compounds, salts, radionuclides, production chemicals and other by-products have been identified in PW; many of which are toxic and can exert among others, lethal, mutagenic, carcinogenic, and endocrine disruption effects, posing significant environmental and public health risks. Given the water scarcity in oil and gas producing regions, increasing disposal costs, and the current efforts to mitigate fluid injection-induced seismicity, there is an urgent need for sustainable PW management solutions outside the O&G industry. However, a comprehensive understanding of the chemical composition (identification and quantification) of raw and treated PW constituents and their potential toxicological impacts on key aquatic organisms remains elusive and hinders the development of effective management strategies and regulatory guidelines. This research aims to contribute to the filling of this critical knowledge gap by employing state-of-the-art analytical techniques and advanced *in vivo* toxicity studies using the zebrafish (*D. rerio*) model to unravel the chemical and toxicological behavior of PW constituents across a scalable a PW management treatment train.

The objectives of the research are to (i) identify and quantify the constituents of untreated oilfield PW (raw) and treated PW and (ii) conduct *in vivo* embryonic development, gene expression, and cardiotoxicity on the PW effluents using the zebrafish model.

4. Description of the methodology employed:

Phase 1 - Evaluation of effluents from Pilot-scale treatment systems

4.1.1. PW collection.

The present study used oilfield-produced water generated in Permian Basin (Sample 1) and San Juan Basin (Sample 2) O&G operations. Sample 1 was collected at an existing deep well injection site located near Orla, Texas; and Sample 2 was collected from operations in the Eagle Springs Field, New Mexico.

4.1.2. PW treatment.

Both Produced Water (PW) samples from the Permian Basin and San Juan Basin underwent treatment at a pilot-scale level after preliminary pre-treatment. The sample from the Permian Basin was treated using thermal distillation, while the San Juan Basin sample was processed through Salt Water Reverse Osmosis (SWRO). The average flow rates for the treatments were 633 barrels per day (bbl/d) for thermal distillation and 291 bbl/d for SWRO, respectively.

4.1.3. PW physicochemical characterization

Characterization was based on conventional water quality parameters such as total dissolved solids (TDS), electrical conductivity (EC), pH and other parameters of special interest such as total organic carbon (TOC), ammonia (NH_3).

4.1.4. Toxicological characterization, Fish Embryo Acute Toxicity (FET) Test

Zebrafish embryo acute toxicity tests were conducted according to the Organization for Economic Co-operation and Development (OECD) method 236 (OECD, 2013). Survival apical observations were made daily by using an inverted microscope under a 96-h semi-renewal test scheme. Certified male and female adult zebrafish, originally purchased from Aquatic Research Organisms

(Hampton, NH) were maintained separated in two 20-gallon flow-through aquariums containing treated tap water with Tetra AquaSafe Plus. Aquariums were maintained with continuous aeration at 26 ± 1 °C with a 16:8 h light-dark photoperiod and controlled light intensity (10 - 20 $\mu E/m^2/s$). Fish were fed 2-3 times daily with a scientifically formulated diet for adult zebrafish from Zeigler ® (Zeigler Bros., Inc.) Zebrafish embryos were produced in six parallel 1.5 L breeding chambers with spawning groups of 3 adults with a male/female ratio of 2:1. To avoid genetic bias, exposed embryos were collected from at least three breeding groups with fertilization rates greater than 70%, mixed, and randomly selected. Exposure began 0-2 h post-fertilization (hpf) in sterilized 24-well cell culture plates at 26 ± 1°C. A total of 24 embryos per condition (1 embryo per well) with 3 mL of test solution were exposed; test solutions were renewed at 48 h of exposure. Coagulation of embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat were the apical observations used for accounting embryos lethality during the exposure period. The control survival rate was higher than 90% throughout the testing. MHRW was used as negative control (i.e., 0% effluent) and a solution of 4 mg/L of 3,4-dichloroaniline was used as reference toxicant.

Phase 2 - Monitoring water quality throughout an integrated treatment train

4.2.1. PW collection.

Sample for this phase of the study was collected at an existing deep well injection site located in Big Spiring, Texas.

4.2.2. PW treatment train

The integrated treatment train consisted of a mechanical vapor recompression (MVR) distillation system, granular activated carbon (GAC) adsorption and zeolite ion exchange. Post-treatments with GAC and zeolite aimed to mitigate the organic compounds and the residual ammonia identified in phase 1 as potential stressors after the pilot-scale distillation system. The average flow rates for the units were 3 gallon per min for the MVR distillation, and 15 L/d for the GAC and Zeolite treatments.

4.2.3. Chemical Characterization

Key performance indicators of PW treatment and traditional water quality measurements were us ed to characterize the effluents in Phase 2. The parameters and specific methods utilized to characterize the effluents are listed in Table 1.

Table 1 Methods used in the chemical characterization

Characterization	Symbol	Technique	Protocol	Method
Total organic carbon	TOC	Oxidation (heating and	SM	5310B
Dissolved Organic carbon	DOC	Oxidation (heating and	SM	5310B
Gasoline Range Organics	GRO (C_6-C_{10})	GC	SW846	8015B NM
Diesel Range Organics	DRO (C_{10}-C_{28})	GC	SW846	8015B NM
Oil Range Organics	ORO (C_{28}-C_{36})	GC	SW846	8015B NM
Total Petroleum Hydrocarbons	TPH	GC	SW846	8015B NM
Methylene Blue Active	MBAS	Spectroscopy	SM	5540C
Metals (Total and Dissolved)		ICP	SW846	6010D
Potential of Hydrogen	pH	H^+ Activity	SM	4500 H+ B
Total Dissolved Solids	TDS	Gravimetry	SM	2540C
Total Suspended Solids	TSS	Gravimetry	SM	2540D
Electrical Conductivity	EC	Conductivity	SM	2510B
Total Alkalinity	-	Titration	SM	2320B
Total Hardness	-	Ca^{+2} + Mg^{+2} Calculation	SM	2340B
Turbidity	-	Nephelometry	EPA	180.1
Total Ammonia Nitrogen	TAN	Colorimetry	EPA	350.1
Oxygen, Dissolved	DO	Colorimetry	SM	4500-O G
Chemical oxygen demand	COD	Reactor Digestion/Colorimetry	Hach	8000
Halogens, Total Organic	TOX	Combustion	SW846	9020B
Anions	-	IC	EPA	300.0

4.2.4. Toxicological characterization, Fish Embryo Acute Toxicity (FET) Test

Zebrafish embryo acute toxicity tests were conducted following the methodology described in section 4.1.4. In total, 3 samples were characterized, raw PW, distillate PW, and post-treated PW.

4.2.5. Cardiotoxicity and teratogenic effects on early stages of zebrafish.

Cardiac impairments and teratogenic effects were monitoring in parallel with FET test. Heartbeat was measured between 48 and 96 hpf and abnormal phenotypic development was monitored

between 24 and 96 hpf; among others, physical observation included pericardiac and yolk sac edemas, craniofacial malformations, scoliosis and abnormal pigmentation of the embryos.

4.2.6. Alteration of gene expression on early stages of zebrafish.

To evaluate the potential of the final effluent to alter the endocrine system of *D. rerio,* the expression of 3 key genes – *cyp19a1a, cyp19a1b, Vtg1* – associated with the reproductive system was evaluated at 7d of exposure. Please refer to the Table 2 to review more details about the set of genes studied.

Table 2 Genes evaluated for their expression

Gene	Product	Reported Roles / Impacts
elf 1a	Elongation factor 1α	Transcription factor, housekeeping gene (McCurley and Callard, 2008)
cyp19a1a	cytochrome P450 enzyme	Ovarian differentiation (Lau et al., 2016) Male differentiation (Yin et al., 2017)
cyp19a1B	cytochrome P450 enzyme	Converts androgens into bioactive estrogens (Shaw et al., 2023) Mediate reproduction and sexual behavior (Shaw et al., 2023)
Vtg1	Vitellogenin 1	Fish reproduction – Reduced gonadosomatic index, egg production, yolk granules and mature follicles in ovary Development – Diminished hatching rates, cumulative survival rate, swimming capacity and food intake (Sun et al., 2023)

Total RNA was extracted from pooled larvae (5 individuals) exposed to MHRW and post-treated effluent; 2 replicates were considered for each exposure group (MHRW and post-treated effluent). RNA extraction was conducted using the PureLink® RNA Mini Kit (Thermo Fisher Scientific, United States) following the manufacturer's protocol for purification from animal tissues and a tissue homogenizer (Fisherbrand™ 150 Homogenizer). The RNA concentration was measured using the BioTek Epoch Microplate Spectrophotometer with a Take3 microvolume plate. The RNA samples were treated with DNase I, Amplification Grade (Thermo Fisher Scientific) to eliminate DNA through hydrolysis before cDNA synthesis. Reverse transcription was conducted

to synthetize cDNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad) following the kit instructions. qPCR reactions were performed using SsoAdanvedTM Universal SYBR Green Supermix (Bio-Rad) following the manufacturer's instructions. Amplification and detection were carried out in a CFX Connect Real-Time system (Bio-Rad, Hercules, CA) after an initial denaturation of 10 min sec at 95°C, followed by 40 cycles of 95°C for 15 s, primer annealing, and extension at 60 °C for 1 min. The relative fold gene expression was determined using the ΔΔCT (delta-delta-CT) method (Schmittgen and Livak, 2008). The fold change was calculated and normalized relative to the expression of the housekeeping gene *elf1a*. To evaluate statical differences between the controls (MHRW) and the treated PW, the Mann-Whitney test – also called the Wilcoxon rank sum test, was used to compare both groups.

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

Phase 1 - Evaluation of effluents from Pilot-scale treatment systems

5.1.1 PW physicochemical characterization

Table 3 presents the physicochemical characteristics of the water samples from the Permian and San Juan Basins, alongside the removal efficiencies of the treatment processes. Both desalination processes showed exceptional performance in the reduction of the salinity content of the PW. For the Permian Basin samples, the distillation system achieved over 97% reduction in TDS, EC, alkalinity, and hardness. Likewise, the San Juan Basin samples underwent substantial reductions in TDS and EC (>99%) as well as a complete removal of the hardness and alkalinity. This underscores the effectiveness of both the distillation system and the SWRO unit in treating PW from different basins.

Table 3. Physicochemical characterization

Basin	Permian			San Juan		
Analyte	Feed	Treated	Removal (%)	Feed	Treated	Removal (%)
TDS (mg/L)	103552	287.00	99.72	10300	56	99.46
EC (µS/cm)	119056	46.35	99.96	12940	48.30	99.63

Hardness (mg $CaCO_3$/L)	10200	288.00	97.18	530	ND	100.00
Alkalinity (mg $CaCO_3$/L)	134	0.52	99.61	150	ND	100.00
pH	6.58	8.54	NA	6.58	8.53	NA
TOC (mg/L)	74.39	42.32	43.11	ND	ND	NA
NH3 (mg NH_3-N/L)	611.0	44.93	93.08	ND	ND	NA

ND: non-detected; NA: non-applicable

The TOC and NH_3 characterization revealed significant disparities between the two basins. In the Permian Basin, TOC and NH_3 concentrations were measured at 74 mg/L and 611 mg/L, respectively. Conversely, in the San Juan Basin, levels of both TOC and NH3 were non-detected (ND), highlighting notable differences in the chemical composition of PW samples collected from these regions. After treatment, despite the distillation system achieving significant removals of both parameters in the Permian Basin water, the residual levels of TOC and NH_3 exceeded 40 mg/L.

5.1.2 Toxicological characterization, Fish Embryo Acute Toxicity (FET) Test

The results of the acute toxicity tests performed on the treated Permian and San Juan samples are presented in Figure 1. Additionally, the statistical analysis results, along with the toxicity endpoints computed for the zebrafish exposures involved in the dose-response assessment, are summarized in Table 4.

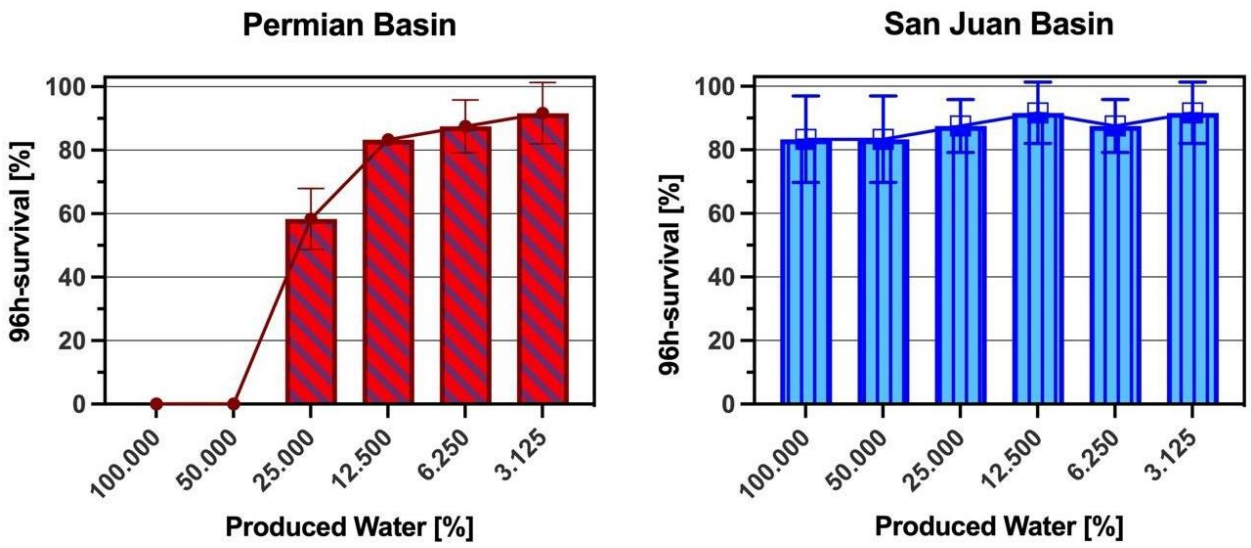


Figure 1. Toxicological characterization of the treated PW samples

After the distillation process, the toxicity tests demonstrated that the effluent produced by the pilot unit was toxic and caused adverse effects on the zebrafish embryos. The results indicate that at critical concentration (100% effluent), the Permian Basin treated PW is highly toxic and caused 100% mortality of the exposed population (24 embryos), even 100% mortality was observed when exposed to the distillate sample diluted 50%. The dose-response assessment showed a dose-response relationship, with NOEC at 6.25%, LOEC at 12.5%, and the LC_{50} estimated at 16.68%.

Table 4. Zebrafish *FET Test,* Dose-response assessment.

Sample	NOEC [%]	LOEC [%]	LC_{50} [%, 95% C.I.]	Hypothesis Test (S – NS)	Point estimate Test	Test acceptability criteria
Permian	6.25	12.5	16.68 (13.24 – 20.12)	S^{SMORT}	P	Pass
San Juan	100	>100	>100 (NA – NA)	NS^{SMORT}	LI	Pass

LC_{50}: concentration causing 50% lethality; 95% CI: 95 % confidence upper and lower intervals;S: 100 % sample concentration is statistically different from the control; NS: 100 % sample concentration is not statistically different from the control; DT: Dunnett's Test.; SMORT: Steel's Many-One Rank Test; LI: Linear interpolation; SK: Spearman-Karber; P: Probit.

Conversely, the embryos exposed to the treated water from San Juan Basin PW exhibited an overall high survival rate across all concentrations. After the dose-response assessment, values for NOEC, LOEC and LC_{50} were estimated to be at least 100%. Therefore, the toxicity results suggest that the SWRO was effective in reducing toxicants in the San Juan Basin PW.

When comparing the toxicity results with the physicochemical characterization, the adverse responses are consistent with the physicochemical data. In contrast with the results observed for the San Juan Basin, the high mortality rates in zebrafish embryos observed in the Permian Basin water can be attributed to the detected residual levels of organics and ammonia. Numerous organic compounds commonly found in PW, including benzene, toluene, phenol, and phenanthrene, are known to be highly toxic to zebrafish (Butler et al., 2013; Hedgpeth et al., 2019; Incardona et al., 2004). Given the measured residual levels of TOC (~42 mg/L), it is plausible that these organics significantly contributed to the high mortality observed in the FET test. Furthermore, the NH_3 concentration detected in the distillate (~44 mg/L) is significantly higher than the reported LC50 values for zebrafish embryos, which range from 0.05 to 0.1 mg/L (Mariz Jr et al., 2023). Therefore, the data suggests that ammonia, at the levels present in the distillate, likely played a critical role in the observed adverse effects on *D. rerio* embryos.

In conclusion, the data suggests that the pilot-scale treatment technologies are effective in reducing the concentrations of the determined parameters. Nevertheless, the differential toxicity observed between the PW samples and the physicochemical characterization, emphasizes the role that organics and ammonia play as stressors in treated PW. This observation underscores the need for tailored treatment strategies, specifically designed to mitigate these critical constituents.

Phase 2 - Monitoring water quality throughout an integrated treatment train

5.2.1. PW physicochemical characterization

Table 5 presents the selected physicochemical parameters of the 3 effluents: feed, distillate, and post-treated effluent. While data analysis related to chemical characterization is still undergoing, the removal of constituents after each of the treatment stages highlights the effectiveness of the complete treatment train to address these parameters. Particularly, salinity indicators (TDS and EC) and the main ions (Na^{+}, Ca^{+2}, Mg^{+2}, K^{+}, Br^{-}, Cl^{-}, F^{-}, SO_4^{-2}, PO_4^{-3}) were successfully reduced after the distillation system. Similarly, the levels of total ammonia nitrogen (TAN) and the parameters used for organic characterization such as TOC, DOC, HEM, TOX, TPH, GRO (C_6-C_{10}), DRO (C_{10}-C_{28}), and ORO (C_{28}-C_{36}) were further reduced after posttreatments with GAC and Zeolite. These results show the how the integrated treatment train reduced consistently all these parameters of interest of PW beneficial reuse.

5.2.2 Toxicological characterization, Fish Embryo Acute Toxicity (FET) Test

The results of the acute toxicity tests performed on the three studied effluents are presented in Figure 2. Additionally, the statistical analysis results, along with the toxicity endpoints computed for the zebrafish exposures involved in the dose-response assessment, are summarized in Table 6.

The dose-response assessment showed not survivors in of the feed water concentrations studied (6.25 to 100%) at 24 hpf, indicating the presence of numerous stressors in the raw PW. The chemical analysis suggests that ammonia, organics, and salinity levels could be contributing factors to this adverse response. After the distillation system, these adverse effects were significantly mitigated in the exposures considering distillate concentrations of 6.25%, 12.5%, 25% and 50%. However, at a 100% concentration, full mortality was observed in the exposed population. These results evidence that despite the distillation unit reduced many of the stressors in the water, there are residual constituents in the distillate that can exert serious adverse effects in the fish embryos. These findings are consistent with phase 1 results, where the effluent after the low-temperature distillation system still exerted adverse effects on the embryo population. Considering the salinity and the major ions were successfully reduced after the distillation system and the residual levels of organics and ammonia presented in Table 3, it is anticipated multiple organics, ammonia and other constituents exerted adverse effects on the embryos. As shown in Table 5 and Figure 2-C, after the post-treatment

with GAC and Zeolite, no adverse effects were observed in the dose-response assessment. 100% survival was observed at 100% effluent, showing that observable adverse effects were completely mitigated. Chemical and toxicological characterization data shows that the reduction of organics and ammonia was consistent with the reduction of mortality in the fish embryos.

Table 5. Physicochemical characterization of the effluents from phase 2.

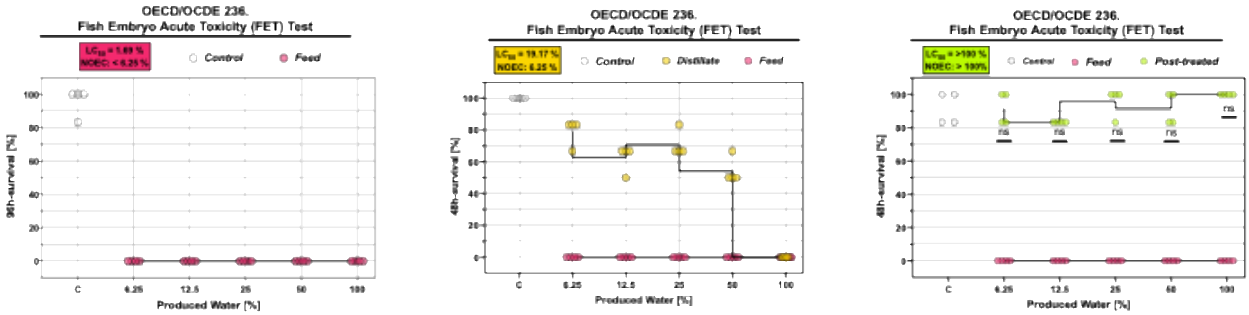
Parameter	Units	Feed	Distillate	Post treated	MDL	RE (%) *D	RE (%) *F
TDS	mg/L	172000	79.5	106	<5.00	+33.33	99.94
TSS	mg/L	165	<8.00	<6.67	<6.67	16.63 - 100	95.96 - 100
EC	umho/cm	177000	161	223	<10.0	+38.51	99.87
pH	S.U.	6.1	10.2	8.2	0.1	19.61	-34.43
Hardness	mg $CaCO_3$/L	25000	2.44	7.93	<4.0	+225.00	99.97
Alkalinity	mg $CaCO_3$/L	11025	93.2	27.3	<4.0	70.71	99.75
TOC	mg/L	71.5	39.5	9.97	<1.00	91.27	95.17
DOC	mg/L	79.8	40.0	3.45	<0.50	75.08	87.51
HEM	mg/L	751	751	26.4	<1.57	96.48	96.48
TOX	ug/L	26500	<14.0	60	<14.0	+328.57	99.77
TPH	mg/L	59.0	4.61	1.15	<0.988	75.05	98.05
GRO (C_6-C_{10})	mg/L	7.27	<1.02	<0.988	<1.02	3.14 - 100	86.41 - 100
DRO (C_{10}-C_{28})	mg/L	43.1	4.6100	<0.988	<0.988	78.57 - 100	97.71 - 100
ORO (C_{28}-C_{36})	mg/L	8.65	<0.980	1.1500	<0.980	+17.35	86.71
Na^+	mg/L	47600	0.626	28.9	<0.152	+4516.61	99.94
Ca^{+2}	mg/L	6350	0.916	2.88	<0.102	+214.41	99.95
Mg^{+2}	mg/L	1270	0.052200	0.430	<0.0445	+723.75	99.97
K^+	mg/L	349	<0.106	1.90	<0.106	+1692.45	99.46
Br^-	mg/L	550	0.0711	0.116	<0.0711	+63.15	99.98
Cl^-	mg/L	72100	1.50	27.2	<0.250	+1713.33	99.96
F^-	mg/L	28.100	<0.100	<0.100	<0.100	-	99.64 - 100
SO_4^{-2}	mg/L	226	<0.200	8.86	<0.200	+4330.00	96.08
PO_4^{-3}	mg/L	25.1	0.241	0.340	<0.0564	+41.08	98.65
Turbidity	NTU	39	5	1.4	<0.50	72.00	96.41
COD	mg/L	2220	142	64.0	<3.36	54.93	97.12
MBAS	mg/L	0.12700	0.11200	-	<0.0800	-	28.57-100
TAN	mg N-NH_3/L	139	21	<0.0508	<0.0508	99.76 - 100	99.96 - 100
NO_3^-	mg N-NO_3 /L	<3.91	0.08710000	0.116	<0.0391	+33.18	97.03
NO_2^-	mg N-NO_2 /L	<0.0293	<0.0293	0.343	0.0293	+1070.65	+1070.65
CHO_2^-	mg/L	<1.0	<1.0	<1.0	<1.0	-	-
$CH_3CO_2^-$	mg/L	<1.0	<1.0	<1.0	<1.0	-	-
CN^-	ug/L	2.14	<2.00	-	<2.00	+304.00	-
H_2S	mg/L	<5.00	<5.00	<5.00	<5.00	-	-
S_2^-	mg/L	<0.0400	0.0406	<0.0400	<0.0400	1.48	-
SiO_2	mg/L	31.2	<0.370	14.7	<0.370	+3872.97	52.88

MDL: method detection limit; RE: Removal efficiency; *D: respect to distillate; *F: respect to feed; N/A: no applicable; - no calculable

Table 6. Zebrafish *FET Test,* Dose-response assessment of the effluents studied in Phase 2.

Sample	NOEC [%]	LOEC [%]	LC50 [%, 95% C.I.]	Hypothesis Test (S – NS)	Point estimate Test	Test acceptability criteria
Feed	<6.25	6.25	1.69 (1.69 – 1.69)	S^{SMORT}	LI	Pass
Distillate	6.25	12.5	19.17 (19.79 – 24.56)	S^{SMORT}	P	Pass
Post-treated	100	>100	>100 (NA – NA)	NS^{SMORT}	P	Pass

LC_{50}: concentration causing 50% lethality; 95% CI: 95 % confidence upper and lower intervals;S: 100 % sample concentration is statistically different from the control; NS: 100 % sample concentration is not statistically different from the control; DT: Dunnett's Test.; SMORT: Steel's Many-One Rank Test; LI: Linear interpolation; SK: Spearman-Karber; P: Probit.



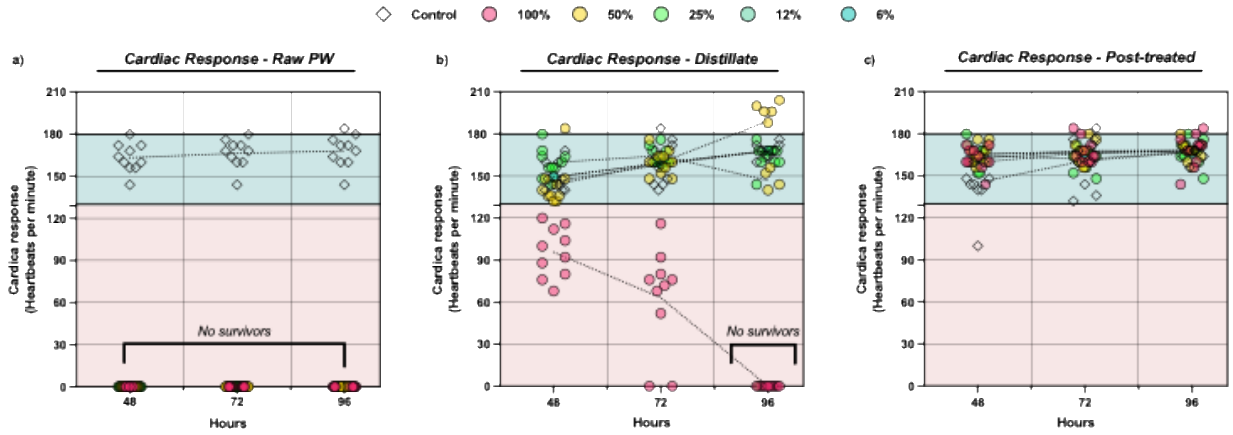
a) feed, b) distillate, and c) post-treated.

Figure 2. Toxicological characterization of the 3 effluents studied in Phase 2

5.2.3. Cardiotoxicity and teratogenic effects on zebrafish embryos.

Figure 3 illustrates the cardiac response of zebrafish embryos to different concentrations of the effluents measured as heartbeats per minute (bpm) over a period of 96 hours. Overall, the control groups (MHRW) consistently maintained a baseline heartbeat rate between 130 and 180 bpm. Exposure to Raw PW resulted in serious impairment of the embryonic development, including cardiac system. As no survivors were observed, nor heartbeats were documented for the raw PW (Fig. 3a). Exposure to the distillate resulted in concentration-dependent changes in cardiac activity.

At 100% concentration, there was a significant reduction in heart rate at 48 h, 72 h and 96 h. In particular, at 96 h, no heartbeats were observed in this concentration. Lower distillate concentrations (50%, 25%, 12%, and 6%) exhibited bpm rates that were comparable to the control, indicating that the compounds in the distillate were not impairing the embryos' heart response at these doses (Fig. 3b). Contrary to raw PW and distillate, the post-treated water showed cardiac responses that were similar to those of the control group (130 and 180 bpm) across all tested concentrations (Fig. 3c). This indicates that the post-treatment processes effectively reduced the constituents causing cardiac alterations observed in the distillate test at 100% (0 bpm).



a) feed, b) distillate, and c) post-treated.

Figure 3. Cardiac response of the embryos exposed to evaluated effluents in study phase 2.

To offer a visual assessment of the developmental impacts associated with the three types of effluents at 100% concentration, Figure 4 provides images of the microscopic observations conducted during the FET test. Exposure to raw PW resulted in a cessation of normal developmental progression, markedly deviating from typical embryonic stages observed in zebrafish (Fig. 4a). These observations show that the raw PW studied contains compounds in concentrations that disrupt normal embryogenesis. While there is a noticeable improvement in the developmental outcomes of the embryos exposure to the distillate compared to raw PW, issues such as spine curvature and pericardial and yolk sac edemas indicate that the distillation process does not entirely remove all harmful components (Fig. 4b). This partial treatment still poses a risk to normal embryonic development and is consistent with the results of the FET test and the cardiac responses, where at 96 h, the embryos exposed to the distillate showed no signs of cardiac function.

The effects of the post-treated effluent on embryo development are presented in (Fig. 4c). These embryos show markedly better development, with more typical morphology and no evidence of developmental stress. This suggests that the post-treatment processes effectively mitigated most of the toxic effects seen with raw PW and the distillate.

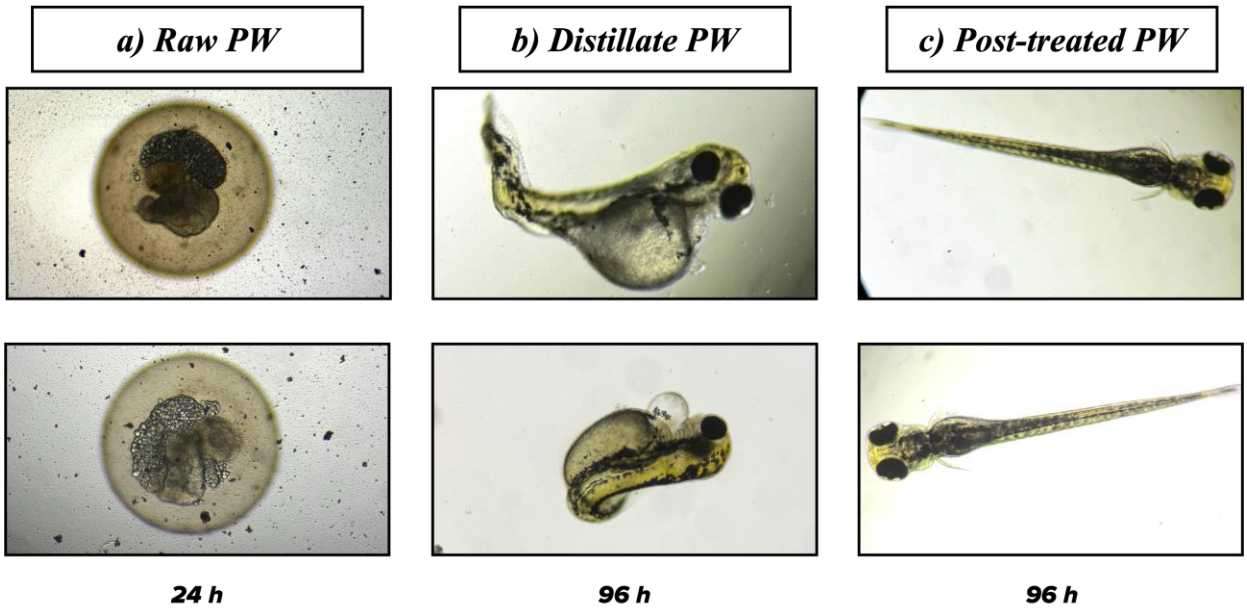


Figure 4. Microscopic observations of embryos exposed to effluents in phase 2.

5.2.4. Impact of post-treated effluent on zebrafish gene expression.

The relative expression of *cyp19a1a*, *cyp19a1B*, and *vtg1* 1 at 7 days post exposure is presented in Figure 5. Normalized expression levels of *cyp19a1a* (2.37), *cyp19a1B* (0.94) in the treated PW groups did not show significant differences compared to the control (MHRW) groups ($P > 0.05$). Similarly, while it appeared that *Vtg1* normalized expression levels (3.23) were elevated in the treated PW group, the ANOVA results revealed no significant differences in expression levels compared to the control group ($P > 0.05$).

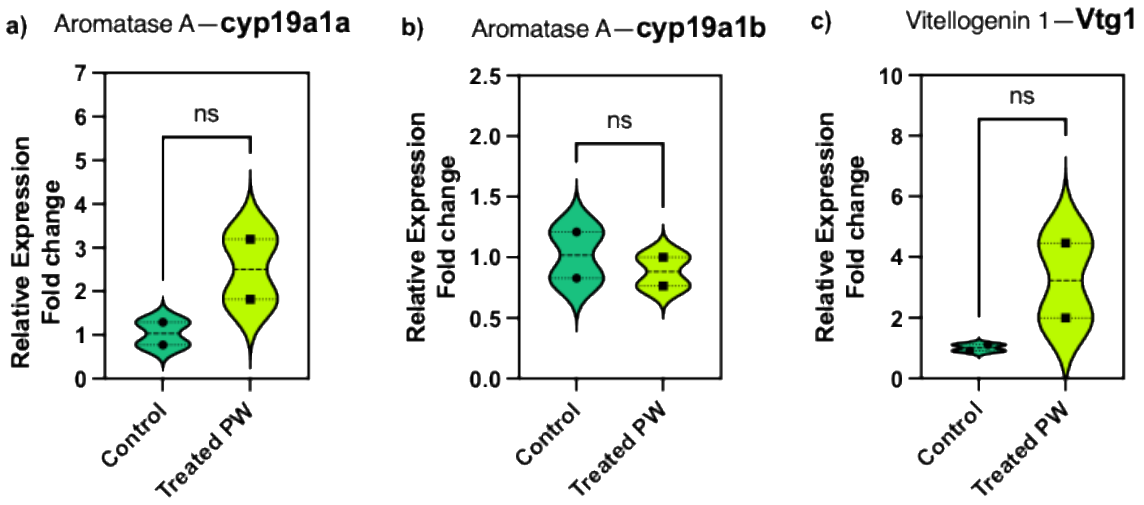


Figure 5. Relative gene expression fold change on embryos exposed to the final effluent.

Compared to similar studies that evaluated the expression of *cyp19a1a*, *cyp19a1b*, and *vtg1* in zebrafish embryos exposed to fractionated PW (organic fractions), the relative gene expression observed in our study is consistently lower. Significant differences were observed for each gene, with fold changes of 10.1 for *cyp19a1a*, 3.2 for *cyp19a1b*, and 6.3 for *Vtg1* (He et al., 2018a). The results suggest that treated PW does not significantly affect the expression of these genes. The absence of statistically significant changes in the expression of *cyp19a1a*, *cyp19a1b*, and *Vtg1* in zebrafish exposed to treated PW indicates that the treatment methods may effectively mitigate the previously reported endocrine-disrupting potential of produced water (He et al., 2018a; He et al., 2018b). Specifically, the findings imply that any compounds capable of altering the expression of these genes present in the treated PW do not significantly impact them at the concentrations found.

In conclusion, the results obtained in Phase 2 showed that both raw PW and its distillate contain constituents that adversely affect *Danio rerio*. In raw PW, embryonic development ceased after 24 hours of exposure at all concentrations studied. These findings demonstrate the significant harm PW can cause to fish and emphasize the need for highly controlled operations during reuse applications to prevent spills, leaks, and overflow of raw PW. After distillation, although toxicity was significantly reduced, several adverse effects persisted, including reduced cumulative survival, impaired cardiac response, and severe morphogenetic alterations. The treatment process, consisting of MVR thermal distillation, GAC, and Zeolite, successfully lowered stressor concentrations in the oilfield PW to levels that are not harmful to *D. rerio*, with no significant

differences compared to the control in FET tests, cardiac response, phenotypic alterations, and the expression of genes associated with the endocrine system. The results highlight the potential of the combined treatment train to reduce the constituents of PW to non-toxic levels and bolster the case to more detailed studies aiming to scale up the treatment strategy while conducting more comprehensive chemical and toxicological characterization of the effluents.

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

The research results collected in this study have several implications for a variety of stakeholders, particularly those involved in the management of PW from the Permian and San Juan Basins. State agencies, such as the New Mexico Environment Department (NMED), and the Office of The State Engineer could significantly benefit from these findings. These agencies are tasked with the regulation and oversight of water quality and could use the data to develop management strategies and treatment guidelines for PW reuse in the state. Furthermore, as PW management regulations evolve as a result of The Produced Water Act (HB 546), the O&G industry could leverage the insights gained to optimize their current water treatment processes to favor PW reuse outside O&G operations. Academic and research institutions focusing on water reuse and environmental risk assessment can also utilize these findings to further explore the ecological implications of PW discharge and reuse, thereby expanding the body of knowledge and fostering innovation in water treatment technologies.

7. Describe how you have spent your grant funds. Also provide your budget balance and how you will use any remaining funds. If you anticipate any funds remaining after August 30, 2024, please contact Carolina Mijares immediately. (575-646-7991; mijares@nmsu.edu)

The grant fund has been used for chemical supplies for water quality and toxicity tests, summer 2024 stipend and the trip to WRRI water conference, Produced Water Society Annual Conference, and New Mexico Groundwater Conference.

8. List presentations you have made related to the project.

- Yeinner Tarazona, Toxicity Reduction Evaluation (TRE) of produced water from the Permian Basin: A step forward on the design of treatment trains for beneficial reuse outside of the oil and gas industry. Texas Desal 2023 Annual Conference, Sep 29, 2023.
- Yeinner Tarazona, Toxicity Reduction Evaluation (TRE) of produced water from the Permian Basin: A step forward on the design of treatment trains for beneficial reuse outside of the oil and gas industry, 68th Annual New Mexico Water Conference, Nov 8, 2023
- Yeinner Tarazona, Toxicity Reduction Evaluation (TRE) of produced water from the Permian Basin, American Groundwater Trust, New Mexico Groundwater Conference, July 10 & 11, 2024.
- Yeinner Tarazona, Toxicity Reduction Evaluation (TRE) of produced water from the Permian Basin, Produced Water Society, Permian Basin Conference 2024, August 12 – 14, 2024

9. 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.

I am enrolled in the Ph.D. program in Civil (Environmental Engineering) and plan to complete my degree in Fall 2024. Post-graduation, I envision myself delving deeper into research and development in either industrial or academic settings to address complex issues related to natural resources, energy, and human and environmental health. Additionally, I am open to exploring opportunities within state or federal government agencies, where I can apply my interdisciplinary background to support the development of environmental regulations that protect water, air, and land.

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