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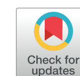
Toxicological characterization of produced water from the Permian Basin



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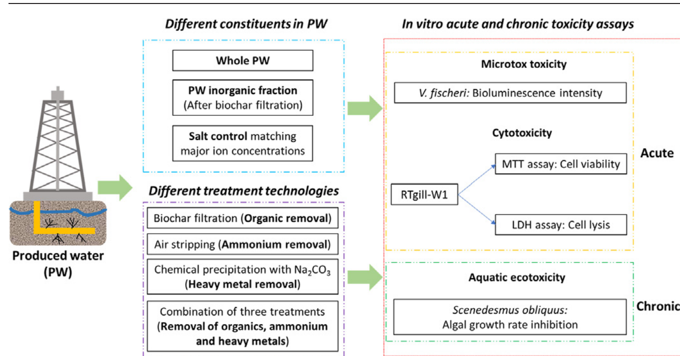
HIGHLIGHTS

- High salinity was the predominant toxicological driver in PW.
- Organic contaminants had a significant impact on PW toxicity.
- Heavy metals and ammonium in PW also contribute to toxicity.
- Toxicity assays had different sensitives to the chemical constituents present in PW.

GRAPHICAL ABSTRACT



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ABSTRACT

Produced water (PW) is a hypersaline waste stream generated from the shale oil and gas industry, consisting of numerous anthropogenic and geogenic compounds. Despite prior geochemical characterization, the comprehensive toxicity assessment is lacking for evaluating treatment technologies and the beneficial use of PW. In this study, a suite of *in vitro* toxicity assays using various aquatic organisms (luminescent bacterium *Vibrio fischeri*, fish gill cell line RTgill-W1, and microalgae *Scenedesmus obliquus*) were developed to investigate the toxicological characterizations of PW from the Permian Basin. The exposure to PW, PW inorganic fraction (PW-IF), and PW salt control (PW-SC) at 30 – 50% dilutions caused significant toxicological effects in all model species, revealing the high salinity was the foremost toxicological driver in PW. In addition, the toxicity level of PW was usually higher than that of PW-IF, suggesting that organic contaminants might also play a critical role in PW toxicity. When comparing the observed toxicity with associated chemical characterizations in different PW samples, strong correlations were found between them since higher concentrations of contaminants could generally result in higher toxicity towards exposed organisms. Furthermore, the toxicity results from the pretreated PW indicated that those *in vitro* toxicity assays had different sensitives to the chemical components present in PW. As expected, the combination of multiple pretreatments could lead to a more significant decrease in toxicity compared to the single pretreatment since the mixture of contaminants in PW might exhibit synergistic toxicity. Overall, the current work is expected to enhance our understanding of the potential toxicological impacts of PW to aquatic ecosystems and the relationships between the chemical profiles and observed toxicity in PW, which might be conducive to the establishment of monitoring, remediation, treatment, and reuse protocols for PW.

1. Introduction

According to the U.S. Energy Information Administration (EIA, 2015; Kuuskraa et al., 2013), approximately 55 billion m^3 of shale oil and 207 trillion m^3 of shale gas are technically recoverable globally, which can satisfy the world's energy supply for over 100 years. The rapid expansion of horizontal drilling and hydraulic fracturing practices has improved the oil and gas production from shale formations, thereby promoting the United

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States to be the largest producer of natural gas and crude oil in the world since 2009 and 2018, respectively (Economides and Wood, 2009; EIA, 2017; EIA, 2018). Along with success in the extraction of shale oil and gas, large volumes of produced water (PW) generated during the production process have recently gained significant public concerns due to their potentially toxicological impacts on aquatic ecosystems (He et al., 2017a; Jia et al., 2017; Mehler et al., 2020). One of the primary risks posed by PW is the accidental spill and release to the surface water and groundwater during the storage and transport, which can adversely affect the aquatic organisms and result in long-term toxicological effects in aquatic ecosystems (Akob et al., 2016; Cozzarelli et al., 2017). In addition, much of shale resources (nearly 38%) are located in severe water shortage areas or arid regions (Reig et al., 2014), whereas hydraulic fracturing needs to consume large amounts of fresh water (up to 42,500 m^3 per well) (Kondash and Vengosh, 2015). For instance, the Permian Basin, as the most prolific unconventional reservoir play in the U.S. (Nadella et al., 2020), located in arid areas of western Texas and southeastern New Mexico, is presenting significant operational challenges due to the shortage of freshwater resources. Thus, the reuse of treated PW is in urgent need for those regions. However, prior to reuse, the toxicological characterizations of PW should be well studied to promote safe and sustainable management and ensure sufficient treatment.

Still, there are significant knowledge deficits on the potential toxicological impacts of PW on aquatic ecosystems. PW composition is often briny, a highly complex mixture of the originally injected hydraulic fracturing fluids (*e.g.*, biocides and surfactants), hypersaline formation water, and even includes secondary byproducts of the downhole reactions associated with the formation environment (Folkerts et al., 2017b; Rodriguez et al., 2020; Stoll et al., 2015). Given the compositional complexity of PW, it is very challenging to precisely identify the chemical profiles and toxicological characterizations associated with individual components. Although the toxicity of individual chemicals has been well studied and documented, there is very limited information regarding the toxicity of the complex compositional mixture of compounds within actual PW. The correlations between chemical composition and toxicity are not clear.

Recently, the high concentrations of salts and organics (*e.g.*, BTEX (benzene, toluene, ethylbenzene and xylene), polycyclic aromatic hydrocarbons) have been identified as the major contributors to the toxicity of PW by several studies (Folkerts et al., 2017b; He et al., 2018). Some of those studies used fish species, including zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), as *in vivo* exposure models to examine the PW toxicity in aquatic ecosystems (Folkerts et al., 2019; He et al., 2017b; He et al., 2018). Correspondingly, significant developmental toxicity, sublethal/lethal toxicity, reproductive effects, oxidative stress, and endocrine disruption were observed in fish after exposure to PW. For example, He et al. (2017b) used the rainbow trout (*Oncorhynchus mykiss*) as the exposure model to evaluate the toxicity of flowback and produced water (FPW), and found that the exposure to 7.5% FPW caused significant induction of ethoxyresorufin-O-deethylase activity in both gill and liver tissues. Another study (Folkerts et al., 2019) revealed that high salinity and organic contaminants were the major contributors to the overall toxicity of PW when using rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*D. rerio*) as aquatic model species. However, those *in vivo* toxicological studies need to consume numerous live fishes, which is a major ethical concern when using live laboratory specimens for the whole effluent toxicity (WET) testing. It was reported that approximately 3–6 million live fish were used for the WET testing annually in the United States (Scholz et al., 2013). Hence, it is essential to develop *in vitro* alternatives to live fish specimens for PW toxicity testing in the private and research sectors. Previous studies revealed that the rainbow trout (*Onchorhynchus mykiss*) gill epithelial cell line RTgill-W1 may be a valuable complement to live fish toxicological studies because of the distinctive role of fish gill as the first organ to take up toxicants in the aquatic environment (Lungu-Mitea et al., 2020; Scott et al., 2021). The damage to gill function will lead to the death of fish. Moreover, it was reported that the RTgill-W1 cell line was quite tolerant of hypersaline conditions and could be easily grown for prolonged periods in the media with varying salinities (Lee et al., 2009). Thus, the RTgill-W1 cell line may be considered an ideal *in vitro* model for assessing PW toxicity.

The RTgill-W1 cell-based cytotoxicity assay alone may not adequately assess the toxicological effects posed by PW. Some additional toxicity assays should also be adopted to provide a short list of toxicity assays for a quick assessment of PW. A previous study employed a bioluminescent dinoflagellate, *Pyrocystis lunula*, to evaluate the potential toxicological effects of endogenous and exogenous chemicals associated with the hydraulic fracturing (Hildenbrand et al., 2015b). It suggested that this bioluminescence assay could be used as a preliminary screening and rapid risk assessment tool since glutaraldehyde and HCl caused significant toxicological responses even at low concentrations. However, this assay has low sensitivity to some heavy metals (*e.g.*, As, Se, Ba, Sr), which have been previously linked to unconventional drilling activities. Our previous studies (Hu et al., 2021; Hu et al., 2020b) have demonstrated that the Microtox® assay using marine luminescent bacterium *Vibrio fischeri* was useful for measuring the acute toxicity of PW due to its adaptation to hypersaline conditions and high sensitivity to multiple contaminants. Moreover, numerous studies have also demonstrated that the Microtox® assay is equally applicable for the toxicity monitoring of diverse chemical substances (*e.g.*, heavy metals, inorganic and organic compounds) and various types of wastewaters (*e.g.*, oil sands process affected water, landfill leachate, pharmaceutical wastewater) (Abbas et al., 2018; Yu et al., 2014b; Zhang et al., 2018). Meanwhile, the Microtox® assay has the advantages of high repeatability and reproducibility compared with other more complex toxicity assays (Garcia-Ordiales et al., 2019). In addition to the Microtox® assay, the ecotoxicity assay with microalgae *Scenedesmus obliquus* may also be a powerful tool to provide the comprehensive toxicity evaluation in aquatic ecosystems (He et al., 2019). In aquatic ecosystems, algae play a crucial role as the primary producers, and algae also have the advantages of easy growing and high sensitivity to environmental stressors when they are used for toxicity assays (Cai et al., 2020). Therefore, the ecotoxicity assay with a freshwater green algae *Scenedesmus obliquus* may also be an appropriate method for toxicity monitoring in PW. A recent review well documented the current treatment technologies (*e.g.*, hydrocyclones, adsorption, bioremediation, coagulation and flocculation) for the removal of inorganic, organic, and biological constituents in PW (Liden et al., 2018). To further clarify the toxicological mechanisms of PW, some common pretreatments from this review paper will be applied to PW to remove the potential toxic components prior to toxicity assessments. Until now, the information regarding the potential toxicological effects of real PW samples on aquatic organisms is very limited. Hence, in this study, the combination of those toxicity assays will contribute towards a better understanding of the toxicological effects of PW in aquatic ecosystems, and the research results subsequently can be used to guide PW treatment and reuse practices in the Permian Basin and others.

The objective of this study is to identify the toxicity signatures of PW samples from the Permian Basin, and investigate the possible relationships between the chemical profiles and observed toxicity in PW. The physicochemical characteristics of PW samples were initially evaluated as delivered from the field. Then, the acute toxicity, cytotoxicity, and aquatic ecotoxicity of PW were examined using three *in vitro* exposure model organisms, including marine luminescent bacterium *Vibrio fischeri*, fish gill epithelial cell line RTgill-W1, and microalgae *Scenedesmus obliquus*. To further understand how water quality parameters affect the toxicity of PW, different pretreatments were applied to raw PW for the removal of organics, ammonium, and heavy metals. After the pretreatments, the toxicity of the treated PW was measured to establish a link between the toxicological behaviors and various contaminant groups in PW. This is one of the first studies to assess the toxicological characterizations of PW from the Permian Basin. Our results will help address the toxicological hazards associated with PW, and develop effective treatment processes to achieve species-specific removal.

2. Materials and methods

2.1. Produced water collection and characterization

Three PW samples (PW-1, PW-2, and PW-3) were collected from the different saltwater disposal wells in the Permian Basin (near Carlsbad, New Mexico) and stored at 4 °C before analysis. To generate the PW inorganic fraction (PW-1-IF, PW-2-IF, PW-3-IF), all raw PW samples were treated by biochar (Wakefield Agricultural Carbon LLC., MO, USA) to adsorb the organic compounds following a previous study (Qin et al., 2019). Prior to the adsorption process, the biochar was washed sequentially with 0.1 mM HCl and NaOH to remove impurities, and then rinsed 5 times using deionized water before drying overnight as described previously (Qin et al., 2019). The main characteristics of the biochar were summarized in our previous studies (Lin et al., 2017; Rodriguez et al., 2020). Additionally, three salt controls (PW-1-SC, PW-2-SC, and PW-3-SC) matching major anion and cation concentrations (Na^+, Mg^{2+}, K^+, Ca^{2+}, and Cl^-) in the raw PW samples were prepared to account for a variety of saline-induced responses. All samples were filtered through a 0.45 μm pore size nylon membrane (Fisher Scientific) to minimize biological activities and stored at 4 °C prior to analysis.

The concentrations of major ions (Na^+, Mg^{2+}, K^+, Ca^{2+}, Li^+, Cl^-, Br^-, and SO_4^{2-}) in all samples were quantified using an ion chromatograph (IC, ICS-2100, Dionex, CA, USA). A pH and conductivity meter (PC800, Apera, OH, USA) was used to measure the pH, electrical conductivity, and total dissolved solids (TDS) of all samples. Total organic carbon (TOC) in PW samples was determined by a carbon analyzer (Shimadzu TOC-L, Kyoto, Japan). Total nitrogen (TN), total phosphorus (TP), and ammonium of PW samples were measured using Hach test kits (Hach Co., CO, USA). An inductively coupled plasma optical emission spectrophotometer (ICP-OES, PerkinElmer, MA, USA) was used to determine the concentrations of other elements and heavy metals (As, B, Ba, Cd, Cr, Co, Fe, Mn, Ni, Si, and Sr) in PW samples.

2.2. Microtox® toxicity assays

The acute toxicity of PW samples towards a marine luminescent bacterium *Vibrio fischeri* was measured by a Microtox® Model 500 Analyzer (Azur Environmental, DE, USA) according to the 81.9% Screening Test Protocol as described previously (Hu et al., 2020b). All PW samples with different dilutions (5% ~ 40%) were filtered through a 0.22 μm pore size membrane, and then adjusted to pH 6 – 8 using 0.5 M NaOH or HCl solution prior to the toxicity test. To check the sensitivity of the luminescent bacterium *Vibrio fischeri*, $ZnSO_4$ solution (10 mg/L) was used as the positive control. The toxic effects were calculated based on the percentage inhibition of the luminescence intensity after 15 min exposure. The EC_{50} (the effective concentration for 50% bioluminescence inhibition) values were determined *via* non-linear regression using SPSS Statistics v25 (SPSS Inc., Chicago, IL).

2.3. Cytotoxicity assays

2.3.1. Cell culture and treatments

The rainbow trout (*Oncorhynchus mykiss*) gill cell line RTgill-W1 was originally obtained from the American Type Culture Collection (CRL-2523, ATCC, VA, USA). Cells were cultured in the basal medium, Leibovitz's L-15 (Cytiva, MA, USA), supplemented with 10% fetal bovine serum (FBS, ATCC® 30–2020™, VA, USA) and 100 I.U./mL penicillin and 100 μg/mL streptomycin (ATCC® 30–2300™, VA, USA). RTgill-W1 cell line was grown in the 75 cm^2 sterile tissue culture flask (Thermo Fisher Scientific, MA, USA) at 18 to 21 °C without CO_2, subcultured or harvested (once a week) for the following cytotoxicity assays at approximately 95% confluency using 0.25% (*w/v*) Trypsin-0.53 mM EDTA solution (Gibco, Thermo Fisher Scientific, MA, USA) as described previously (Brinkmann et al., 2020). To examine the cytotoxicity of raw PW, PW-IF and PW-SC, cells were exposed to those PW samples with different dilutions (5% ~ 50%). Untreated cells and blanks containing medium only were also carried out as controls for each experiment when conducting the following cytotoxicity assays. All treatments and controls were performed in triplicate.

2.3.2. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

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 (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to purple formazan crystals by metabolically active cells (Fig. S1). In this study, the MTT Cell Proliferation Kit (Roche, Mannheim, Germany) was used to evaluate the cell viability after PW exposures according to the manufacturer's instructions with slight modifications. Briefly, confluent cells were detached and re-suspended in the fresh medium. After cell counting by a hemocytometer, RTgill-W1 cells (100 μL) were seeded at a concentration of 5×10^5 cells/mL in 96-well plates (Coring, NY, USA), and left to recover and adhere at 18–21 °C for 24 h. Afterward, the medium was removed and replaced with 100 μL 5% ~ 50% PW samples (diluting samples with different volumes of the cell culture media to achieve 5% ~ 50% PW, PW-IF, and PW-SC). As described in Section 2.2, raw PW samples (PW-1, PW-2, and PW-3) with the same salinity levels (TDS 30 g/L, 50 g/L, and 60 g/L) were also used for the MTT assay. Cells were then incubated for 48 h at 18–21 °C followed by the addition of 10 μL MTT labeling reagent. After the incubation period (4 h), 100 μL solubilization solution was added to each well and allowed to stand overnight in the incubator in a humidified atmosphere. Finally, the optical density (OD) was recorded at 550 nm and 690 nm using Epoch™ Microplate Spectrophotometer (BioTek, VT, USA). The OD value of each sample was calculated by subtraction of 690 nm measurement from 550 nm measurement. The percentage of cell viability was calculated by the following formula:

$$\text{Cell viability } (\%) = \left[\frac{\text{Mean OD of experimental samples}}{\text{Mean OD of controls}}\right] \times 100$$

2.3.3. LDH (lactate dehydrogenase) release assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different cell types and is a well-defined and reliable indicator of cytotoxicity (Fig. S2). Thus, the Pierce™ LDH Cytotoxicity Assay Kit (Thermofisher Scientific, MA, USA) was used to measure the cell lysis after PW exposures according to the manufacturer's protocol with slight modifications. Briefly, RTgill-W1 cells (100 μL) were seeded at a density of 5×10^4 cells/mL in 96-well plates and allowed to attach for 24 h before treatment with different PW samples. A complete medium control without cells was included to determine LDH background activity present in the sera, while the spontaneous LDH activity control (treated with ultrapure water) and maximum LDH activity control (treated with 10× Lysis Buffer) were also prepared. Then, the culture medium was discarded and replaced with different diluted PW samples (5–50%, diluting PW samples with different volumes of the cell culture media) to induce cytotoxicity and subsequent LDH release. The supernatants (50 μL) were then transferred to a new 96-well plate and mixed with Reaction Mixture. After 30 min incubation in the dark, reactions were stopped by adding 50 μL Stop Solution. Absorbance at 490 nm and 680 nm was measured using Epoch™ Microplate Spectrophotometer (BioTek, VT, USA) to determine the LDH activity. The LDH activity was calculated by subtraction of 680 nm measurement from 490 nm measurement. Finally, the cytotoxicity level (%) was determined using the following formula:

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

2.4. Aquatic ecotoxicity assays

2.4.1. Algal culture

Aquatic ecotoxicity assays were conducted with a unicellular green algae *Scenedesmus obliquus* (UTEX 393), which was purchased from the Culture Collection of Algae at the University of Texas (UTEX, TX, USA). *Scenedesmus obliquus* was cultured in 250 mL Erlenmeyer flasks containing sterilized BG-11 medium (Sigma-Aldrich, MO, USA) at 20–23 °C to ensure that the algae were in the exponential growth phase as described previously (Pan et al., 2018). The algae were pre-cultured and subjected to the following experiments in an illuminated incubator under the standard conditions: a photocycle of 12:12 h light/dark, and illumination intensity of approximately 3000 lx with cool-white fluorescent lamps at 20–23 °C. To achieve a homogenous cell distribution, all Erlenmeyer flasks were manually shaken five times per day.

2.4.2. Growth inhibition tests

The growth inhibition tests were carried out according to the Organization for Economic Co-operation and Development (OECD) Guideline 201 (OECD, 2011). The algal concentration was determined in two ways: measurement of the optical density (OD) using a spectrophotometer (DR 6000, Hach Co., CO, USA) at 680 nm and direct counting under an inverted microscope by a hemocytometer. Then, a linear curve between the algal cell number and optical density (OD_{680}) value was established (Fig. S3). For the aquatic ecotoxicity assays, algal cells in the exponential growth phase were used for all tests, with an initial cell density of approximately 6×10^5 cells/mL. First, *Scenedesmus obliquus* cells were exposed to PW samples (PW, PW-IF, and PW-SC) with different dilutions (5–50%) for 7 days. A corresponding control was prepared with ultrapure water instead of PW. The flasks were manually shaken five times daily. All treatments and controls were performed in triplicate with the same concentration of nutrient solution (BG-11 medium). Then, the optical density (OD) of algal culture at 680 nm was measured using a spectrophotometer at the beginning of the assay and every day until 7 days following previously published methods (Fan et al., 2019; Pan et al., 2018; Sun et al., 2020). The corresponding algal cell numbers were determined based on the linear relationship between the cell density and OD_{680} value. Finally, the growth inhibition rate induced by PW was calculated as described previously (Cai et al., 2020; Lin et al., 2020).

2.5. Comparison of toxicity in various samples under the same salinity

To further study the contribution of contaminant groups to toxicity, the toxicity of three PW samples (PW-1, PW-2, and PW-3) was compared after offsetting saline-influenced responses. The salinity of three PW samples was adjusted to the same levels by dilution. Under TDS 30 g/L, 50 g/L, and 60 g/L, the toxicity data towards marine luminescent bacterium *Vibrio fischeri*, fish gill epithelial cell line RTgill-W1, and microalgae *Scenedesmus obliquus* were compared and linked to the chemical characterization results.

2.6. Effect of different pretreatments on the toxicity of PW

To identify the main constituents responsible for toxicological behaviors in PW and explore the effective treatment technologies, the Microtox® toxicity, cytotoxicity, and aquatic ecotoxicity of PW-3 were measured after different pretreatments.

Our previous studies (Hu et al., 2021; Hu et al., 2020b) have demonstrated that the pretreatments of air stripping and chemical precipitation (by Na_2CO_3 addition) could effectively remove more than 90% of the ammonium and heavy metals in PW, respectively. Thus, in this study, four different pretreatments were applied to PW-3, including the i) abiotic biochar filtration for the removal of organics; ii) air stripping for the removal of ammonium; iii) chemical precipitation for the removal of heavy metals; and iv) the combination of biochar filtration, air stripping, and chemical precipitation for the removal of organics, ammonium and heavy metals. The detailed experimental procedures were similar to those described in our previous study (Hu et al., 2021; Hu et al., 2020b). Briefly, for the abiotic biochar filtration process, the flow velocity was set at 0.41 mL/min (empty bed contact time of 180 min) to ensure the maximum organic removal. For the air stripping process, the pH of PW was firstly adjusted to around 9.5 by 0.5 M NaOH, after which PW was placed in a conical flask with the airflow rate of 1.72 L/min for 330 min. The Na_2CO_3 stock solution was used for the chemical precipitation process with the Ca^{2+}:CO_3^{2-} molar ratio of 1:1.2. Then, the pH of treated PW samples was adjusted to around 7 using 0.5 M NaOH or HCl before the toxicity assays. Due to the high salinity of PW-3, the treated PW-3 samples were diluted five times (20% PW, similar to the salinity level of seawater) to measure the Microtox® toxicity, cytotoxicity, and aquatic ecotoxicity as described above.

2.7. Statistical analyses

All experiments were conducted at least three times in this study, and the data in all figures were reported as the mean ± standard error of the mean (SEM). The statistical analyses were performed using SPSS Statistics v25 software (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by Tukey test was employed to evaluate the statistical differences between treated and control groups (or different treated groups). The statistical differences at p-value < 0.05 were considered significant.

3. Results and discussion

3.1. Physicochemical characteristics of PW

The physicochemical characteristics of three PW samples collected from the Permian Basin are summarized in Table 1. The pH of all PW samples was relatively neutral with a range between 6.6 and 6.9, which was consistent with the previous study in the Permian Basin (Nadella et al., 2020). All PW samples were characterized with very high TDS (160.4–219.6 g/L, approximately 4.6–6.3 times of seawater), indicating that high salinity is still the dominant problem associated with PW during the shale oil and gas extraction in the Permian Basin. As reported previously, PW salinity exhibits a wide range of 1–400 g/L in the United States (Shaffer et al., 2013), which was largely dependent on the shale geological conditions, the composition of fracturing fluid, and fracture features (Sun et al., 2019). PW from the Permian Basin usually has a high TDS load of more than 100 g/L based on the previous studies (Hu et al., 2021; Jiang et al., 2021; Liden et al., 2019; Nadella et al., 2020), which is consistent with the TDS results reported herein. Results from PW quality analysis revealed that chloride, sodium, calcium, magnesium, potassium, and strontium were the primary constituent ions in PW (Chaudhary et al., 2019). Among those ions, chloride and sodium were major contributors to the PW salinity, accounting for 53.1–55.5% and 24.2–25.8% of the TDS, respectively. These data suggest that the high salinity of PW potentially originated from the evaporation of paleo-seawater, ion exclusion in compacted shale minerals, and equilibration with formation mineralogy (*e.g.*, evaporite minerals) (Engle et al., 2016; Nadella et al., 2020; Rosenblum et al., 2017; Shih et al., 2015). Additionally, high concentrations of calcium (6319– 8159 mg/L), magnesium (932–1327 mg/L), strontium (723–925 mg/L), and silica (122–213 mg/L) were measured in PW, which are attributed to their geologic origins in sandstone, mixed silicate, and limestone reservoirs (Engle et al., 2016). It should be noted that high levels of alkaline earth metals (Ca, Mg, Sr) are considered a major concern in reusing PW since they can cause scaling issues in pipelines and wells (Akob et al., 2015; Hu et al., 2021). Moreover, itwas found that three PW samples have similar major elements, suggesting that PW from the Permian Basin may be treated with similar strategies for reuse and disposal.

On the other hand, it should be noted that some highly toxic heavy metals, such as arsenic (1.66– 1.83 mg/L), cadmium (0.75–0.83 μg/L), and chromium (1.52–2.31 μg/L), were detected in PW (Table 1). Although they only make up a small percentage of components in PW, they may exhibit significant biological effects by inducing acute/chronic toxicity,

Table 1
The main characteristics of three produced water samples collected from the Permian Basin.

Parameter	Symbol	Unit	Mean value		
			PW-1	PW-2	PW-3
Physiochemical characteristics					
pH	–	–	6.8	6.9	6.6
Electrical conductivity	–	mS cm^{-1}	226.4	241.4	310.5
Total organic carbon	TOC	mg/L	52.1	72.5	139.7
Total dissolved solids	TDS	g/L	160.4	172.2	219.6
Total nitrogen	TN	mg/L	381.2	507.8	691.5
Total phosphorus	TP	mg/L	< 0.1	< 0.1	< 0.1
Major ions and elements					
Ammonium	NH_4^+	mg/L	483.4	654.4	879.3
Boron	B	mg/L	51.3	53.6	51.9
Bromide	Br^-	mg/L	651.7	916.8	972.3
Calcium	Ca	mg/L	6319	7027	8159
Chloride	Cl^-	mg/L	88,276	95,576	116,582
Lithium	Li	mg/L	22.3	25.7	20.1
Magnesium	Mg	mg/L	932.1	1167.5	1327.3
Potassium	K	mg/L	687.2	893.7	1201.5
Silica	SiO_2	mg/L	121.6	213.2	136.7
Sodium	Na	mg/L	38,755	41,867	56,629
Strontium	Sr	mg/L	805.7	924.6	723.1
Sulfate	SO_4^{2-}	mg/L	409.3	615.8	758.2
Heavy metals					
Arsenic	As	mg/L	1.69	1.66	1.83
Barium	Ba	mg/L	2.513	2.758	2.612
Cadmium	Cd	μg/L	0.76	0.75	0.83
Chromium	Cr	μg/L	1.52	2.31	1.68
Cobalt	Co	μg/L	7.52	7.96	7.53
Iron	Fe	mg/L	12.08	21.95	22.08
Manganese	Mn	mg/L	1.12	1.36	1.52
Molybdenum	Mo	mg/L	0.1035	0.1206	ND
Nickel	Ni	mg/L	0.2395	ND	ND

ND: Non-Detect.

carcinogenicity, developmental toxicity, mutagenicity, and genotoxicity in animals and human beings (Hu et al., 2020a; Sun et al., 2019; Zou et al., 2020). As shown in Table 1, the total organic carbon (TOC) of PW was in the range of 52.1–139.7 mg/L, and those organic compounds in PW may pose significant risks to aquatic life and human health. Previous studies identified that BTEX (benzene, toluene, ethylbenzene, and xylenes), naphthalene, and polycyclic aromatic hydrocarbons (PAHs) were the primary organic components present in PW (He et al., 2017a; Khan et al., 2016; Zhang et al., 2019), which may also pose a great threat to the aquatic environment. Our previous and current study also demonstrated that BTEX, phenol, naphthalene and petroleum organics are abundant in PW from the Permian Basin (Khan et al., 2016). Still, the toxicological characterizations associated with those potentially toxic contaminants in PW are very limited to date. Hence, further research should be conducted to provide a shortlist of toxicity assays for PW.

3.2. Microtox® toxicity of PW

Microtox® assay using *Vibrio fischeri* has been widely applied for the acute toxicity measurement since it has multiple advantages, including shorter test duration, cost-effectiveness, high sensitivity, and ease of operation (Abbas et al., 2018; El-Din et al., 2011). Fig. 1 shows the toxicity results of different PW samples towards *Vibrio fischeri*. In the current study, to link the toxicological characterizations with the individual group of compounds, each PW was divided into three groups, including whole PW (PW), PW inorganic fraction (PW-IF), and PW salt control (PW-SC). As illustrated in Fig. 1a~c, exposure to 5–10% PW did not cause significant bioluminescence inhibition effect (< 9%) on *Vibrio fischeri*, indicating that 5–10% PW was not very toxic for *Vibrio fischeri*. However, the bioluminescence inhibition level increased significantly when *Vibrio fischeri* was exposed to 20% PW or more. Enhanced toxicity towards *Vibrio fischeri* was probably attributed to the increased salinity in PW. For PW-1 (Fig. 1a), exposure to 20% PW caused a higher inhibition effect (38.6%) compared to 20% PW-IF (35.9%). In this study, biochar treated PW was used as the PW inorganic fraction (PW-IF) since this treatment has been previously demonstrated to remove more than 90% organic compounds, leaving behind the major ions (Table 1 and Table S1) (Hu et al., 2021). This result suggests the organics present in PW may be partially responsible for the acute toxicity. This was supported by the reported studies (He et al., 2017a; He et al., 2018), which revealed that the presence of organic compounds, especially polycyclic aromatic hydrocarbons (PAHs) in PW, might be a significant contributor to the toxicity. Besides, exposure to 20% PW-SC also caused a similar inhibition effect (33.2%) (Fig. 1a), indicating that the high salinity in PW was the predominant toxicological driver. Moreover, it was found that the bioluminescence inhibition level was over 85% when *Vibrio fischeri* was exposed to 40% PW, PW-IF, and PW-SC (Fig. 1a), which further confirmed that high salinity in PW was a major contributor to the acute toxicity. As expected, similar trends were observed in PW-2 and PW-3 (Fig. 1b~c). However, as shown in Fig. 1, when exposed to different PW samples at the same dilution rate, the inhibition effect was generally decreased in the following order: PW-3 > PW-2 > PW-1. Furthermore, EC_{50} (the effective concentration inducing 50% bioluminescence inhibition) was calculated to compare the difference in Microtox® toxicity between three different PW samples. As presented in Table 2, PW-3 was the most toxic one since it showed the lowest EC_{50} value (21.6 $\pm$ 0.3%). One reason for the difference in Microtox® toxicity was that PW-3 had the highest ammonium concentration (879.3 mg/L) compared to PW-1 and PW-2 (483.4 and 654.4 mg/L, respectively), since our previous studies reported that the toxicity of PW towards *Vibrio fischeri* was significantly and positively correlated with ammonium concentration (Hu et al., 2021; Hu et al., 2020b). Additionally, the concentrations of TDS and TOC in PW-3 were higher than those in PW-1 and PW-2 (Table 1), which may be a second possible explanation for the difference in toxicity (Hull et al., 2018).

To further clarify the salinity-influenced responses, different diluted raw PW samples (PW-1, PW-2, and PW-3) with same salinity levels (TDS 30, 50, and 60 g/L) were used for the toxicity assay (Fig. 1d). We found that raw PW samples with same salinity levels caused similar bioluminescence inhibition effects on *Vibrio fischeri*. For example, when exposed to different PW samples (PW-1, PW-2, and PW-3) at TDS 50 g/L, the bioluminescence inhibition effects varied slightly in the range of 60.6–67.5% (Fig. 1d). Thus, no significant statistical difference (p-value > 0.05) was found between PW samples with the same salinity levels, which further confirmed that high salinity in PW was a major contributor to the Microtox® toxicity.

3.3. Cytotoxicity toxicity of PW

3.3.1. MTT viability assay

Several studies have proven that the use of fish cell cultures (*e.g.*, RTgill-W1) is an effective and reliable tool for aquatic toxicology (Brinkmann et al., 2020; Bussolaro et al., 2019; Zeng et al., 2016). In the current work, the cell viability following different PW exposures was examined using the MTT assay, which is a colorimetric method and widely used for the cytotoxicity measurement (Franco et al., 2019; Fu et al., 2017). Fig. 2a~c illustrates the effect of different PW (5–50% PW, PW-IF, and PW-SC) exposures on the RTgill-W1 cell viability. It was found that no remarkable cytotoxicity towards RTgill-W1 cell line was observed for any of the samples at 5–10% dilutions, since the cell viabilities were all above 85% (Fig. 2a~c). The cell viability significantly decreased with increasing PW fraction from 20% to 50%. As shown in Fig. 2a~c, when exposed to 20–40% PW (PW-1, PW-2, and PW-3), the cell viability was generally increased in the following order: PW < PW-IF < PW-SC, indicating that the raw PW was the most toxic one compared to PW-IF and PW-SC. For instance, when the RTgill-W1 cells were exposed to 30% PW-2, the cell viabilities were 26.9%, 43.1%, and 53.2% for PW, PW-IF, and PW-SC, respectively (Fig. 2b). Exposure to PW caused a lower cell viability compared to PW-IF, suggesting a stronger lethal effect on RTgill-W1 cells as result of organic fraction exposure in raw PW (He et al., 2018). Additionally,

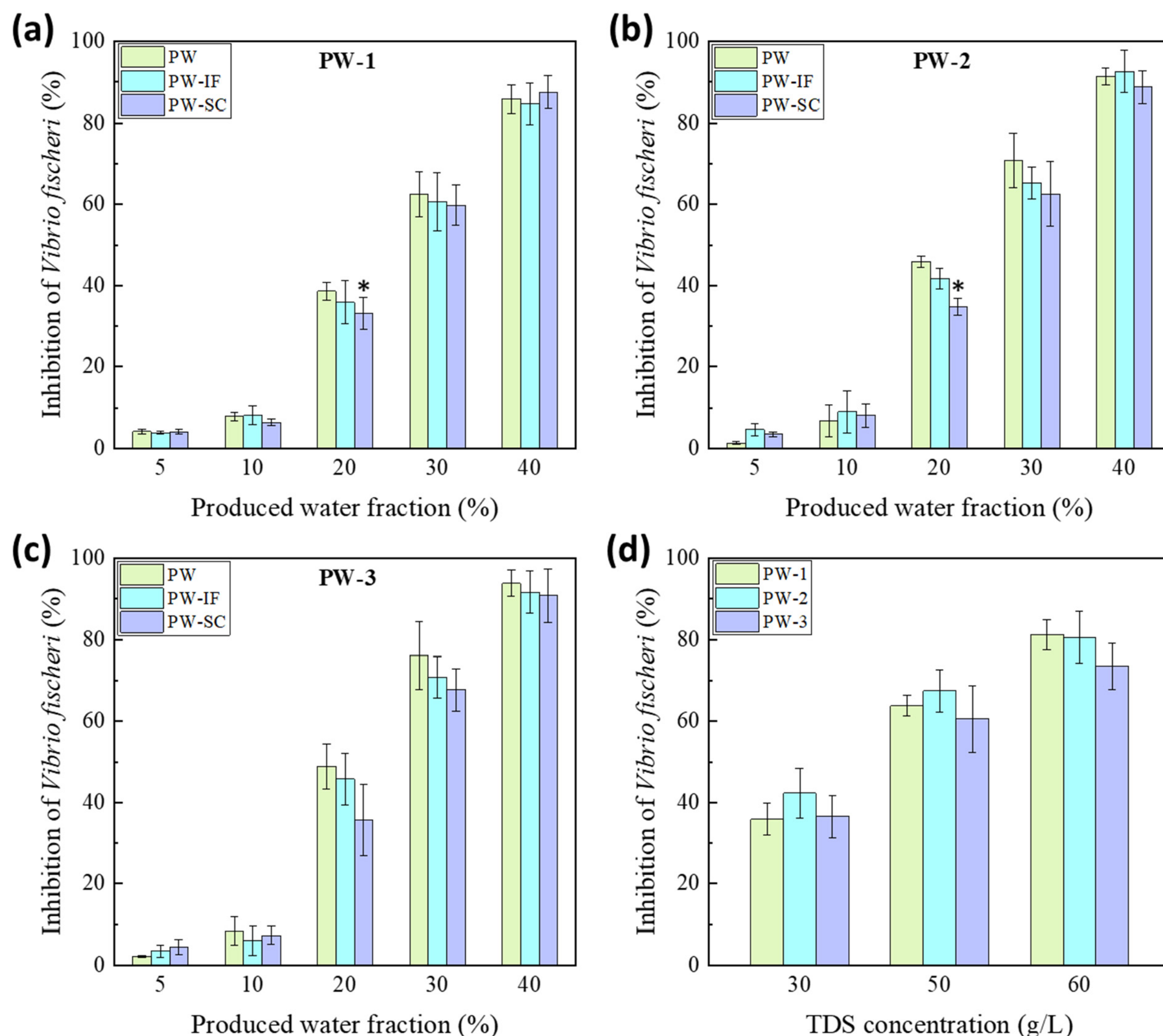

Fig. 1. The acute toxicity results of different PW samples and PW salt controls using Microtox® assay: (a) PW-1; (b) PW-2; (c) PW-3; (d) diluted raw PW samples with same salinity levels (TDS 30 g/L, 50 g/L, and 60 g/L). The error bars in all graphs represent the standard error of the mean (SEM). The asterisk (*) indicates the statistically significant difference (p-value < 0.05) when comparing PW-IF/PW-SC to raw PW at the same produced water fractions.

exposure to PW-IF also resulted in a lower cell viability compared to PW-SC, indicating that the inorganic contaminants present in PW (*e.g.*, heavy metals and other unidentified chemicals) might also be responsible for the cytotoxicity (Mehler et al., 2020; Scott et al., 2021). Those results were supported by the EC_{50} values as presented in Table 2. For PW-2, the EC_{50} values of PW, PW-IF, and PW-SC were 23.6 ± 0.4%, 27.3 ± 0.2%, and 31.5 ± 1.2%, respectively, demonstrating that the exposure to organic components in PW could induce greater cytotoxicity towards RTgill-W1 cells. As expected, similar trends were observed in PW-1 and PW-3. However, for any of the samples at 50% dilution, the cell viabilities were all less than 10% (Fig. 2). Since the results in PW exposures were not statistically different (p-value > 0.05) from the PW-IF and PW-SC exposures, suggesting that the high salinity should be the predominant toxicological driver at 50% dilution. Furthermore, similar to results obtained in the Microtox® toxicity assay (Fig. 1d), no significant difference (p-value > 0.05) on the cell viability was observed between diluted PW samples (PW-1, PW-2, and PW-3) with same salinity levels (Fig. 2d). Hence, the high salinity may mask the toxicological effects posed by other hazardous constituents in PW, thereby making the determination of potentially toxic contaminants challenging (He et al., 2017a).

3.3.2. LDH release assay

To determine whether PW could cause damage of the cell plasma membrane, the cytotoxic effects of all samples (PW, PW-IF, and PW-SC) were

Table 2
EC_{50} values (the effective concentration inducing 50% inhibition/mortality) of different PW samples and salt controls towards various organisms.

Organisms	Toxicity assay	Exposure time	PW-1 (%)			PW-2 (%)			PW-3 (%)		
			PW	PW-IF	PW-SC	PW	PW-IF	PW-SC	PW	PW-IF	PW-SC
Vibrio fischeri	Microtox®	15 min	25.9 ± 0.3	26.5 ± 0.5	26.7 ± 0.6	22.7 ± 0.2	23.9 ± 0.5	25.1 ± 0.3	21.6 ± 0.3	22.8 ± 0.1	24.3 ± 0.5
RTgill-W1	MTT	48 h	26.1 ± 0.3	28.5 ± 0.1	30.3 ± 0.2	23.6 ± 0.4	27.3 ± 0.2	31.5 ± 1.2	20.6 ± 0.3	22.9 ± 0.6	23.7 ± 0.4
	LDH	24 h	33.8 ± 1.1	32.1 ± 0.5	35.6 ± 0.3	30.8 ± 0.5	32.5 ± 0.3	32.7 ± 0.8	27.2 ± 0.5	28.1 ± 0.6	28.8 ± 1.1
Scenedesmus obliquus	Growth inhibition	7 d	27.9 ± 0.6	33.9 ± 0.3	28.3 ± 0.4	25.3 ± 0.7	28.5 ± 0.4	24.1 ± 0.9	11.5 ± 0.3	16.9 ± 0.5	11.2 ± 0.3

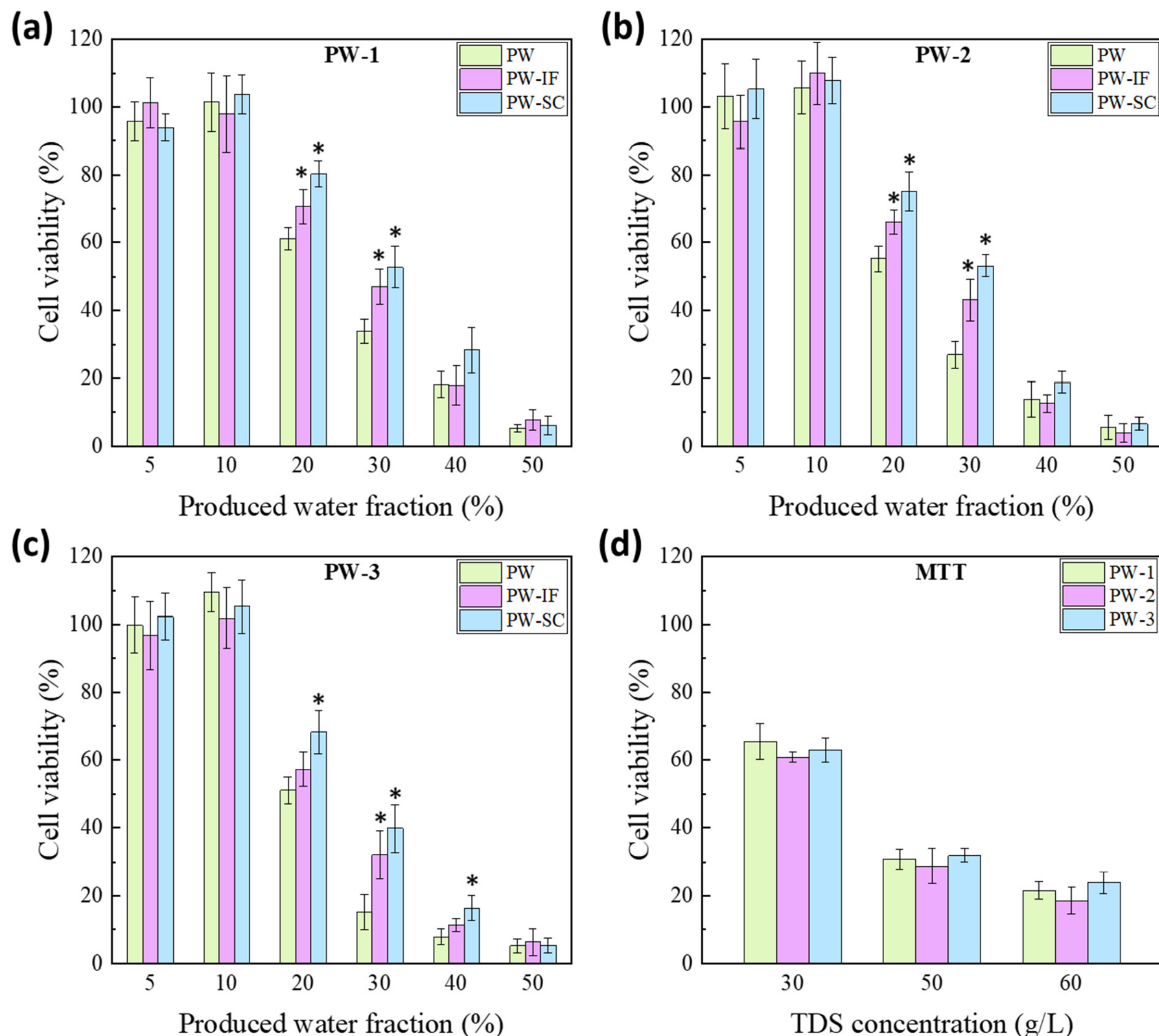

Fig. 2. The effect of different PW samples and PW salt controls on RTgill-W1 cell viability using MTT assay: (a) PW-1; (b) PW-2; (c) PW-3; (d) diluted raw PW samples with same salinity levels (TDS 30 g/L, 50 g/L, and 60 g/L). The error bars in all graphs represent the standard error of the mean (SEM). The asterisk (*) indicates the statistically significant difference (p-value < 0.05) when comparing PW-IF/PW-SC to raw PW at the same produced water fractions.

evaluated with RTgill-W1 cells using the LDH release assay (Fig. 3). Lactate dehydrogenase (LDH) is a cytosolic enzyme present in RTgill-W1 cells. Exposure to hazardous compounds can result in the plasma membrane damage of RTgill-W1 cells, thereby releasing LDH into the cell culture media. Extracellular LDH in the media can then be quantified by a coupled enzymatic reaction, which is a reliable indicator of cytotoxicity (McLaughlin et al., 2021; Ragazzo et al., 2017). As illustrated in Fig. 3a∼c, for the exposure to all samples at 5–10% dilutions, the levels of cytotoxicity were relatively low (< 9.2%), which were consistent with the results obtained from the MTT viability assay (Fig. 2a∼c). However, the exposure to any of the samples at 20–50% dilutions caused a significant increase in the LDH release (up to 93.1%). For example, when exposed to PW-3 at 30% dilution (Fig. 3c), the cytotoxicity levels of PW, PW-IF, and PW-SC were 65.3%, 56.5%, and 53.8%, respectively. As expected, similar trends were observed when exposed to PW-1 and PW-2, which further confirmed that the organic compounds present in PW had a substantial impact on the cell membrane damage. These results are consistent with the findings recorded previously (He et al., 2017a), which characterized the organic signatures in PW samples from the Duvernay Formation and found that the presence of organic components, especially polycyclic aromatic hydrocarbons (PAHs) in PW, might be the main contributor to the acute toxicity in zebrafish embryos. However, it is also notable that the exposure to 40–50% PW resulted in significantly higher cytotoxicity levels for all samples, and almost no statistical differences (p-value > 0.05) were found in PW, PW-IF, and PW-SC (Fig. 3a∼c). Thus, the high salinity can be considered the major contributor to the cytotoxicity in RTgill-W1 cells. These results were supported by a previous study (He et al., 2018), which investigated the potential effects of PW on zebrafish (*D. rerio*) embryo development and demonstrated that the high salinity caused a significant adverse impact in developing fish embryos. Moreover, it was found that the LDH release was similar when exposed to diluted PW samples (PW-1, PW-2, and PW-3) with the same salinity levels (Fig. 3d), suggesting that the high salinity in PW is the predominant contributor to cell lysis. Furthermore, as shown in Table 2, PW-3 was the most toxic one because it had the lowest EC_{50} value (27.2 ± 0.5%) compared to PW-1 and PW-2 (33.8 ± 1.1% and 30.8 ± 0.5%, respectively), which was possibly due to the highest concentrations of TOC, TDS, and TN (Table 1). However, the results in the LDH assay were slightly different from the results in the MTT assay. One possible explanation is the MTT and LDH assays with different responses to PW due to their different test mechanisms (Fu et al., 2017; McLaughlin et al., 2021). A second reason may be the diversity and complexity of the mixture of chemicals found in PW resulting in some minor differences in the cytotoxic responses (He et al., 2017a).

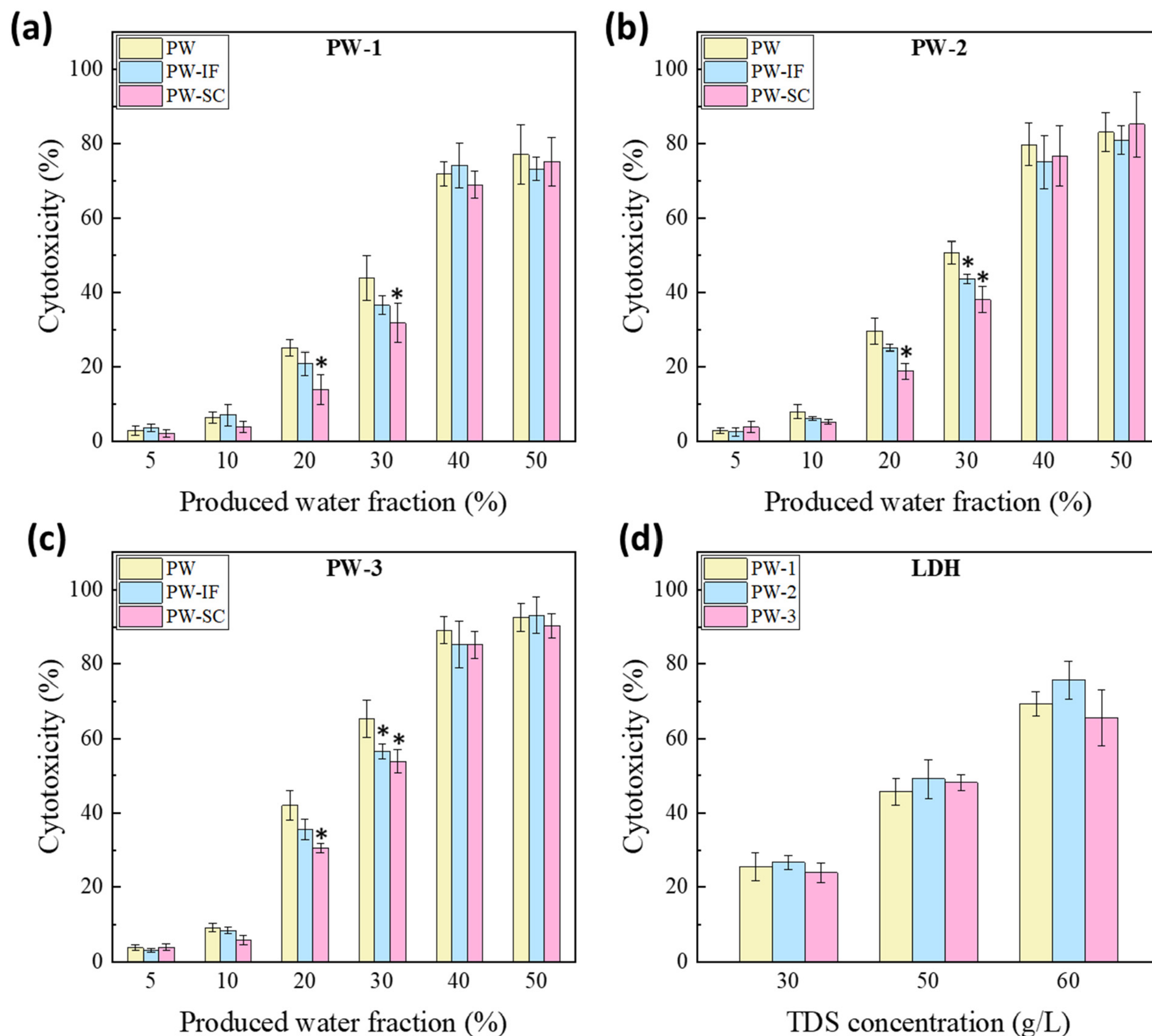

Fig. 3. The cytotoxic response of RTgill-W1 cell line exposed to different PW samples and PW salt controls using LDH assay: (a) PW-1; (b) PW-2; (c) PW-3; (d) diluted raw PW samples with same salinity levels (TDS 30 g/L, 50 g/L, and 60 g/L). The error bars in all graphs represent the standard error of the mean (SEM). The asterisk (*) indicates the statistically significant difference (p-value <0.05) when comparing PW-IF/PW-SC to raw PW at the same produced water fractions.

Several *in vivo* toxicity assays have been applied previously to characterize the toxicological behaviors of PW using various live vertebrates and invertebrates, such as zebrafish (*D. rerio*), *Lumbriculus variegatus*, water flea (*Daphnia magna*), fathead minnow, and rainbow trout (*Oncorhynchus mykiss*) (Blewett et al., 2017; Folkerts et al., 2019; Folkerts et al., 2017a; Folkerts et al., 2017b; He et al., 2017b; McLaughlin et al., 2020; Mehler et al., 2020). Those studies have also shown that exposure to PW could induce significantly toxic responses in zebrafish and rainbow trout through several mechanisms, including oxidative stress, biotransformation, and endocrine disruption (Folkerts et al., 2017b; He et al., 2017b). However, the current work is the first study to apply the RTgill-W1 cell-based cytotoxicity assay to explore the toxicological characterizations of PW, which can be served as a valuable *in vitro* alternative to the live fish specimens for the WET tests to reduce the number of animals sacrificed.

3.4. Aquatic ecotoxicity of PW

The aquatic ecotoxicity of PW towards the green microalgae *Scenedesmus obliquus* was evaluated by the growth inhibition assay, which has been proven to be sensitive for the WET testing, especially when multiple contaminants are present simultaneously (He et al., 2019; Yu et al., 2014b). As presented in Fig. 4a~c, for all samples, the growth inhibition rate significantly increased with increasing PW fractions, showing a dose-response relationship. The growth of *Scenedesmus obliquus* was significantly inhibited (inhibition rate > 85%) when exposed to 50% PW-1 and PW-2 (Fig. 4a and 4b), while the exposure to 30% PW-3 could inhibit the algal growth completely (inhibition rate > 91%, Fig. 4c). Hence, PW-3 seems more toxic than PW-1 and PW-2, which was further supported by the EC_{50} values as shown in Table 2. The EC_{50} value of PW-1 was 27.9 ± 0.6%, which was slightly higher than that of PW-2 (25.3 ± 0.7%), suggesting a comparable level of aquatic ecotoxicity for these two PW samples towards the microalgae *Scenedesmus obliquus*. In contrast, PW-3 showed the highest aquatic ecotoxicity towards *Scenedesmus obliquus*, with a much lower EC_{50} value (11.5 ± 0.3%) than PW-1 and PW-2 (Table 2). In the current work, the significant inhibition effect posed by PW was probably because the high salinity caused irreversible damage to *Scenedesmus obliquus* and resulted in the cell lysis.

It is noteworthy that the exposure to PW salt control (PW-SC) sample caused a slightly higher toxicity level compared to raw PW (Fig. 4a~c), which was quite different from the results in Microtox® and cytotoxicity assays (Fig. 1~3). For example, when exposed to 30% PW-2, the growth inhibition rates were 68.4% and 72.9% for PW and PW-SC (Fig. 4b), respectively, indicating that the PW-SC was more toxic to the microalgae *Scenedesmus obliquus*. A possible explanation for this noted discrepancy is

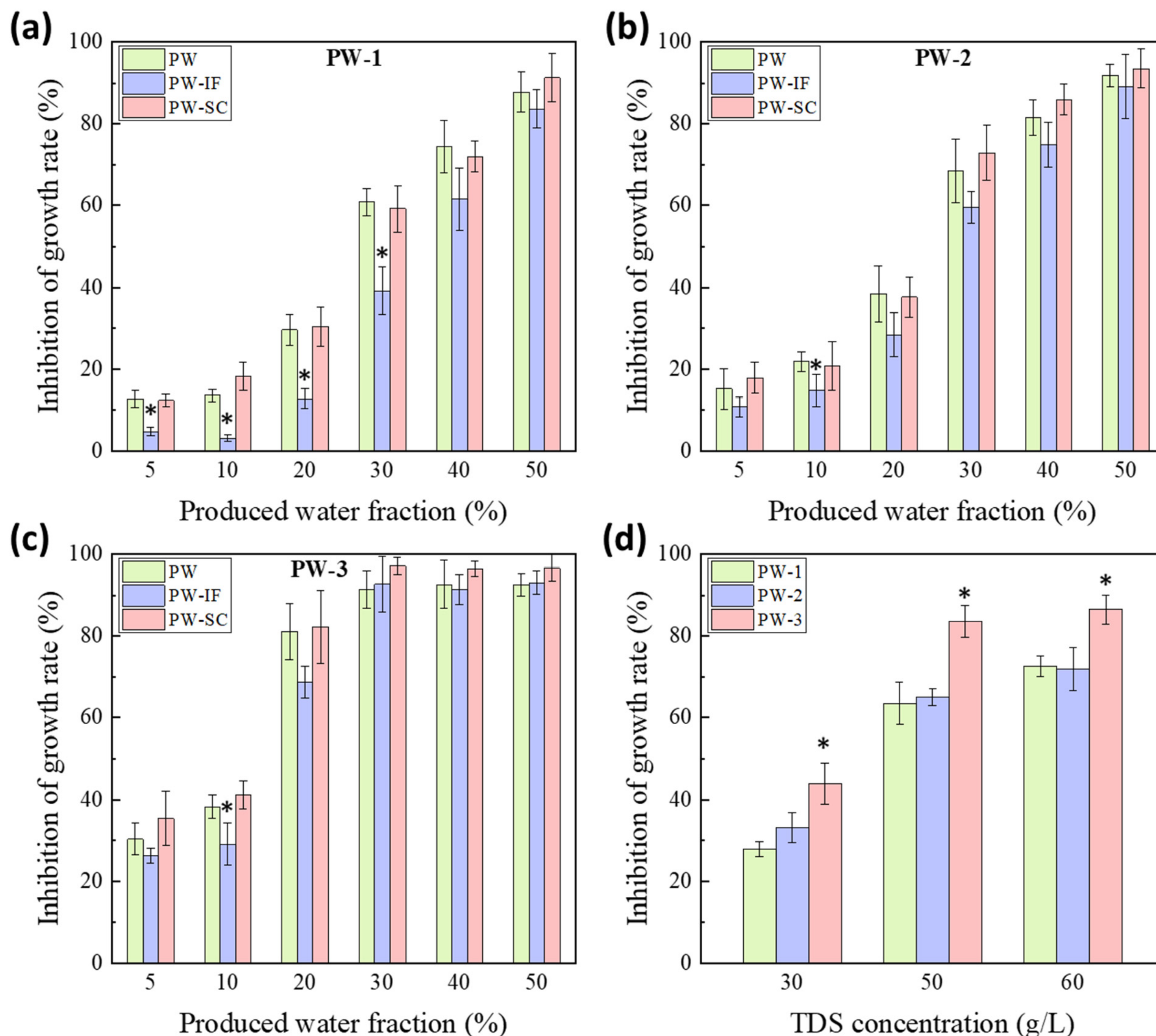

Fig. 4. The aquatic ecotoxicity results of different PW samples and PW salt controls: (a) PW-1; (b) PW-2; (c) PW-3; (d) diluted raw PW samples with same salinity levels (TDS 30 g/L, 50 g/L, and 60 g/L). The error bars in all graphs represent the standard error of the mean (SEM). The asterisk (*) indicates the statistically significant difference (*p*-value <0.05) when comparing PW-IF/PW-SC to raw PW at the same produced water fractions (or comparing PW-2/PW-3 to PW-1 at the same TDS concentrations).

that ammonium present in raw PW at high concentration (483.4–879.3 mg/L, Table 1) can promote the algal growth (Liu et al., 2019; Martınez et al., 2000) after dilution, thereby alleviating the adverse effects of raw PW on *Scenedesmus obliquus*. Moreover, as shown in Fig. 4a, when exposed to 30% PW-1, the growth inhibition rates were 60.8% and 39.2% for PW and PW-IF, respectively. Similar results were also observed for other samples. Thus, in addition to salinity, organic contaminants present in PW have the most significant impact on the aquatic ecotoxicity towards *Scenedesmus obliquus*. These results were consistent with the findings of He et al. (2019, who found that the aquatic ecotoxicity of the flowback and produced water towards *Scenedesmus obliquus* decreased markedly after removing organics (*i.e.*, the growth inhibition rate decreased from 98.7–100% to 63.6–78% after 72 h exposure). In line with our results, another study (Sambusiti et al., 2020) showed that the synthetic PW with various organic compounds but low salinity (1.5 g/L) was highly toxic to microalgae *Pseudokirchneriella subcapitata*, with the EC_{50} value of 1%.

Furthermore, as presented in Fig. 4d, after PW samples were diluted to the same salinity level, the exposure to PW-3 caused a much higher growth inhibition rate (43.9–86.5%) than PW-1 and PW-2 (27.9–72.6%), which was slightly different from the results in other toxicity assays. When different PW samples were diluted to the same salinity level, PW-3 still had the highest TOC concentration compared to PW-1 and PW-2 (Table S2). Given that the diluted PW-3 had similar ammonia and heavy metal concentrations as the diluted PW-2, a possible explanation for this noted discrepancy is that the aquatic ecotoxicity assay towards *Scenedesmus obliquus* was more sensitive to organics in PW compared to the Microtox® toxicity and cytotoxicity assays.

3.5. Effect of different pretreatments on the toxicity of PW

To date, the toxicological behaviors of different constituents in PW have not been evaluated. However, given the complexity of PW, it would be challenging to identify the toxicological characterizations of each individual compound in PW (Sun et al., 2019), and toxicological characteristics would likely be altered with chemical mixture combinations. Hence, in this study, to pinpoint target contaminants, different pretreatments were applied to remove the typical groups of contaminants (*e.g.*, ammonium, heavy metals, and organics) from PW-3 (Table S3). Then, the Microtox® toxicity, cytotoxicity, and aquatic ecotoxicity of the treated PW-3 (diluted to 20%) were measured. Fig. 5a illustrates the acute toxicity of raw and treated PW towards *Vibrio fischeri*. It was found that both organic removal and heavy metal removal could lead to a slight decrease in the bioluminescence inhibition level (from 48.9% to 45.8% and 45.3%, respectively),

while the ammonium removal resulted in a greater decrease in the acute toxicity towards *Vibrio fischeri* (inhibition effect decreased from 48.9% to 37.5%), suggesting that high ammonium concentration was one of the main contributors to the acute toxicity. These results are supported by the findings published previously (Yu et al., 2014a; Yu et al., 2014b), which revealed that the acute toxicity towards *Vibrio fischeri* was positively correlated with ammonium concentration in antibiotic and pharmaceutical wastewaters. For the results of the cytotoxicity assays (Fig. 5b and 5c), we found that all pretreatments could slightly reduce the cytotoxicity towards RTgill-W1 cells, and no statistical differences ($p > 0.05$) were observed between those pretreatments. Thus, the contaminants, including ammonium, organics, and heavy metals, were all considered responsible for the cytotoxicity in PW. These results were consistent with a previous study (Scott et al., 2021), which used the RTgill-W1 cell line as an *in vitro* model to evaluate the cytotoxicity of toxicants commonly found in wastewaters and found that RTgill-W1 cells were very sensitive to ammonium, metalloids, metal, and water-soluble organic compounds. On the other hand, as shown in Fig. 5b, the combination of organic, ammonium, and heavy metal removal could lead to a greater increase in the cell viability of RTgill-W1 (from 50.8% to 70.5%), indicating that a higher reduction of cytotoxicity was achieved after the combination of different pretreatments. As expected, similar results were obtained with the LDH release assay (Fig. 5c). A possible explanation for this discrepancy is that the mixture of contaminants in PW may result in the synergistic induction of cytotoxicity compared to the individual compound (He et al., 2017a; He et al., 2018), thereby exhibiting a more severe impact on RTgill-W1 cells. For the aquatic ecotoxicity assay (Fig. 5d), the ammonium removal slightly increased the algal inhibition rate from 81.1% to 86.2%, which was totally different from the results in the Microtox® toxicity cytotoxicity assays (Fig. 5a~c). One possibility for this discrepancy is that the high ammonium concentration in PW-3 (879.3 mg/L) could enhance the growth of microalgae *Scenedesmus obliquus* as discussed in Section 3.4 (Liu et al., 2019). As presented in Fig. 5d, the organic removal could result in a greater decrease in the growth inhibition rate (from 81.1% to 68.6%) compared to the heavy metal removal (from 81.1% to 72.5%). Hence, in addition to salinity, organic contaminants have the most significant impact on the aquatic ecotoxicity of PW towards *Scenedesmus obliquus*. Overall, owing to the different sensitivity associated with those assays, toxicity assays should be selected based on the target compounds in PW for practical applications.

3.6. Environmental implications

This work is one of the first studies to assess the toxicological characteristics of PW from the shale plays in the Permian Basin. Such toxicity information is essential because of the complexity of chemical composition in PW and the confidential nature of operations in the shale oil and gas

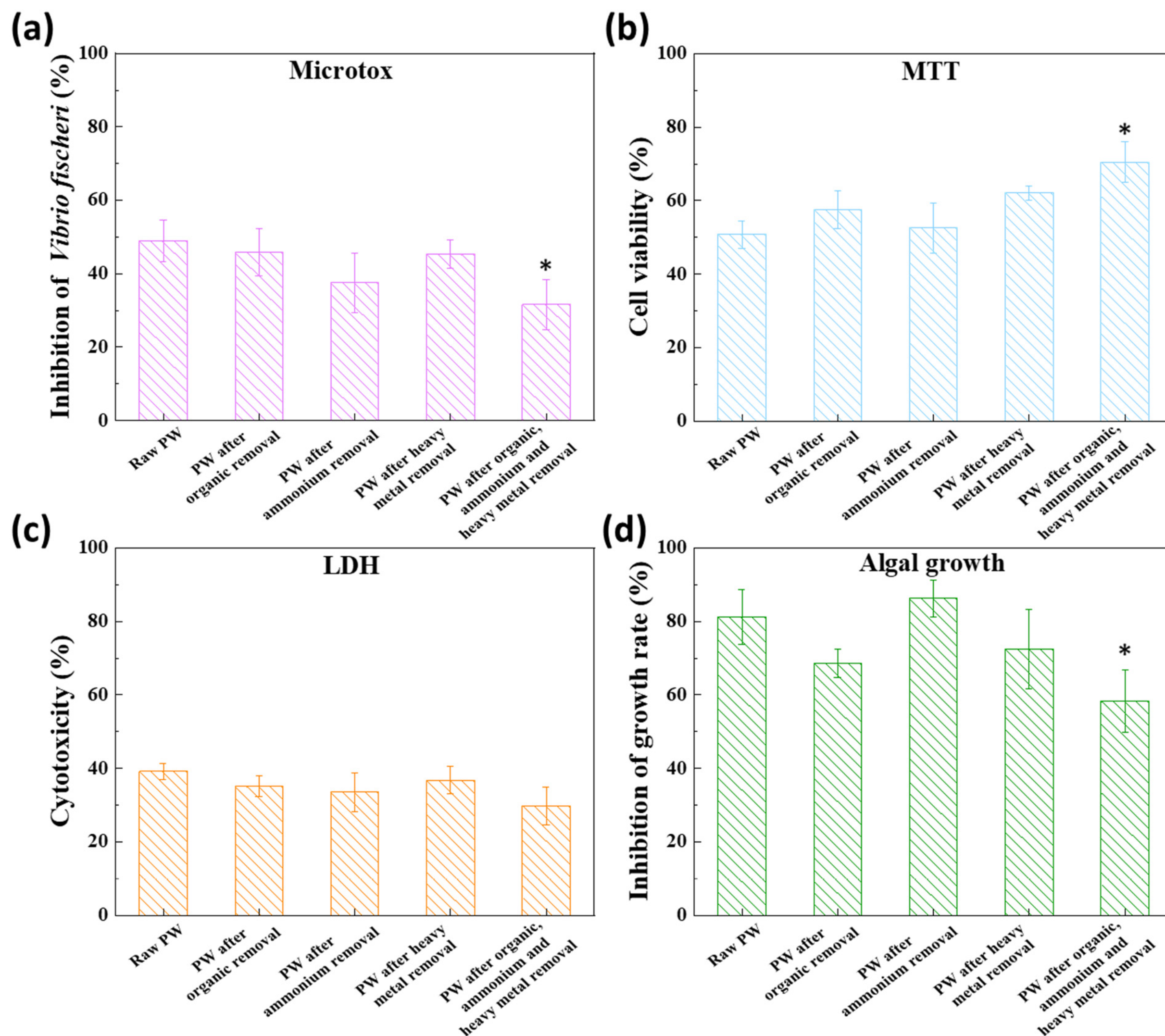

Fig. 5. The effect of different pretreatments on the (a) acute toxicity towards *Vibrio fischeri*; (b) RTgill-W1 cell viability based on MTT viability assay; (c) RTgill-W1 cell cytotoxicity based on LDH release assay; and (d) aquatic ecotoxicity towards *Scenedesmus obliquus* of PW. The error bars in all graphs represent the standard error of the mean (SEM). The asterisk (*) indicates the statistically significant difference (p-value <0.05) when comparing treated PW to raw PW.

industry. Several published papers (Burton et al., 2016; Hildenbrand et al., 2015a; Hildenbrand et al., 2016; Hildenbrand et al., 2017; Hildenbrand et al., 2020) tried to establish a link between the groundwater quality and the activity of shale oil and gas extraction in different shale regions. Although the data did not always identify the activities of shale oil and gas exploration as the source of groundwater contamination since the contamination processes and pathways are complex, variable, and uncertain. Various toxic compounds (*e.g.*, bromide, alcohols, chlorinated species, and BTEX compounds) associated with the shale oil and gas extraction techniques have been previously detected in the groundwater in the Barnett Shale region and Permian Basin (Hildenbrand et al., 2015a; Hildenbrand et al., 2016), which might be evidence of episodic groundwater contamination events potentially attributed to the shale oil and gas development. Hence, due to the public health concern, it is critical to monitor PW quality regularly utilizing targeted toxicity assays to characterize the environmental impacts of PW spills or illegal discharge and support evaluation of spill mitigation strategies by considering the dilution factors. The *in vitro* toxicity assays used in this study have the potential to serve as cost-effective and rapid health risk assessment tools, which should also be helpful to evaluate the effect of putative contamination events potentially attributed to shale oil and gas production. Furthermore, with detected toxicity from various fractions and contaminant groups in PW, appropriate PW treatment and management strategies can be identified to minimize the risk of PW discharge and reuse.

4. Conclusions

In the current study, the chemical profiles and toxicological signatures of PW samples from the Permian Basin were characterized and linked for the first time. Our results demonstrated that the exposure to PW, PW-IF, and PW-SC at 30–50% dilutions could induce significant toxicity in all toxicological model species (bacterium *Vibrio fischeri*, fish gill cell line RTgill-W1, and microalgae *Scenedesmus obliquus*), suggesting that the high salinity was the predominant toxicological driver in PW. For all *in vitro* toxicity assays, it was observed that PW exhibited a higher toxicity level than PW-IF. Thus, in addition to salinity, organic contaminants has an important impact on the toxicity of PW. It was found that microalgae *Scenedesmus obliquus* has the highest sensitivity to the organic contaminants in PW, and it should be used to evaluate the toxicity of organic compounds in raw and treated PW. Moreover, correlations were found between chemical components and toxicity results since PW samples that had the lowest EC_{50} values had the highest TDS, TOC, and TN concentrations. Furthermore, the combination of multiple pretreatments could lead to a more significant decrease in toxicity compared to a single pretreatment, suggesting that the mixture of components in PW might exhibit synergistic toxicity. However, work to evaluate the potential toxicological effects of PW in aquatic ecosystems is still in its infancy. Therefore, extensive future studies are needed to further investigate species sensitivity and exposure scenarios in the Permian Basin and other oil and gas production basins, which could provide valuable information for the regulatory agencies and industry to monitor and mitigate the toxicological effects of PW releases to the environment.

CRediT authorship contribution statement

Lei Hu: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Wenbin Jiang:** Investigation. **Xuesong Xu:** Investigation. **Huiyao Wang:** Conceptualization, Writing – review & editing. **Kenneth C. Carroll:** Conceptualization, Writing – review & editing. **Pei Xu:** Writing – review & editing, Project administration, Funding acquisition. **Yanyan Zhang:** Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.152943.

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