THESIS

REUSE OF OIL AND GAS PRODUCED WATER FOR IRRIGATION OF SPRING WHEAT (*TRITICUM AESTIVUM L.*): PLANT PHYSIOLOGICAL AND IMMUNE SYSTEM RESPONSE

Submitted by

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ABSTRACT

REUSE OF OIL AND GAS PRODUCED WATER FOR IRRIGATION OF SPRING WHEAT (*TRITICUM AESTIVUM L.*): PLANT PHYSIOLOGICAL AND IMMUNE SYSTEM RESPONSE

Water resources for agricultural irrigation in the semiarid western United States are challenged due to increased oil and gas (O&G) activity and increasing water scarcity. Produced water (PW) generated from the O&G industry has been considered as an alternative source for crop irrigation, but there are few studies on the topic. Thus, here a greenhouse study was conducted to evaluate the impacts of PW irrigation on spring wheat (*Triticum aesticum L.*) with respect to plant morphology, physiology, and immunity to bacterial and fungal pathogens. Plants were irrigated with the following types of water: 100% tap water (TW), 10% and 50% PW (PW10 & PW50) and a salt (NaCl) solution (SW50 control; NaCl concentration is equal to PW50). Furthermore, pathogen treatments containing bacteria (*Xanthomonas campestris*) and fungi (*Septoria tritici*) were applied to the wheat plants to test plant immune response. In comparison with the TW control, plants irrigated with PW50 exhibited developmental delay and premature senescence, significant loss of yield, and significant decline in photosynthetic efficiency and immune function. The PW10 and SW50 control both resulted in reduced plant yield and photosynthesis, but PW10 was more damaging than SW50 to plant immune system, despite the high salt contents in SW50. These findings indicate that constituents (e.g., organic contaminants) other than NaCl in PW are contributing to plant stress, and they may play a far greater role in affecting plant immune function than salt stress.

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1. INTRODUCTION

1.1 Global Water Use and Scarcity

Water is essential for sustaining life (Ercin & Hoekstra, 2014); human civilization originated around river basins because the water contributed to fertile soils, which enabled the emergence of agriculture (Macklin & Lewin, 2015). Agriculture consumes around 70% of the global water supply (Figure 1) and currently, water resources are limited for food production (Shiklomanov, 2000). Moreover, almost half of all agricultural land in the world is located in semiarid regions, which produce 44% of the world's foods (Echchelh, Hess, & Sakrabani, 2018). Presently, four billion people are experiencing severe water scarcity monthly, and half a billion people are suffering from it throughout the year (Mesfin M. Mekonnen & Hoekstra, 2016). It is predicted that in 2025, two-thirds of the global populations will suffer from water stress and 90% will be considered water vulnerable (Arnell, 2004; Raskin, Gleick, Kirshen, Pontius, & Strzepek, 1997). Although scarcity of agricultural water is a problem throughout the globe, this study focuses on the U.S., specifically Colorado.

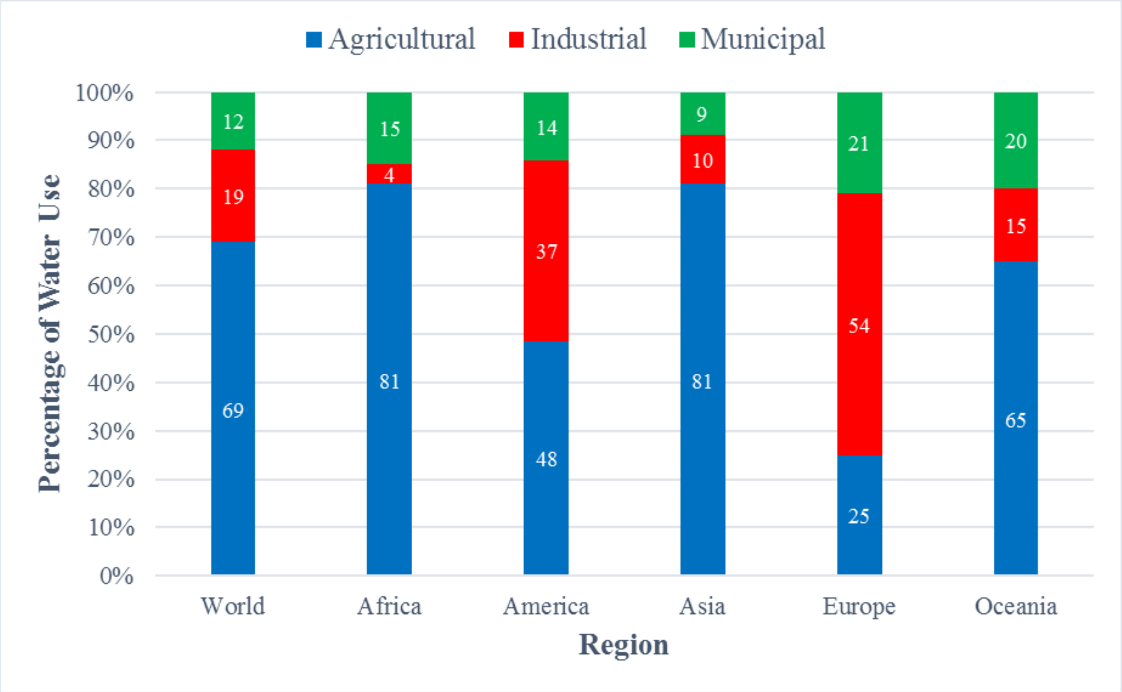

Figure 1. Global water withdrawal by sectors around 2010. Six regions from left to right include World, Africa, America, Asia, Europe, and Oceania. Three sectors from top to bottom are municipal (green), industrial (red), and agricultural (blue). (source: http://www.fao.org/nr/water/aquastat/tables/WorldData-Withdrawal_eng.pdf)

1.2 Water Use and Scarcity in the United States

Water use in the U.S. is dominated by thermoelectric power and irrigation. In 2000, 2005, 2010, and 2015, the estimated amount of water used for irrigation was 137,000, 128,000, 115,000, and 118,000 million gallons per day, which accounts for 34%, 31%, 33%, and 37% of the total water use in the U.S., respectively (Figure 2). Furthermore, western contiguous U.S. used around 70% of the irrigation water nationally, particularly in California, Idaho and Colorado, top three states that use the most water for irrigation (Figure 3).

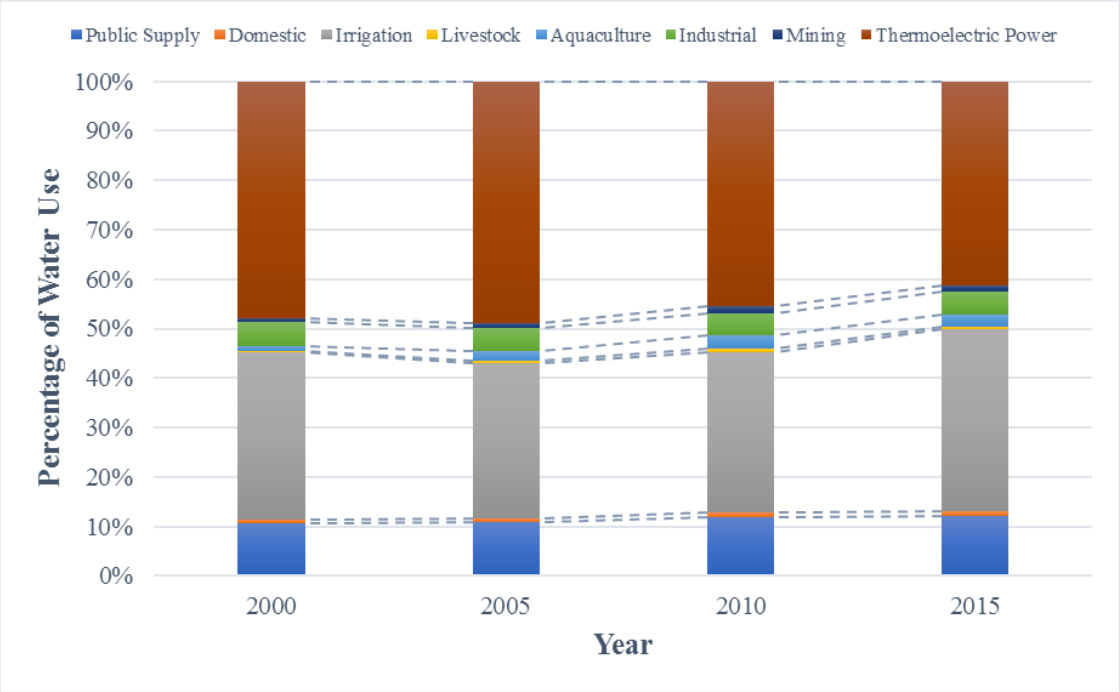

Figure 2. Estimated use of water in the United States in 2000, 2005, 2010, and 2015 (Dieter et al., 2018; Hutson et al., 2004; Kenny et al., 2009; Maupin et al., 2014)

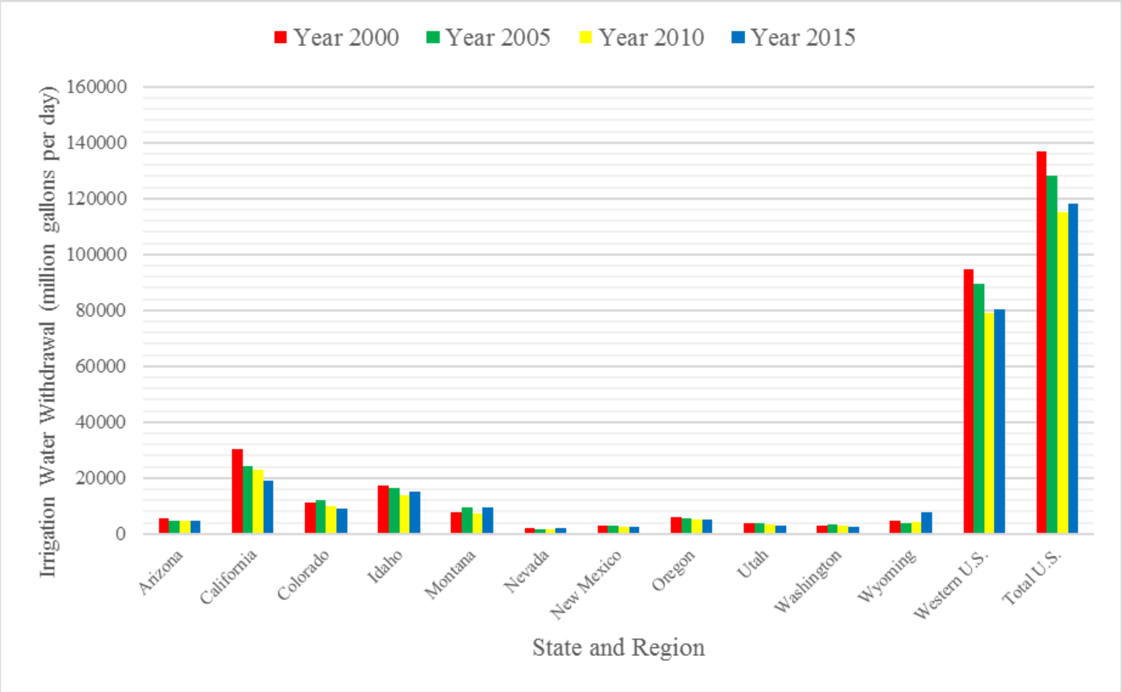

Figure 3. Estimated irrigation water uses of western contiguous United States in 2000, 2005, 2010, and 2015 (Dieter et al., 2018; Hutson et al., 2004; Kenny et al., 2009; Maupin et al., 2014)

Agricultural water supply is under threat by climate change and population growth. Climate change will reduce water availability in the semiarid U.S. due to inconsistent precipitation and increasing temperature (Islam et al., 2012; Schewe et al., 2014). Additionally, population growth and economic development also affect water demand (Ercin & Hoekstra, 2014; Vörösmarty, Green, Salisbury, & Lammers, 2000). The commonly used water stress index informs that 1,700 m^3 of fresh water per capita per year as a threshold level to meet basic needs. Regions whose water supplies cannot meet this requirement are under water stress, and water scarcity occurs if the supplies are lower than 1,000 m^3, such as the High Plains in the United States (Mesfin M. Mekonnen & Hoekstra, 2016; Rijsberman, 2006). In the 2010s, as a result of rapid population growth and intensive irrigation water use, the western U.S. experienced moderate to severe water scarcity during the spring and summer seasons, and the Colorado River Basin is struggling with groundwater depletion (Mesfin M. Mekonnen & Hoekstra, 2016). To solve these problems, the Colorado Water Plan gathers information and estimates from stakeholders of major basins, but neither conservation strategies nor reservoir projects can fulfill the water supply gap, thus forcing Colorado to find alternative water sources for agricultural use (Dolan, Cath, & Hogue, 2018).

1.3 Irrigated Agriculture in the United States

Agricultural irrigation is one of the major consumers of water in the U.S., and it plays an important role in U.S. development and economy. In 2012, 50% of the market value of crops sold in the U.S. comes from irrigated farmlands, and over 70% of these farms are in the 17 western states. Between 2008 to 2013, around 500,000 acres of farmland across the U.S. could not get sufficient water for irrigation, due to surface and groundwater shortages. This problem is particularly severe in the western U.S. because of increasing demands on water supplies, thereby raising concerns about irrigation sources and efficiency (Stubbs, 2016).

1.3.1 Water Sources for Irrigation

There are three major sources of water for crop irrigation: (1) stored rainwater, (2) groundwater and surface water, and (3) reclaimed wastewater (M. M. Mekonnen & Hoekstra, 2011; STEELE & ODUMERU, 2016; Wada, Van Beek, & Bierkens, 2012). Fresh surface water and groundwater are used as primary sources for agricultural irrigation in the U.S., which accounts for more than 99% of the U.S. irrigation water use over the past 20 years. In contrast, neither rainwater nor reclaimed wastewater are commonly used for irrigation. The USGS just start collecting the irrigation data of reclaimed wastewater reuse, and the amount is negligible (< 1%) compared to the total amount of irrigation water use (Table 1).

Table 1. Estimated groundwater, surface water, reclaimed wastewater, and total water used for irrigation in the United States in 2000, 2005, 2010, and 2015 (Dieter et al., 2018; Hutson et al., 2004; Kenny et al., 2009; Maupin et al., 2014)

Year	**Groundwater (million gallons per day)**	**Surface Water (million gallons per day)**	**Reclaimed Wastewater (million gallons per day)**	**Total (million gallons per day)**
2000	56,900	80,000	Not available	137,000
2005	53,500	74,900	Not available	128,000
2010	49,500	65,900	Not available	115,000
2015	57,200	60,900	669	118,000

In recent years, irrigation use of groundwater has been significantly increased in semiarid regions due to technological advancements (Garrido, Martínez-Santos, & Llamas, 2006). At the same time, depletion of aquifers occurred in the U.S. High Plains and Central Valley (Mesfin M. Mekonnen & Hoekstra, 2016; Scanlon et al., 2012). However, the application of efficient irrigation technology does not help with groundwater conservation but leads to increased extraction, which exacerbates water shortage in High Plains aquifer (Pfeiffer & Lin, 2014).

1.3.2 Irrigation Water Managements and Challenges

Due to increasing demand and decreasing supply of agricultural water, management of irrigation water resources has become an important topic in semiarid regions (Wallace, 2000). Agricultural water management strategies in drylands include water conservation, wastewater reusing, cropping pattern modification, and irrigation managements (Pedrick, 2012; Pereira, Oweis, & Zairi, 2002). In the past several decades, the U.S. has begun using less agricultural water by growing less water intensive crops and using more efficient irrigation technologies (Donnelly & Cooley, 2015; Stubbs, 2016).

Subsurface, sprinkler, and trickle or drip irrigations lead to higher crop productivities while reducing water use as compared to the conventional surface and furrow irrigation (Sammis, 2010). The irrigation techniques have been practiced with various crops such as onion, cotton and kale (Al-Jamal, Ball, & Sammis, 2001; Ibragimov et al., 2007; Maisiri, Senzanje, Rockstrom, & Twomlow, 2005). However, these efficient irrigation methods have disadvantages. Sprinkler irrigation is sensitive to wind and is limited to specific field and irrigation requirements, also has potential to decrease soil water infiltration capacity thereby causing soil erosion (Claude H. Pair, 2013; D. L. Bjorneberg & J. K. Aase, 2013). Although subsurface drip irrigation is regarded as the system with high water use efficiency and good yield response, this system is difficult to monitor and evaluate, has clogging and leaking problem, and expensive (C. R. Camp, 2013; Martínez & Reca, 2014).

In recent years, agricultural scientists have started modifying old irrigation methods and developing new technologies such as alternative subsurface drip irrigation, an innovation that can not only improve crop yields, but avoid most of the disadvantages listed above (Martínez & Reca, 2014). Deficit irrigation, which refers to irrigation applied during drought sensitive growth stages of crop, is one of the most commonly practiced strategies that provides a good compromise between crop performance and water use when full irrigation is not applicable (Fereres & Soriano, 2007; Geerts & Raes, 2009). The U.S. government and producers has raised interest in the adoption of irrigation technologies and management practices due to water shortages, and the application of new technologies can further increase irrigation efficiency (Stubbs, 2016).

Although most abovementioned new irrigation strategies can save large amount of water, these practices are not widely used (Levidow et al., 2014). An important problem is relevant stakeholders such as farmers, scientists, and regulators have different understanding of water use efficiency. For example, regulators want to balance water use therefore improving environmental sustainability, whereas most farmers' perspectives of irrigation efficiency mean maximizing business and economic productivity instead of saving water (Knox, Kay, & Weatherhead, 2012). Scientists are also failing to provide and distribute easily understood information, causing most farmers lack of general knowledge about crops' water use and yield response to new irrigation practices (Knox et al., 2012; Levidow et al., 2014). In addition, financial consideration, labor requirement, and soil and crop type, can also be barriers to the implementation of new technologies (Stubbs, 2016).

1.4 Water in Oil and Gas Industry

Water plays a vital role in the oil and gas (O&G) industry as an essential element for drilling and fracturing as well as promoting and refining production in many sites. On the other hand, water is also naturally present in the formations and is extracted along with the O&G production as a byproduct or waste stream in large quantities. The quality and quantity of water used and wastewater generated during the O&G process varies widely depending on type of O&G

production, geographic location of site, and geological characteristic of formation (C. E. Clark & Veil, 2009; Veil, 2015; Veil, Puder, Elcock, & Redweik Jr, 2004).

1.4.1 Water Usage

The amount of water needed in the O&G industry mainly depends on the type of production. Compared to the conventional O&G operation, the unconventional shale O&G requires around 40% to 300% more water over the life cycle due to more frequent use of hydraulic fracturing (HF) (Table 2) (Corrie E. Clark, Horner, & Harto, 2013).

Table 2. Comparison of water use and hydraulic fracturing application for both conventional and unconventional O&G industry (Corrie E. Clark et al., 2013)

Type of Oil and Gas Production	Water Use Intensity (water used/energy produced)	Hydraulic Fracturing Application
Conventional Oil and Gas	9.3-9.6 Liter/GigaJoule	Sometimes
Unconventional Shale Oil and Gas	13-37 Liter/GigaJoule	Always

The unconventional shale O&G industry, which allows energy to be extracted from low permeability tight and shale rock formations (Gallegos, Varela, Haines, & Engle, 2015; Gandossi & Von Estorff, 2013), has expanded significantly in the U.S. since 2006 due to technological advances in HF (Coughlin, Arthur, Bohm, Cornue, & Layne, 2009; Vengosh, Jackson, Warner, Darrah, & Kondash, 2014). Currently, it is widespread throughout the U.S., and comprises nearly half of U.S. oil production and approximately two-thirds of U.S. gas production in 2015 (Nicot, Scanlon, Reedy, & Costley, 2014; US Environmental Protection Agency, 2016). As a result of rapid growth in unconventional shale O&G industry, a substantial amount of water has been consumed by HF. Although the volumes of water used for HF vary from well to wells (Corrie E. Clark et al., 2013), most shale plays in the U.S. require around 2.5 to 8.0 million gallons of water per well (Gallegos et al., 2015; A. Kondash & Vengosh, 2015). HF water use in western U.S. such as California and Colorado has been studied (Goodwin et al., 2013; Tiedeman, Yeh, Scanlon, Teter, & Mishra, 2016), and there is also a state-level analysis of HF water use in the U.S. from 2008 to 2014 (H. Chen & Carter, 2016). For most western states, the amount of water consumed by HF is insignificant compared to their irrigation water use (Table 3). However, an increased HF water use intensity is reported (A. Kondash & Vengosh, 2015; Tiedeman et al., 2016), and more than half of the fractured wells are in water scarce regions that experience water stress, leading to competition of water sources between the O&G industry and irrigated agriculture in this area (Freyman, 2014; Hitaj, Boslett, & Weber, 2014).

Table 3. Comparison of 2014 hydraulic fracturing water use and 2015 irrigation water use in the United States (H. Chen & Carter, 2016; Dieter et al., 2018)

State	2014 Hydraulic Fracturing Water Use (million gallons per day)	2015 Irrigation Water Use (million gallons per day)
Arkansas	6.43	11,600
California	0.18	19,000
Colorado	15.71	9,000
Kansas	0.89	2,680
Louisiana	3.51	1,050
Montana	0.94	9,450
New Mexico	2.70	2,370
North Dakota	20.69	233
Ohio	8.95	55
Oklahoma	22.78	931
Pennsylvania	26.65	34.3
Texas	120.54	5,490
West Virginia	11.06	4.15
Wyoming	1.79	7,790
Total U.S.	242.82	118,000

1.4.2 Wastewater Generation and Reuse

During HF process, substantial amount of HF fluids, mainly water, are injected and wastewater is generated as a byproduct (Gallegos et al., 2015; Veil et al., 2004). The waste stream returns to the surface at a high flowrate during the flowback period, which is usually the first two or three weeks after HF is completed. This flowback water has similar chemical properties to the injected

HF fluids. The amount of wastewater recovered during the flowback period varies greatly from 5% to 85%, typically the range is 10% to 50% (K. B. Gregory, Vidic, & Dzombak, 2011; King, 2012; Stringfellow, Domen, Camarillo, Sandelin, & Borglin, 2014). The wastewater that originates in the rock formation and is coproduced with O&G production at low flowrate over the entire well lifetime is defined as produced water (PW) (Barbot, Vidic, Gregory, & Vidic, 2013; K. B. Gregory et al., 2011; King, 2012; Vidic, Brantley, Vandenbossche, Yoxtheimer, & Abad, 2013).

Estimates of the total amount of PW generated from both conventional and unconventional O&G production in the U.S. for 2012 is around 21.2 billion barrels (bbl; 1 bbl = 42 U.S. gallons), which is equivalent to a volume of 2,400 million gallons per day (Veil, 2015). Additionally, over 80% of the nation's PW is generated in the semiarid western U.S. (Katie Guerra, Dahm, & Dundorf, 2011). Recently, studies have begun evaluating the feasibility of reusing treated PW as an alternative water source to irrigate both food and non-food crops in drylands (Dolan et al., 2018; Echchelh et al., 2018; Meng, Chen, & Sanders, 2016; Pica, Carlson, Steiner, & Waskom, 2017). This would be economically desirable when O&G wells are proximate to agricultural fields and when the region experiences agricultural water deficits (Echchelh et al., 2018; Meng et al., 2016). Moreover, current PW disposal options are expensive and challenging (Dolan et al., 2018), thus disposing of the water on cropland is attractive, provided it can be done safely and sustainably.

Currently almost all PW in the U.S. is being injected into underground wells (C. E. Clark & Veil, 2009; Veil, 2015), which is not only expensive but unsafe as they lead to seismic activity (Alessi et al., 2017; Dolan et al., 2018; K. Gregory & Mohan, 2015). Due to quantity of PW generated and disadvantages (e.g., seismic activity) of deep-well injection as well as spatial proximity between O&G plays and farmlands (Ellsworth, 2013; K. Gregory & Mohan, 2015), it is imperative to evaluate the possibility of reuse PW for irrigation, especially in semiarid western U.S. such as Colorado.

Irrigation consumes orders of magnitude more water than produced by O&G operations. Figure 4 and Figure 5 show the amount of PW generated and irrigation water used by each state in 2012 and 2015, respectively. Figure 6 shows the potential for PW to meet irrigation needs. Reusing PW would be most beneficial in states like Oklahoma or Texas that have smaller irrigation needs than states like California but have sufficient O&G activity to contribute to the irrigation water budget. Although reusing treated PW for irrigation does not alleviate significant water pressure, it is a part of the solution to find sustainable water sources for agriculture in the U.S.

Figure 7 and Figure 8 compare the quantity of PW generated and irrigation water used by each county in Colorado in 2018 and 2015, respectively. As expected, the counties with the densest O&G facilities have the largest volumes of PW generation such as Rio Blanco and Weld County, and Washington and Weld county had the highest percentage of agricultural land use and annual irrigation demand (Dolan et al., 2018). Although the amount of PW generated is insignificant compared to the irrigation water need in most counties, reuse of PW would be helpful in counties like Las Animas and Rio Blanco that have larger PW generation and smaller irrigation needs. Also, for the counties that have both large quantities of PW generation and irrigation demands such as Washington and Weld, PW could be an important alternative source for agricultural irrigation to fulfill the water supply gap (Figure 9).

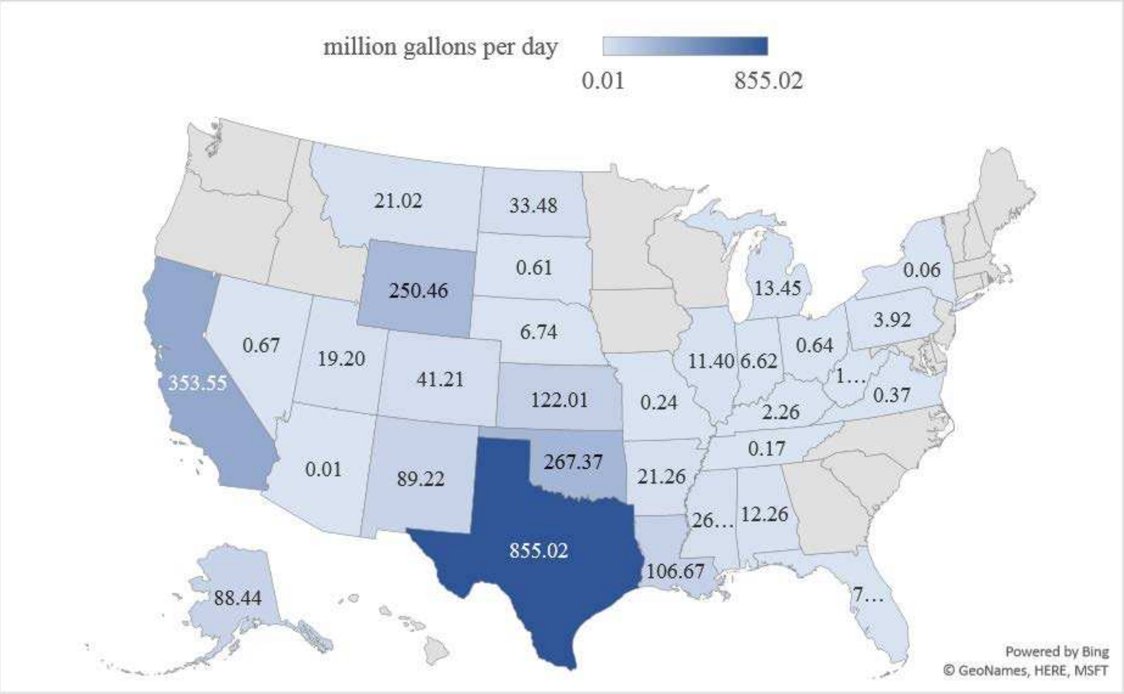

Figure 4. 2012 U.S. produced water generation map (Veil, 2015)

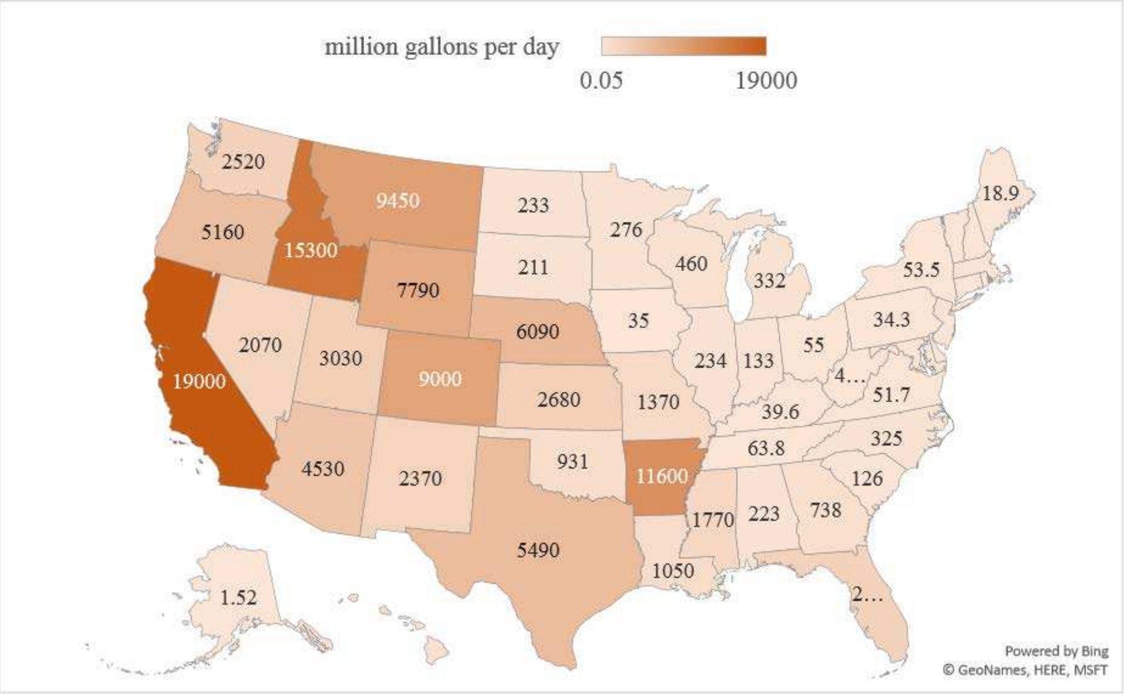

Figure 5. 2015 U.S. irrigation water use map (Dieter et al., 2018)

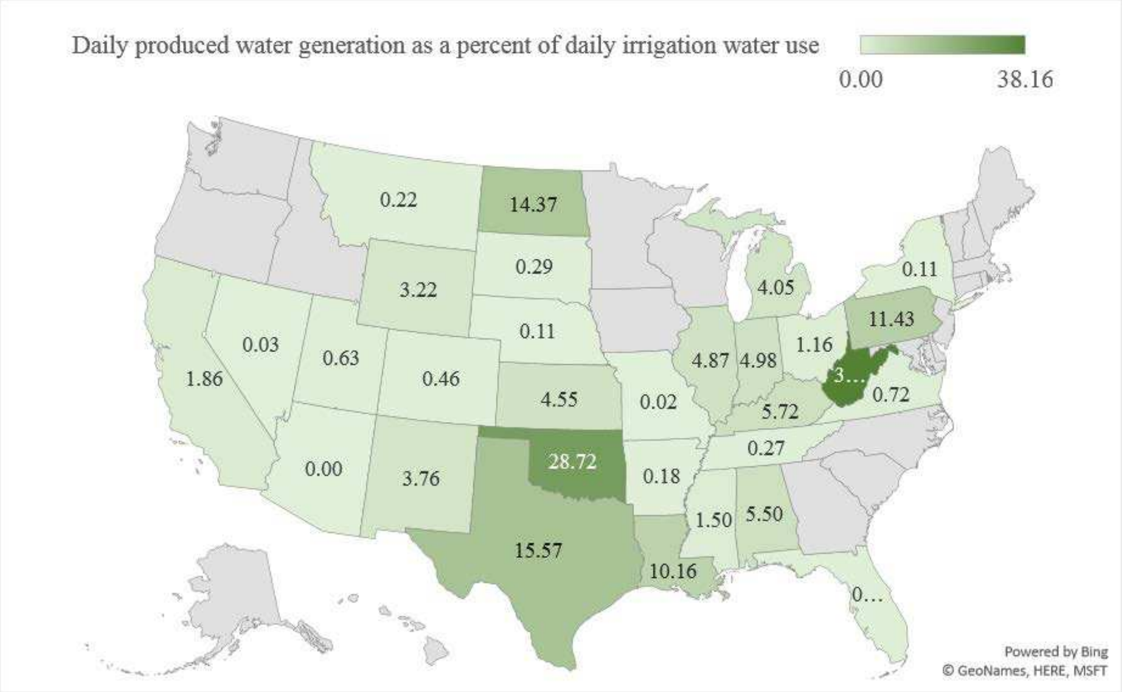

Figure 6. U.S. map of potential for reusing produced water to meet irrigation needs

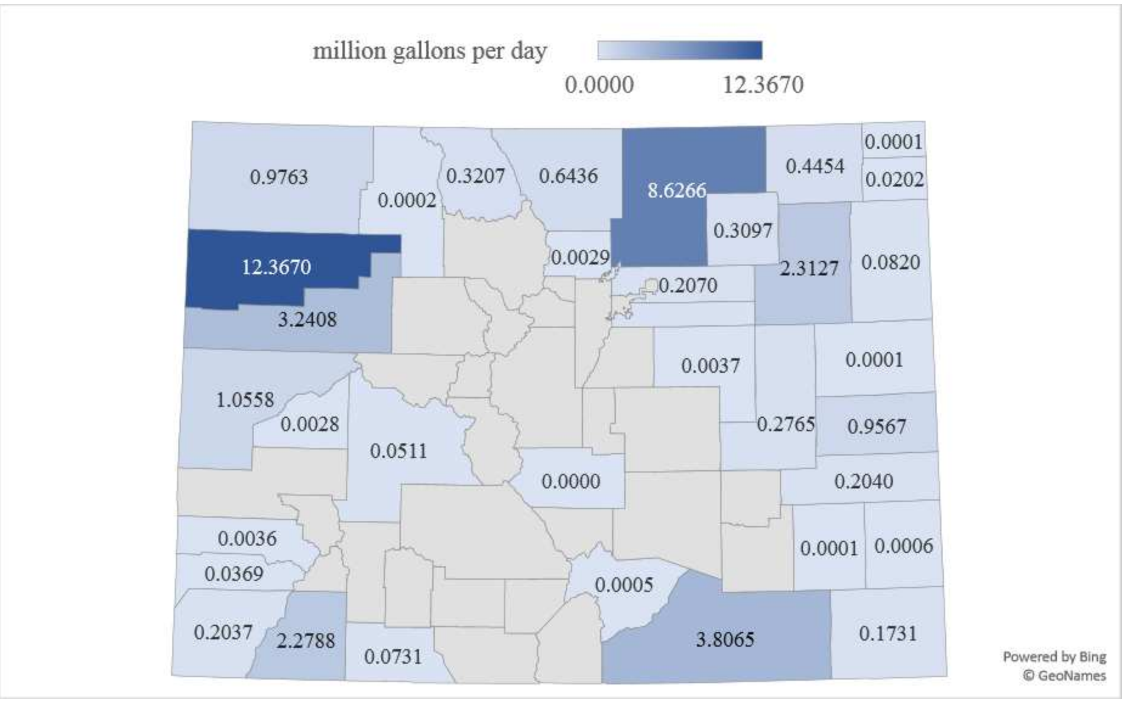

Figure 7. 2018 Colorado produced water generation map (source: https://cogcc.state.co.us/data.html)

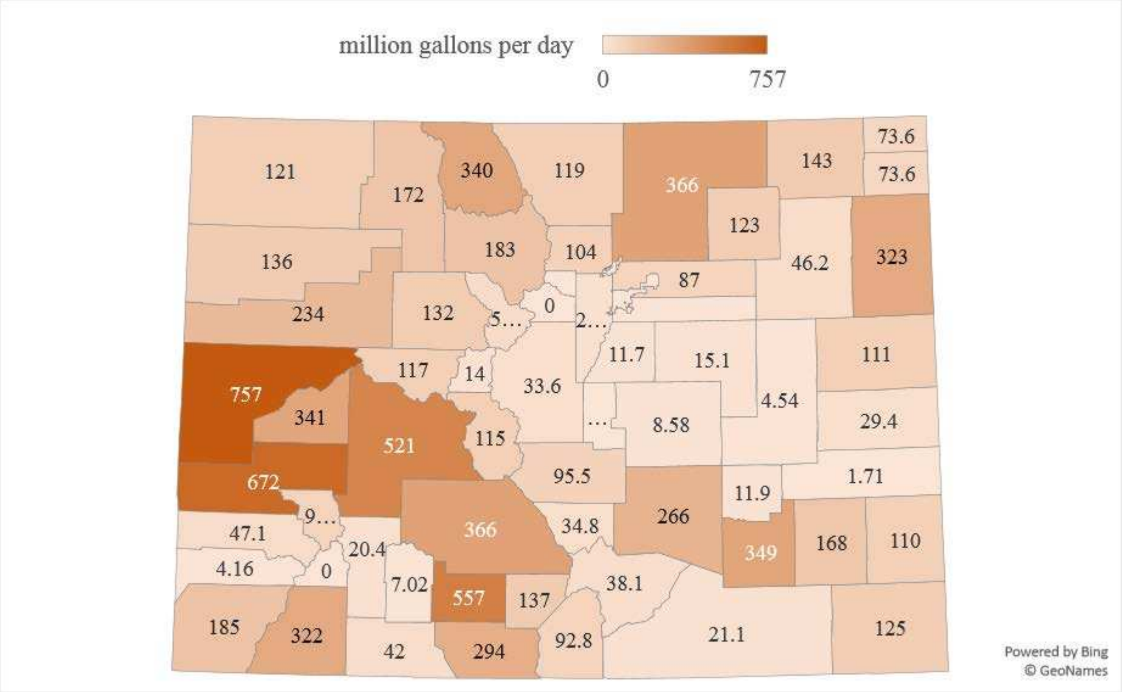

Figure 8. 2015 Colorado irrigation water use map (Dieter et al., 2018)

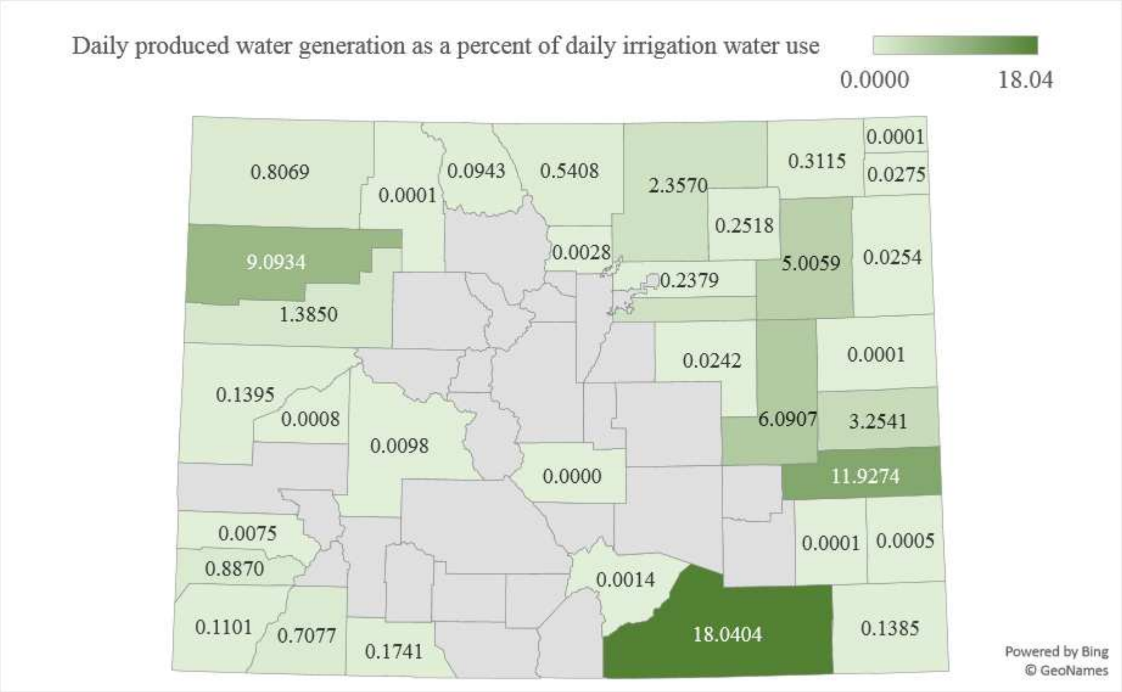

Figure 9. Colorado map of potential for reusing produced water to meet irrigation needs

1.5 Chemicals in Oil and Gas Wastewater

The chemical characteristics of O&G wastewater are complex and vary considerably with time of collection, location of the shale, and constituents in injected HF fluids (Alessi et al., 2017; C. E. Clark & Veil, 2009; K. B. Gregory et al., 2011; King, 2012). Typically, chemicals in flowback water primarily come from HF fluids and PW has more geogenic formation compounds (Akob, Cozzarelli, Dunlap, Rowan, & Lorah, 2015; Stringfellow et al., 2014). However, one study reports that the chemical property of flowback water and injected fluids are significantly different (Zolfaghari, Dehghanpour, Noel, & Bearinger, 2016). Nevertheless, there is no sharp distinction between flowback water and PW since blending of two types of streams may happen during the HF process (Ferrer & Thurman, 2015; Stringfellow et al., 2014). Injected HF fluids constitute less than 10% of flowback and PW; more than 90% of O&G wastewater comes from the native formation water (A. J. Kondash, Albright, & Vengosh, 2017). Producers normally do not distinguish between flowback water and PW, and they are both identified as O&G wastewater by regulators and agencies (Alessi et al., 2017; A. Kondash & Vengosh, 2015). This study focuses on the PW since it has been more frequently studied and is considered the primary environmental concern of the O&G industry (Stringfellow et al., 2014).

1.5.1 Chemical Additives in Injected Fluids

HF fluids are composed of several groups. Water is commonly used as a base carrier fluid; it accounts for more than 90% of total volume and is injected into the target reservoir under high pressure to generate cracks in the rock formation. A support called proppant (often in the form of quartz sand) is subsequently applied to keep the artificial fractures open. Various chemical additives, which only make up 0.5% to 3% of total fluids, are mixed with injected water before or during the injection and serve different functions to optimize HF performance (Elsner & Hoelzer, 2016; Ferrer & Thurman, 2015; Stringfellow et al., 2014).

All additives in the injected fluids can be generalized based on their chemical functions, frequency of use, and hazardous potential (Table 4). Prior to the HF operation, acid is applied to clean the wellbore, dissolve minerals and initiate cracks in the geological formation (Ferrer & Thurman, 2015; K. B. Gregory et al., 2011; Vidic et al., 2013). During the process, surfactants and friction reducers are used to minimize tension and friction, thereby helping injected fluids move down into the well to create fractures and return back to the surface (Ferrer & Thurman, 2015; Stringfellow et al., 2014). Gelling agents and crosslinkers bind together to form polymer molecules to increase the viscosity and elasticity of fluids, therefore allowing proppants to transport and suspend the artificial fractures successfully. Also, pH adjusting agents are added simultaneously to ensure the best results. Subsequent to the HF, breakers are introduced to decrease viscosity by breaking the polymers thus enabling withdrawal of HF fluids and extraction of energy (Elsner & Hoelzer, 2016; Ferrer & Thurman, 2015; Stringfellow et al., 2014). Other chemical additives are also applied to meet the technical requirements throughout the HF process (Elsner & Hoelzer, 2016). Clay stabilizers are used to protect clay structures in shale formation against collapsing, and corrosion inhibitors help prevent corrosion of well surface. In addition, well clogging caused by precipitation and/or biofouling can be avoided through introducing scale inhibitors, iron control and biocides (Elsner & Hoelzer, 2016; Ferrer & Thurman, 2015; Stringfellow et al., 2014).

Table 4. Composition, purpose and frequency of use, range of percentage and concentration, and hazard potential of common chemical additives in hydraulic fracturing fluids (Coughlin et al., 2009; Ferrer & Thurman, 2015; Gordalla, Ewers, & Frimmel, 2013; Stringfellow et al., 2017, 2014; Vidic et al., 2013)

Chemical Additives	Chemical Composition	Purpose of Use	Frequency of Use	Range of Percentage	Range of Concentration	Hazard Potential
Water	H_2O	Base carrier fluid	1,579 of 1,623 treatments	90.6-90.8	Not available	Not classified as hazardous
Proppant	Silica quartz sand	Keep open fractures	1,598 of 1,623 treatments	8.5-8.95	Not available	Not classified as hazardous
Acid	Acetic acid	Clean wellbore, dissolve minerals Initiate cracks in formation	Not available	0.11-0.15	Not available	Causes severe burns and eye damage Flammable liquid and vapor
Biocide	5-Chloro-2-methyl-2H-isothiazol-3-one	Prevent bacteria growth and biofouling	1,516 of 1,623 treatments	0.001-0.06	10-800 mg/L	Very toxic to aquatic life with long lasting effects Toxic if swallowed
Breaker	Sodium bromate	Breaking crosslinking polymers Allowing gas exploitation	1,599 of 1,623 treatments	0.009-0.06	1-400 mg/L	Harmful if swallowed Causes skin irritation Causes serious eye irritation May cause respiratory irritation May cause fire or explosion
Clay stabilizer	Tetramethyl ammonium chloride	Prevent clay swelling and collapse	1,184 of 1,623 treatments	0.05-0.12	500-2,000 mg/L	Toxic if swallowed Harmful in contact with skin Causes skin irritation Causes serious eye irritation May cause respiratory irritation
Corrosion inhibitor	Acetaldehyde	Prevent corrosion of pipe and well surface	102 of 1,623 treatments	0.001-0.002	10-7,000 mg/L	Not available
Crosslinker	Borate salt	Promote fluid viscosity as temperature increases	1,503 of 1,623 treatments	0.006-0.007	0.5-250 mg/L	May damage fertility May damage unborn child
Friction reducer	Polyethylene glycol-octylphenyl ether	Minimize friction between fluid and pipe	43 of 1,623 treatments	0.07-0.08	Not available	Causes serious eye irritation
Gelling agent	Guar	Promote fluid viscosity Allowing better suspend and transport of proppant	1,593 of 1,623 treatments	0.05	10-1,000 mg/L	Not available
Iron control	Acetic acid	Prevent precipitation of iron oxides	262 of 1,623 treatments	0.004-0.006	50-200 mg/L	Causes severe burns and eye damage Flammable liquid and vapor
pH adjusting agent	Sodium hydroxide	Maintain efficacy of crosslinking polymer	Not available	0.01	100-300 mg/L	Causes severe skin burns and eye damage
Scale inhibitor	Polycarboxylate	Prevent scale deposition on pipe	971 of 1,623 treatments	0.04-0.09	75-400 mg/L	Not available
Surfactant	Glycol ether	Increase fluid viscosity Decrease surface tension	1,546 of 1,623 treatments	0.075-0.08	500-1,800 mg/L	Harmful by inhalation, in contact with skin and if swallowed Risk of serious damage to eyes

A wide range of chemical additives used in the HF process can pose a threat to the environment and public health, thus raising concern in their toxicity evaluation and frequency of use (Table 4) (Elliott, Ettinger, Leaderer, Bracken, & Deziel, 2017; Gordalla et al., 2013; Stringfellow et al., 2017). For example, Elliott et al. identified that 67 compounds are potential reproductive or developmental toxicants in a total of 1,021 fracturing chemicals, and Kargbo et al. concluded that many chemical additives are carcinogenic and can cause severe human health problems (Elliott et al., 2017; Kargbo, Wilhelm, & Campbell, 2010). Biocides are of special concern due to their high toxicity and potential formation of more toxic degradation compounds (Kahrilas, Blotevogel, Stewart, & Borch, 2015). Common pathways of HF fluids exposure have been also delineated, including potential migration, inappropriate disposal, accidental spill and/or leak, and failure of subsurface well casing (Howarth, Ingraffea, & Engelder, 2011; Kargbo et al., 2010; Vidic et al., 2013).

1.5.2 Geogenic Chemicals in Reservoir Formation

Substances native to the target reservoir formation are the other source of chemicals in the O&G wastewater (Alessi et al., 2017; Fakhru'l-Razi et al., 2009). Commonly reported constituents include total dissolved solids (TDS), total suspended solids (TSS), total dissolved organic matter (TOC), chemical oxygen demand (COD), naturally occurring radioactive materials (NORM), inorganic ions, metalloids and heavy metals, and oil and grease (Table 5).

Table 5. Typical range of concentrations for common constituents of flowback and produced water in Marcellus Shale (Abualfaraj, Gurian, & Olson, 2015; Barbot et al., 2013)

Constituent	Minimum	Maximum	Average	Number of Samples
TDS (mg/L)	680	345,000	106,390	129
TSS (mg/L)	4	7,600	352	156
TOC (mg/L)	1.2	1,530	160	55
COD (mg/L)	195	36,600	15,358	89
Oil and grease (mg/L)	4.6	802	74	62
Alkalinity (mg/L as $CaCO_3$)	7.5	577	165	144
pH	5.1	8.42	6.56	156
CN^- (mg/L)	0	0.954	0.036	86
NO_2^- (mg/L)	0	146	8.4	46
NO_3^- (mg/L)	0	15.9	0.76	37
SO_4^{2-} (mg/L)	0	763	71	113
F (mg/L)	0	58.3	1.8	20
Cl (mg/L)	64.2	196,000	57,447	154
Br (mg/L)	0.2	1,990	511	95
Se (mg/L)	0	0.35	0.033	98
Al (mg/L)	0	47	0.81	170
Na (mg/L)	69.2	117,000	24,123	157
Ca (mg/L)	37.8	41,000	7,220	159
Mg (mg/L)	17.3	2,550	632	157
As (mg/L)	0	0.151	0.049	97
Ba (mg/L)	0.24	13,800	2,224	159
Cd (mg/L)	0	0.0625	0.017	88
Cr (mg/L)	0	0.704	0.03	115
Cu (mg/L)	0	116	1.2	101
Mn (mg/L)	0	29	3.3	216
Pb (mg/L)	0	0.97	0.052	138
Sr (mg/L)	0.59	8,460	1,695	151
Zn (mg/L)	0	250	2.2	196
Fe dissolved (mg/L)	0.1	222	40.8	134
Fe total (mg/L)	2.6	321	76	141
Ra^{228} (pCi/L)	0	1,360	120	46
Ra^{226} (pCi/L)	2.75	9,280	623	46
U^{234} (pCi/L)	0	3.8	1.03	11
U^{235} (pCi/L)	0	20	1	14
U^{238} (pCi/L)	0	497	42	14

The salinity of PW is usually high, due to the nature of geological formation, which makes sodium and chloride the most abundant elements in PW (Ferrer & Thurman, 2015). Other commonly found inorganic ions include calcium, magnesium, bromide, carbonate (Table 5) (Barbot et al., 2013; K. B. Gregory et al., 2011). Also, cyanide, fluoride, nitrite, nitrate, ammonium, boron, and aluminum are identified in some flowback water samples (Table 5) (Abualfaraj et al., 2015; Lester et al., 2015). Sulfate usually present low concentrations in PW, due to barite precipitation (Barbot et al., 2013; Neff, Lee, & DeBlois, 2011). Heavy metals such as arsenic, lead, iron, barium, manganese, chromium, cadmium, strontium, and zinc are reported (Table 5), but their concentrations vary with geology and age of the formation (Fakhru'l-Razi et al., 2009; Neff et al., 2011). In addition, the strontium isotopic ratio (^{87}Sr/^{86}Sr) has been used as a fingerprint to evaluate the risk of PW contamination of water resources (Chapman et al., 2012; Warner et al., 2012).

PW from many O&G sites around the world contains NORM (Neff et al., 2011). ^{228}Radium, ^{226}radium, and their parent isotopes ^{235}uranium, ^{238}uranium, are the most frequently detected NORMs (Table 5) (Abualfaraj et al., 2015; Barbot et al., 2013; Neff et al., 2011). For example, high levels of radium isotope activities has been found in PW samples from Marcellus shale (Rowan, Engle, Kirby, & Kraemer, 2011). Precipitation of solid NORM can occur via interacting with barite to form a scale in the equipment (K. Gregory & Mohan, 2015; Kargbo et al., 2010), or being accumulated in carbonate rich discharge sediments for disposal purpose (McDevitt et al., 2019). Exposure of NORM can also happen due to accidental leaks and/or human activities, which poses a threat to the environment and public health (K. Gregory & Mohan, 2015; Kargbo et al., 2010).

The organic chemicals in PW are very complicated, and more than 30,000 compounds are identified in shale oil formation (Luek & Gonsior, 2017). In most cases, the organic contents are reported as TOC instead of listing individual species (Maguire-Boyle & Barron, 2014). PW from shale gas normally has fewer organic chemicals than that from shale oil (Luek & Gonsior, 2017), but volatile gases such as methane, usually present in higher concentration and has been used as a trace element fingerprinting of specific geologic formation (Fakhru'l-Razi et al., 2009; Ferrer & Thurman, 2015). Generally identified organic chemicals in PW include aliphatic, aromatic, and heterocyclic compounds (Luek & Gonsior, 2017; Maguire-Boyle & Barron, 2014; Orem et al., 2014). Aliphatic compounds are major organics present in PW, and their abundance typically follows an order of linear > branched > cyclic (Maguire-Boyle & Barron, 2014). Aromatic organics such as benzene, toluene, ethylbenzene, and xylene (BTEX) are the most common one-ring aromatic hydrocarbons (Luek & Gonsior, 2017; Neff et al., 2011), which are often reported as volatile organic chemicals (VOCs) in PW study (Abualfaraj et al., 2015; Lester et al., 2015). Polycyclic aromatic hydrocarbons (PAHs) are the main group of chemicals that cause health and environmental hazards, due to their toxicity and persistence (Maguire-Boyle & Barron, 2014; Neff et al., 2011). Furthermore, most found PAHs in PW are 2- and 3-rings small molecules such as naphthalene, phenanthrene and their alkyl homologous. PAHs with higher molecular weight are rarely observed because of their low aqueous solubility (Neff et al., 2011; Orem et al., 2014).

Atypical organic chemicals such as halogenated organics are also identified in the O&G wastewater, although the source and detailed reaction mechanism is still under debate (Hoelzer et al., 2016; Maguire-Boyle & Barron, 2014; Thomas, 2009). One study found polychlorinated biphenyls (PCBs) and pesticides in flowback water but they were present in very low concentrations, hence the author believes that it is not necessary to raise concerns about these chemicals for future studies (Thomas, 2009). In contrast, others think that the appearance and elevated level of organo-halogen compounds in flowback water is a result of biotic and abiotic oxidation of halides with dissolved organic matter, which should be taken into consideration due to their potential hazards to the environment and health (Hoelzer et al., 2016; Luek, Harir, Schmitt-Kopplin, Mouser, & Gonsior, 2018; Maguire-Boyle & Barron, 2014).

1.6 Management and Treatment of PW

Major factors to be considered when managing PW include economic costs, environmental impacts, human health hazards, and public perceptions (Boudet et al., 2014; Burnett, 2004; K. B. Gregory et al., 2011; Kargbo et al., 2010). Improper management can lead to serious environmental issues (K. B. Gregory et al., 2011; Vidic et al., 2013). For example, a widespread mortality of aquatic species in Acorn Fork Creek in Kentucky was observed after an unapproved disposal of HF fluids (Vengosh et al., 2014). In regards to environmental preferences, a hierarchical organization of PW management with three tiers are introduced; top tier management is environmentally preferred while bottom tier is not (Figure 10) (Jiménez, Micó, Arnaldos, Medina, & Contreras, 2018; Veil, 2015).

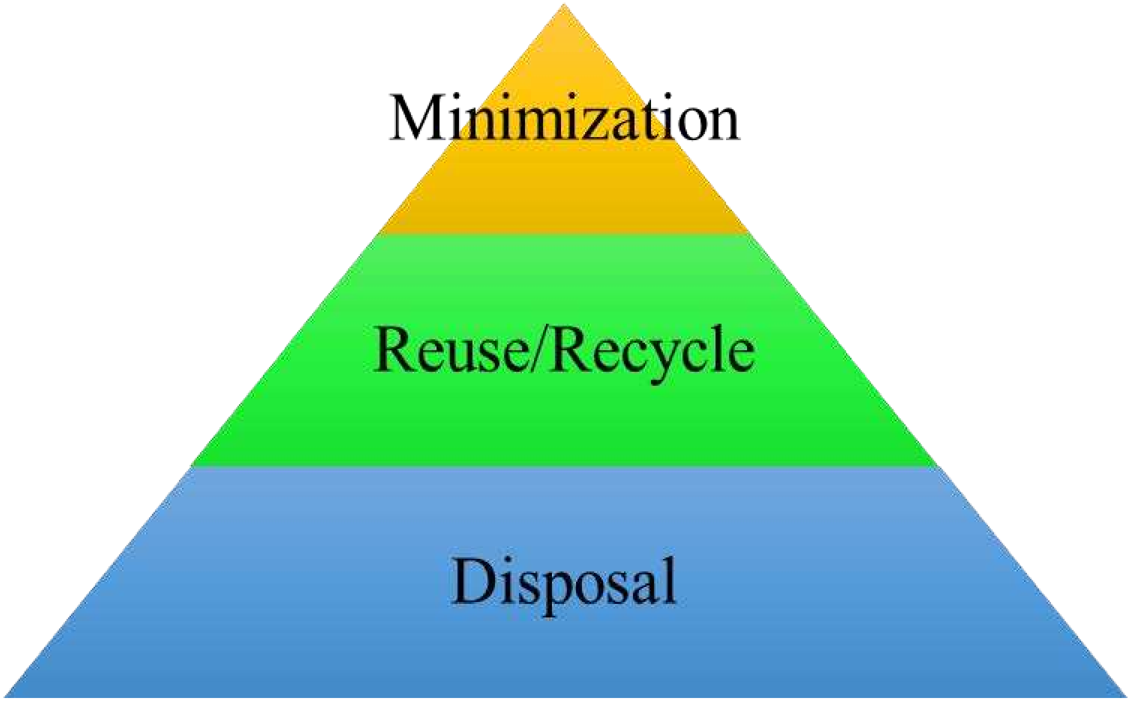

Figure 10. Three tiers of produced water management (Jiménez et al., 2018; Veil, 2015)

1.6.1 Minimization

Minimization is tier 1 in PW management, which refers to reduce the volume of wastewater generated during the O&G extraction and production process. PW amount can be decreased by either reducing the volume of formation water brought to the surface or preventing the HF fluids entering the well. Minimization results in good protection of the environment, but it requires high-level of technology and can be very expensive (Jiménez et al., 2018; Veil, 2015).

1.6.2 Reuse or Recycle

Reuse or recycle is tier 2 in the management for PW that cannot be managed by minimization approaches. Options of PW recycle include injection reuse, agricultural reuse, and industrial reuse (Jiménez et al., 2018; Veil, 2015). PW re-injection for enhanced oil recovery is the most common way for recycling (C. E. Clark & Veil, 2009; Jiménez et al., 2018). Agricultural reuse for crop irrigation or livestock watering can be a great benefit for handling water shortage and has been practiced in California, Texas and Wyoming, but treatment is required to ensure satisfactory level of water quality (U.S. Environmental Protection Agency, 2019). Industrial reuse, such as on-site internal recycling of HF fluids for future O&G operations, is an emerging and attractive practice that is preferred by engineers and operators, because it is economically desirable and environmentally favorable (Boschee, 2015; Estrada & Bhamidimarri, 2016; Shaffer et al., 2013). However, limiting factors such as chemical compatibility between recycled PW and HF fluids, and potential damage to the well and formation, impeding the reuse process (K. B. Gregory et al., 2011; Vidic et al., 2013). Nonetheless, beneficial reuse of PW is getting popular, and reuse for agricultural irrigation is becoming an important strategy for reducing water shortages in drylands include semiarid western U.S. (Dolan et al., 2018; Echchelh et al., 2018).

1.6.3 Disposal

Disposal is tier 3 in PW management; it is environmentally unpreferable and the last available option (Veil, 2015). Also, it brings additional challenges to the operators (Coughlin et al., 2009). Common disposal methods include deep-well underground injection (other than for enhanced oil recovery) and surface discharge (Veil, 2015).

Currently in the U.S., more than 90% of PW is injected underground in to Class II wells either for enhanced oil recovery or for disposal (Veil, 2015). One of the major risks related to this method is earthquake hazards, but the detailed mechanism responsible for seismic activity is still not fully understood (Guglielmi, Cappa, Avouac, Henry, & Elsworth, 2015; Rubinstein & Mahani, 2015). The central and eastern U.S. has experienced a drastic increase in earthquakes during the past few years, which may result from deep-well injection (Ellsworth, 2013). Injection disposal of PW resulted in small earthquakes in Texas and Arkansas (Frohlich, 2012; Horton, 2012), and several significant seismic activities with five moment magnitude (M_w) > 5.0 were also observed in Oklahoma and Colorado (Keranen, Savage, Abers, & Cochran, 2013; Van Der Elst, Savage, Keranen, & Abers, 2013). Other challenges associated with deep-well injection include chemical compatibility between injected PW and receiving formation (C. E. Clark & Veil, 2009; Veil, 2015), availability of disposal well capacity (K. B. Gregory et al., 2011; Vidic et al., 2013), and high transportation costs (Boschee, 2015; Vidic et al., 2013). The injected PW should be treated to ensure that receiving formation does not get damaged (C. E. Clark & Veil, 2009; Veil, 2015). Compared to Texas where there are approximately 12,000 Class II disposal wells, there are no more than 10 wells in the entire state of Pennsylvania. Furthermore, construction of new disposal wells is complex, time-consuming, and economically unfavorable (Boschee, 2015; K. B. Gregory et al., 2011; Vidic et al., 2013). As a consequence, wastewater generated in Pennsylvania is sent to neighboring states that have more wells such as Ohio (Lutz, Lewis, & Doyle, 2013; Torres, Yadav, & Khan, 2016), but expensive transportation often hinders this approach (Boschee, 2015; Vidic et al., 2013).

Discharge of PW is forbidden for most onshore O&G facilities by state agencies or the U.S. Environmental Protection Agency (EPA), but is allowed in the semiarid western U.S. if the wastewater meets quality requirements after treatment (US Environmental Protection Agency, 2016; Veil, 2015). In 2008-2009, discharge of Marcellus shale PW into publicly owned treatment works or municipal wastewater treatment plants was permitted in Pennsylvania, due to the impossibility of deep-well injection (K. B. Gregory et al., 2011; K. Gregory & Mohan, 2015). However, contamination of receiving and downstream water has been observed, including increased concentrations of TDS and pollutants such as toxic metals, technologically enhanced naturally occurring radioactive materials, and carcinogenic trihalomethanes (Burgos et al., 2017; Ferrar et al., 2013; Vengosh et al., 2014; Warner, Christie, Jackson, & Vengosh, 2013).

1.6.4 Treatment Technology and Cost

Physical, chemical, biological, thermal, and membrane technologies are commonly used for PW treatment (Fakhru'l-Razi et al., 2009; Igunnu & Chen, 2014). Typical constituents that need to be removed from PW include dissolved and suspended organics, TDS and TSS, NORM, toxics, and heavy metals (Arthur, Langhus, & Patel, 2005; Estrada & Bhamidimarri, 2016). PW is difficult and expensive to treat, since the chemical characteristics of PW are very complex and their compositions change over time (Shaffer et al., 2013). In consequence, a combination of two or more technologies are applied simultaneously in a hybrid system to get better performance (Fakhru'l-Razi et al., 2009; Igunnu & Chen, 2014). Scientists recently developed a hybrid membrane bio-system, which can remove more than 90% of contaminants in PW (Freedman et al., 2017; Riley, Oliveira, Regnery, & Cath, 2016). Emerging technology for PW treatment is a hot topic of research, due to their high efficacy and low cost. For example, mechanical vapor compression and forward osmosis, which are shown high efficiency of desalination and contaminants removal as well as low energy cost (Coday et al., 2014; Shaffer et al., 2013).

1.7 PW Irrigation Concerns

Reuse of reclaimed wastewater for agricultural irrigation has potential to reduce irrigation water costs and alleviate global water scarcity, and it is the most established application by far (Scheierling, Bartone, Mara, & Drechsel, 2011). However, wastewater has to meet quality standards before it can be reused for irrigation (Table 6), to prevent environmental and public health issues (Hanjra, Blackwell, Carr, Zhang, & Jackson, 2012). Treated municipal wastewater is the most common alternative source for irrigation when freshwater is limited (Cirelli et al., 2012). In contrast, treated industrial wastewater, is rarely used due to accumulation of nondegradable hazardous chemicals such as heavy metals (Vergine et al., 2017). Besides that, miscellaneous contaminants present in PW also make it challenging for agricultural irrigation reuse. Salinity, sodicity, and toxicity are key concerns that need to be dealt with to prevent damage to soil, plant, and human health via consumption of food crops (C. E. Clark & Veil, 2009; Veil, 2015; Veil et al., 2004).

Table 6. Irrigation water quality criteria (Pedrero, Kalavrouziotis, Alarcón, Koukoulakis, & Asano, 2010)

Irrigation Problem	Parameter		Recommended Concentration				
Salinity	Electrical conductivity (dS/m)		< 0.7				
	Total dissolved solids (mg/L)		< 450				
Infiltration	Electrical conductivity (dS/m)		> 0.7	> 1.2	> 1.9	> 2.9	> 5.0
	Sodium adsorption ratio		0-3	3-6	6-12	12-20	20-40
Specific ion toxicity	Sodium (mg/L)	Surface irrigation	< 70				
		Sprinkler irrigation	< 3.0				
	Chloride (mg/L)	Surface irrigation	< 140				
		Sprinkler irrigation	< 100				
	Boron (mg/L)		< 0.7				
Other effects	Nitrogen (NO_3-N or NH_4-N) (mg/L)		< 5.0				
	Bicarbonate (mg/L)	Overhead sprinkling only	< 90				
	Residual chlorine (mg/L)	Overhead sprinkling only	< 1.0				
	pH		Normal range 6.5-8.4				

1.7.1 Soil Concerns of PW Irrigation

Irrigation with treated PW might have negative impacts on soil properties since induced salinity and sodicity can cause severe damages to soil structure, such as reduction of hydraulic conductivity and infiltration rate (Halliwell, Barlow, & Nash, 2001; Toze, 2006). A short-term land disposal of treated PW shows that soil physical and chemical properties are damaged by high salt contents, which implies that irrigation reuse can be a difficult challenge (Al-Haddabi & Ahmed, 2007). Furthermore, research conducted in Wyoming indicated that PW irrigation results in significant increase of electrical conductivity (EC) and sodium adsorption ratio (SAR) in soils (Ganjegunte, Vance, & King, 2005; Johnston, Vance, & Ganjegunte, 2008; Joyce et al., 2015). Irrigation induced salinity and sodicity also has deleterious effects on soil microbial property (Rietz & Haynes, 2003; K. Singh, 2016). Heavy application of treated PW may cause unfavorable conditions for soil microbes and enzymes (Nwaugo, Onyeagba, Azu, & Nworie, 2018; Oliveira et al., 2014), and soil fauna function can be greatly affected due to PW irrigation (Ferreira, Weber, & Crisóstomo, 2015).

Toxicity of PW is mainly related to HF fluid additives, PAHs, NORMs, and toxic metal(loids). Heavy metals are nonbiodegradable and easily accumulate in soil, and their toxicity is mostly depending on the physiochemical properties of the soil such as soil organic matter level (Dube, Zbytniewski, Kowalkowski, Cukrowska, & Buszewski, 2001). NORMs such as radium and uranium can sorb to organic matter, but their behavior varies widely depending on soil pH and redox potential (Koch-Steindl & Pröhl, 2001; Rachkova, Shuktomova, & Taskaev, 2010). For example, radium and uranium applied as mine wastewater irrigation have shown high accumulation and sorption in highly weathered acidic surface soil (Willett & Bond, 2010). HF fluids additives may also accumulated in agricultural topsoil layers when conditions are unfavorable for degradation (McLaughlin, Borch, & Blotevogel, 2016). However, PAHs are believed to present low risk to soil since they can be subjected to biodegradation processes (Pichtel, 2016). Nevertheless, effects of PW irrigation on soil health in terms of toxicity including salinity is poorly understood.

1.7.2 Plant Concerns of PW Irrigation

PW irrigation can also have adverse effects on plants. Abiotic stress such as salts, heavy metals, and pollutants can negatively affect plant growth, physiology, and molecular biology (Rao, Raghavendra, & Reddy, 2006). Acceptable TDS concentration for crop irrigation is 500 mg/L, and some high tolerant plants can grow when irrigated with 2,000 mg/L saline water (Beltrán & Koo-Oshima, 2006). However, TDS level of most PW ranges from 5,000 to 400,000 mg/L (Echchelh et al., 2018; Neff et al., 2011; Shaffer et al., 2013), which is orders of magnitude higher than the maximum acceptable threshold. Excessive salt contents accumulate in the root zone and prevent plants from taking up cation nutrients and water (Bernstein, 2003), causing plant wilting or even death (Ayers & Westcot, 1985). For example, irrigation with PW that has an elevated level of salinity can cause a decline in biomass yields and morphological parameters of non-food biofuel plants (Pica et al., 2017). Also, PW irrigation negatively affects the biomass, photosynthetic efficiency, and reproductive growth of wheat plants (Sedlacko et al., 2019).

Boron should be addressed when reusing PW for irrigation (C. E. Clark & Veil, 2009; Veil et al., 2004), since it is an important factor in determining plant health (Katie Guerra et al., 2011), and boron toxicity to plants commonly occurs in drylands all over the world (Nable, Bañuelos, & Paull, 2011). Heavy metals are the most commonly studied chemicals in terms of wastewater irrigation because they can easily build up in soils and plant matter (Khan, Cao, Zheng, Huang, & Zhu, 2008; Muchuweti et al., 2006; A. Singh & Agrawal, 2010; Tiwari, Singh, Patel, Tiwari, & Rai, 2011). Radium and uranium can be taken up by various plants such as wheat and lupine, while the uptake varies with plant species (S. B. Chen, Zhu, & Hu, 2005; Pulhani, Dafauti, Hegde, Sharma, & Mishra, 2005). Plant uptake of organic pollutants in PW is possible and may result in poor plant health (Jackson & Myers, 2002; Pica et al., 2017). Plant bioaccumulation and translocation of PAHs and other homologous chemicals include pharmaceuticals and personal care products can happen, which poses negative health consequences for food consumption (Blaine et al., 2014; Chaîneau, Morel, & Oudot, 2010;

Goldstein, Shenker, & Chefetz, 2014; Malchi, Maor, Tadmor, Shenker, & Chefetz, 2014; Samsøe-Petersen, Larsen, Larsen, & Bruun, 2002; Shenker, Harush, Ben-Ari, & Chefetz, 2011; Tao, Zhang, Zhu, & Christie, 2009; Wu, Conkle, Ernst, & Gan, 2014). In addition, heavy metals and organic pollutants in soil can be both taken up by some plants, such as zucchini and spinach simultaneously bioaccumulate and translocate of chlordane and cadmium (Mattina, Lannucci-Berger, Musante, & White, 2003).

Although there are many studies about plant uptake of sewage pollutants, plant immune system response to PW-derived chemicals has rarely been studied. When plants respond to pathogen attacks, they rapidly produce reactive oxygen species, strengthen cell walls, and experience localized cell death at the sites of infection (Dodds & Rathjen, 2010; Jones & Dangl, 2006; Nakagami, Pitzschke, & Hirt, 2005). Heavy metals are highly toxic to plant in higher concentrations since they block the functional groups or essential metal ions in biomolecules, thereby impeding production of reactive oxygen species (Nakagami et al., 2005). Hormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) are key factors in plant immune system response to pathogens (Robert-Seilaniantz, Grant, & Jones, 2011). PAHs (phenanthrene) have shown the ability to elevate ET and SA level in plant tissues (Weisman, Alkio, & Colón-Carmona, 2010). Although excessive boron is toxic to plants (Nable et al., 2011), boron is essential for the formation of an important enzyme in plant cell wall that plays a vital role in defense of bacterial and fungal pathogens (Goldbach & Wimmer, 2007; Hunt, 2003; Pelloux, Rustérucci, & Mellerowicz, 2007). For example, boron was found to increase the resistance of oat and barley to fungal pathogen (*Erysiphe graminis*), and significantly affect the colonization and symptom expression of soilborne pathogens (*Plasmodiophora brassicae*) in field mustard (Webster & Dixon, 1991). Moreover, salt also contribute to plant immune functions, since it has been shown to greatly reduce the tomato susceptibility to biotrophic fungal pathogen (*Oidium neolycopersici*) (Achuo, Prinsen, & Höfte, 2006). Regardless, the effects of PW on plant immune response to pathogens have not been studied so numerous questions remain unanswered in this field.

1.7.3 Human Health Concerns of PW Irrigation

When toxic chemicals in wastewater are taken up and translocated to the edible part of food crops, the risk for human consumption should be taken into account. Currently the research directly related to human exposure of wastewater derived chemicals in food crops is rare, but most plant uptake studies point out that this question has to be addressed in the future. There is only one study to the best of our knowledge that has evaluated the exposure of humans to contaminants via a route of reclaimed wastewater → soil → plant → human, and results show that healthy individuals who consume reclaimed wastewater irrigated food crops excreted significantly higher levels of xenobiotics in their urine (Paltiel et al., 2016). From the public health standpoint, the safety assessment of reusing wastewater for crop irrigation is urgent since human exposure to xenobiotics by consumption of wastewater irrigated produce has already been demonstrated.

1.8 Research Hypotheses and Objectives

In this study, the effects of diluted PW irrigation on wheat plant physiology, yield, and immune function were evaluated to advance our current understanding of plant responses to PW irrigation-induced abiotic stresses.

Hypothesis: Irrigation with diluted PW will adversely impact the physiology, yield, and immune function of wheat plants due to residual organic constituents.

Objective 1: Evaluate the physiological and yield response of wheat plants to PW irrigation.

Objective 2: Determine the change in immune system response of wheat plants towards bacterial and fungal pathogens when irrigated with PW versus a traditional water source.

Objective 3: Reveal the potential chemical groups in PW that affect the responses of wheat plants.

Objective 4: Discuss study implications and recommendations for future PW irrigation studies.

2. EXPERIMENTAL METHODS

2.1 Material Selection

2.1.1 Soil Selection

Soil organic matter significantly impacts the bioavailability of contaminants. Thus, we used Field & Fairway Soil (Profile Products LLC, Buffalo Grove, IL, 60089, USA), an inorganic profile porous ceramic soil amendment, as a worst-case soil matrix to minimize sorption of chemicals to soil thereby maximizing contaminant delivery to plants (Table A10).

2.1.2 Plant Selection

Wheat was selected for this experiment because it is one of the most consumed grain crops by humans and livestock globally (Pimentel & Pimentel, 2007), and it is economically and nutritionally important in Colorado. Despite its high salinity tolerance (Table 7), wheat will be irrigated with greater frequency due to increasing drought under global warming (Sedlacko et al., 2019). Specifically, USU-Apogee, a full-dwarf hard red spring wheat (*Triticum aestivum L.*) was selected as the model crop for this study due to its fast growth rate and hardiness (Bugbee, Koerner, Albrechtsen, Dewey, & Clawson, 1997). In addition, the Feekes Scale, a measure of development of cereal crops, was employed to describe the growth of wheat plants in this study (Figure 11).

Table 7. Potential yield reduction from saline water irrigation for common Colorado field crops (Bauder, Waskom, Sutherland, & Davis, 2011)

Crop	**% of Yield Reduction**			
	0%	**10%**	**25%**	**50%**
EC_w = electrical conductivity of the irrigation water in dS/m at 25ºC				
Barley	5.3	6.7	8.7	12.0
Wheat	4.0	4.9	6.4	8.7
Sugarbeet	4.7	5.8	7.5	10.0
Alfalfa	1.3	2.2	3.6	5.9
Potato	1.1	1.7	2.5	3.9
Corn (grain)	1.1	1.7	2.5	3.9
Corn (silage)	1.2	2.1	3.5	5.7
Onion	0.8	1.2	1.8	2.9
Dry Bean	0.7	1.0	1.5	2.4

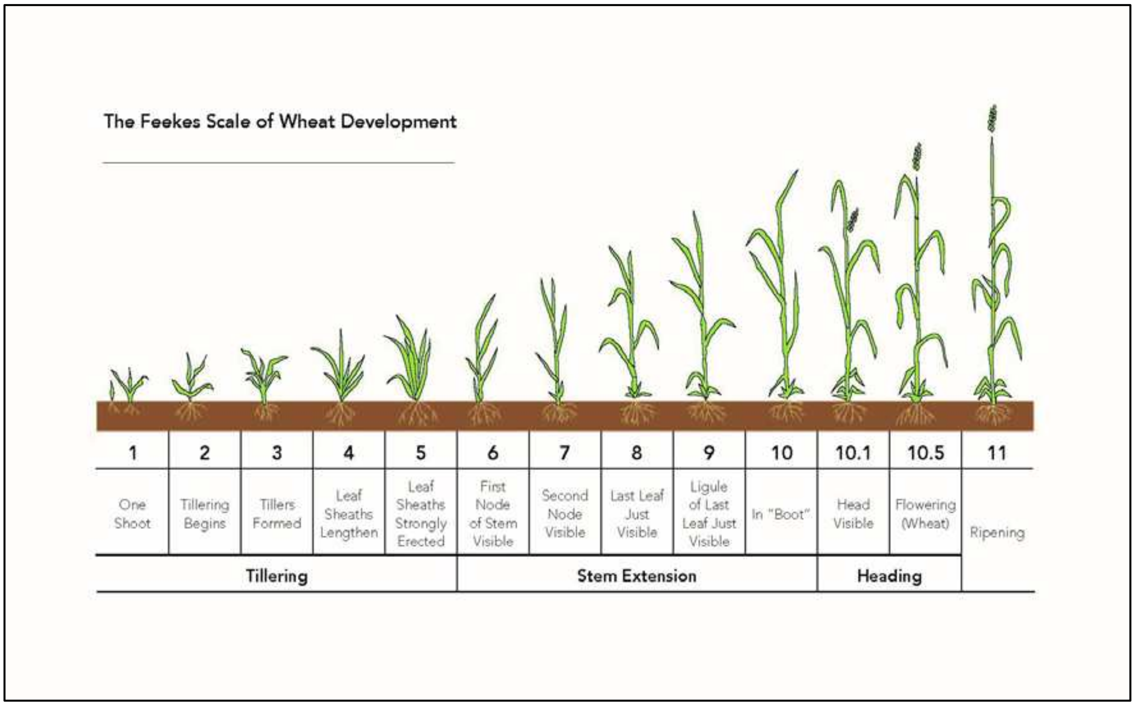

Figure 11. The Feekes Scale of wheat development (source: https://extension.unl.edu/statewide/enre/Wheat%20Production.pdf)

2.1.3 Material Information

The materials used in this experiment served various functions include supporting plant growth, measuring data, helping with inoculation, irrigation, and sampling harvest (Table 8). All supplies were either bought from The Home Depot retail market (The Home Depot, Inc., Atlanta, GA, USA) or provided by the greenhouse facility.

Table 8. Supplementary materials and functions

Supply	Function
Potting bench	Placing pot
Nursery pot	Holding soil for plant growth
Trowel/shovel	Filling pot with soil
Geotechnic fabric liner	Preventing leakage of soil from drainage hole
Plant saucer	Collecting extra water and soil leak
Plant label	Labeling pot and plant
Electronic scale/balance	Weighing soil, water and plant matter
Grow light	Helping plant growth
Fertilizer	Providing nutrient for plant growth
Pesticide	Excluding pest
Sulfur lamp	Excluding pathogen
Soil meter	Measuring soil pH and electrical conductivity
Metric stick	Measuring plant height
MultiSpeQ meter	Measuring photosynthesis parameter
Samsung galaxy phone	Exporting photosynthesis data and taking picture
qRT-PCR meter	Analyzing pathogen colonization
Aquatainer	Storing irrigation stock solution
Graduate cylinder	Using for hand irrigation
Bottle sprayer	Spraying inoculum on plant
Mesh cover	Preventing accidental cross contamination of pathogen
Nitrile gloves	Preventing hand cross contamination of pathogen
Ethanol	Preventing cross contamination of pathogen by third-party source
Scissor	Dissecting plant
Ziploc/paper bag	Preserving plant
Dewar with liquid nitrogen	Quenching plant
Permanent marker	Marking to indicate differences

2.2 Site Selection and Growth Condition

We grew the wheat in a greenhouse to provide a consistent growth condition. The Colorado State University (CSU) Horticulture Center (1707 Centre Ave, Fort Collins, CO, 80526, USA) was selected as test site for this controlled greenhouse experiment. The CSU Plant Growth Facility provides high quality greenhouses, growing facilities and professional support for soil, plant and water research. In this experiment, consistent greenhouse environment was maintained at optimal condition for wheat plant growth. Important environmental conditions such as temperature (68-75F during the day, 60-65F at night) and humidity (50-70%) were recorded periodically during the entire period of experiment. Also, photoperiod (16:8 hours) and air circulation were adjusted by supplemental growth light and greenhouse electric fan, respectively (Sedlacko et al., 2019).

2.3 Method Selection

2.3.1 Pilot Study

Since the soil matrix used for plant growth in this experiment represented a worst-case scenario, and the irrigation water was of low quality, there was the risk that wheat would not grow. Thus, we conducted a small-scale preliminary pilot study prior to the full-scale experiment to verify wheat germination and growth under the described conditions.

In the pilot study, two types of soil (GG, Greens Grade Soil as worst-case scenario; P, Pro-mix Bx Soil as regular potting soil) were used (Figure 12, Figure 13) with three treatments of irrigation water (TW, tap water control; 10S and 50S, 10% and 50% of NaCl salt water control). There were three replicates for each water treatment and soil type, for a total of 18 pots (Table 9). The Greens Grade Soil (Profile Products LLC, Buffalo Grove, IL, 60089, USA) was replaced by Field & Fairway Soil for the full-scale experiment, to avoid using a green dyed soil matrix.

Nine weeks after planting the seeds, all seeds successfully germinated, and the wheat plants were developed enough for analysis, which indicated that selected plant, soil and designed irrigation water treatments were eligible for further research. The full-scale experiment started immediately after the small-scale pilot study.

Figure 12. Sample pot filled with Greens Grade Soil

Figure 13. Sample pot filled with Pro-mix Bx regular potting soil

Table 9. Description of label, soil type, and irrigation water treatment of pilot study

Label	Soil Type	Irrigation Water Treatment
GG_TW_1-3	Greens Grade	Tap water (control)
GG_10S_1-3	Greens Grade	10% NaCl (1.7 g/L) salt water (control for the NaCl concentration in the 10% PW)
GG_50S_1-3	Greens Grade	50% NaCl (8.5 g/L) salt water (control for the NaCl concentration in the 50% PW)
P_TW_1-3	Pro-Mix Bx	Tap water (control)
P_10S_1-3	Pro-Mix Bx	10% NaCl (1.7 g/L) salt water (control for the NaCl concentration in the 10% PW)
P_50S_1-3	Pro-Mix Bx	50% NaCl (8.5 g/L) salt water (control for the NaCl concentration in the 50% PW)

2.3.2 Full Scale Experiment

In the full-scale experiment, wheat plants were grown from seeds to mature plants for harvest in a greenhouse. Plant were watered regularly with each irrigation water treatment, and their growth stages and physiological parameters were measured periodically. Additionally, pathogens were inoculated when plants reached specific development stages. All plants were harvested when they reached maturity and leaves were stored in a freezer for future analysis of immune responses. Detailed information about the full-scale greenhouse experiment is described below.

2.4 Experimental Design

2.4.1 Experimental Treatment Design

We had four irrigation water treatments and two pathogen inoculation groups with one control. Four water treatments include: (1) 100% of municipal tap water (TW) as a control; (2) PW diluted 1:9 (PW10) with TW, (3) PW diluted 1:1 (PW50) with TW, and (4) synthetic salt water (NaCl) as a salinity control (SW50) for the NaCl concentration in the PW50 treatment (Figure 14). Based on the electrical conductivity level (EC_w) of PW and information of potential yield reduction from saline water irrigation for common Colorado field crops, we expected that irrigation with PW50 (EC_w = 18 dS/m) will cause more than 50% yield reduction (EC_w = 8.7 dS/m), while theoretically PW10 (EC_w = 2.4 dS/m) irrigation should have no effect on wheat yield (EC_w = 4.0 dS/m) (Table 7). The SW10 treatment was omitted since SW50 matched the worst-case scenario and the selected four water treatments were adequate to address the objectives of this study.

Two pathogen inoculations and one control group were: (1) non-infection as a control; (2) infection of bacterial pathogen (*Xanthomonas translucens* pv. *undulosa*); and (3) infection of fungal pathogen (*Zymoseptoria tritici*) (Figure 15). This bacterial pathogen is known as bacterial leaf streak, which is the most widely distributed bacterial disease for small grains that can cause yield losses of up to 40 percent (Wegulo, 2006). The inoculated fungal pathogen causes one of the most important foliar wheat diseases, and is among the top two or three most economically damaging diseases of wheat in the U.S. (Ponomarenko, Goodwin, & Kema, 2011).

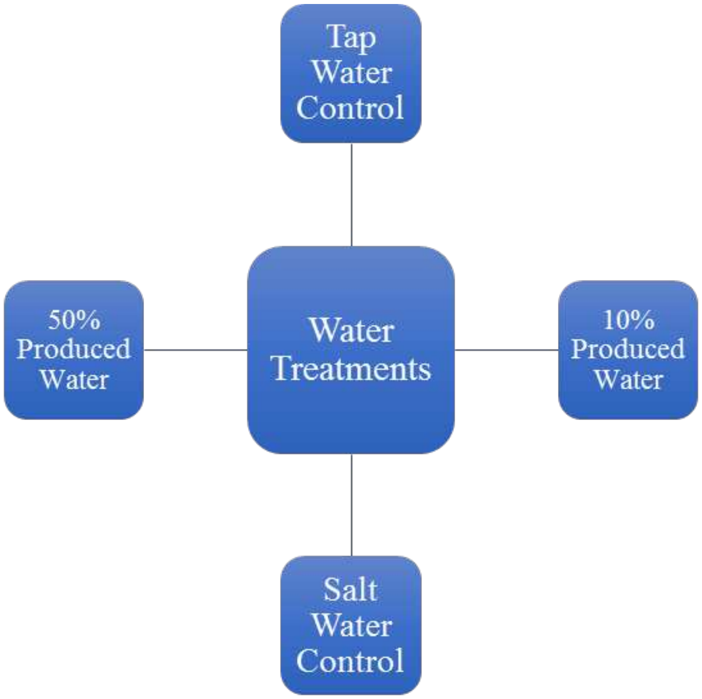

Figure 14. Description of the four irrigation water types used in this study

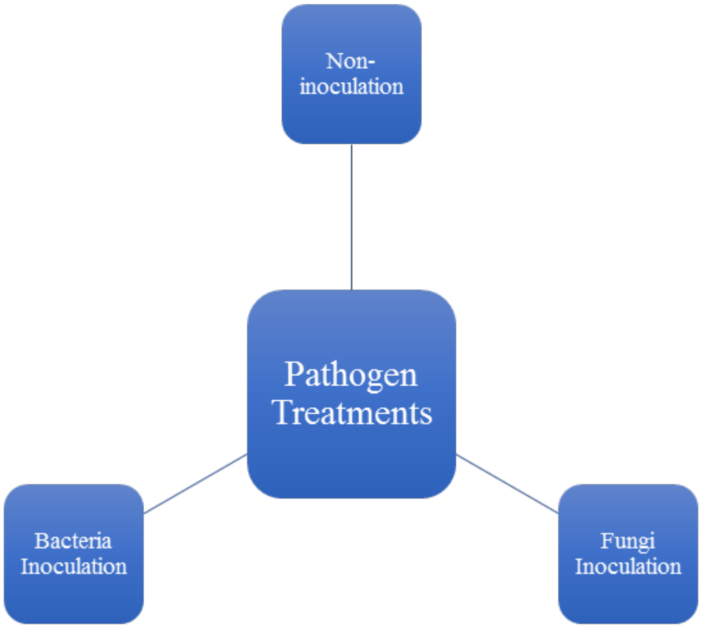

Figure 15. Overview of the pathogen inoculation study

2.4.2 Plant Density Determination

Five wheat seeds were directly sown in each one-gallon nursery pots (2.84L; 7 inches height, 6 ½ inches diameter) for germination. The seedlings were selectively thinned to three plants when they reached three leaves stages (Feekes Scale 3, Figure 11) so that the plants were visibly uniform. In addition, all pots were spaced around six to eight inches apart, so the leaves of adjacent plants did not overlap when they grew.

2.4.3 Replicate Determination

To account for biological, environmental, and experimental variation, six replicates were grown for each water treatment and pathogen inoculation. Therefore, in this experiment, we had a total of 72 pots of wheat, with six pots per unique treatment (Table 10).

Table 10. Description of labels, pathogen inoculation groups, and irrigation water treatments of full-scale greenhouse experiment

Label	Pathogen Inoculation Group	Irrigation Water Treatment
TW_1-6	Non-inoculation	Tap water
TW_b_1-6	Bacteria inoculation	Tap water
TW_f_1-6	Fungi inoculation	Tap water
SW50_1-6	Non-inoculation	NaCl salt water
SW50_b_1-6	Bacteria inoculation	NaCl salt water
SW50_f_1-6	Fungi inoculation	NaCl salt water
PW10_1-6	Non-inoculation	10% produced water
PW10_b_1-6	Bacteria inoculation	10% produced water
PW10_f_1-6	Fungi inoculation	10% produced water
PW50_1-6	Non-inoculation	50% produced water
PW50_b_1-6	Bacteria inoculation	50% produced water
PW50_f_1-6	Fungi inoculation	50% produced water

2.4.4 Bench Randomization

To account for greenhouse climate differences, all pots were randomly situated on the bench. All 72 pots were randomized as four pots per row and 18 pots per column (Table 11, Table A11)

Table 11. Greenhouse layout of pot positions

N (farthest from door)																			
W (wall)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	E (wall)
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	
	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	
	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
S (closest from door)																			

2.4.5 Timeline Planning

We created a timeline to record the important activities throughout the study (Table 12). Day 1 was the day we planted the seeds. Other important activities listed include starting and ending day of irrigation, day of each measurement, inoculation and sampling, and day of harvest.

Table 12. Timeline of full-scale greenhouse experiment

Activity	Day
Planting seeds	1
First day of initial watering	3
Day of first soil electrical conductivity measurement	8
Last day of initial watering	15
First day of water treatment irrigation	17
Day of first physiological measurement	30
Day of second soil electrical conductivity measurement	36
Day of second physiological measurement	45
Day of bacteria inoculation	48
Day of fungi inoculation	56
Day of first sampling of bacteria and fungi leaf	57
Day of second sampling of bacteria and fungi leaf	59
Day of third sampling of bacteria and fungi leaf	64
Last day of water treatment irrigation	71
Day of harvest	91
Day of third soil electrical conductivity measurement	106
Day of plant yield measurement	111
Day of greenhouse cleaning	133

2.5 Planting Protocol

We cut fabric pot liners that kept the soil matrix from spilling out of the plastic pot drainage holes. The pots were then filled with 1,500 ± 2.0 grams of Fields & Fairway Soils. After this, soils were rinsed five times with tap water to promote soil purity and to remove any remaining water-soluble constituents. The draining water from potting soils became cleaner after several rinses, which indicated that the highly water-soluble constituents were leaching out. A plastic saucer was placed underneath each pot when finished with rinsing.

Because there were no nutrients in the selected soil, one teaspoon of Osmocoat^{TM} 19-5-8 time release fertilizer (The Scotts Company, Marysville, OH, USA) was added and distributed evenly within the top two inches of the soil. Each pot was watered for about four seconds with tap water to ensure wet soil when the seeds were sowed. When sowing seeds, we used our fingers to dig five 1.5 inches holes and put seeds in a systematic crosswise fashion, then covered the seeds (Figure 16). In this experiment, a total of 360 seeds were planted in 72 pots with five seeds per pot (Figure 17).

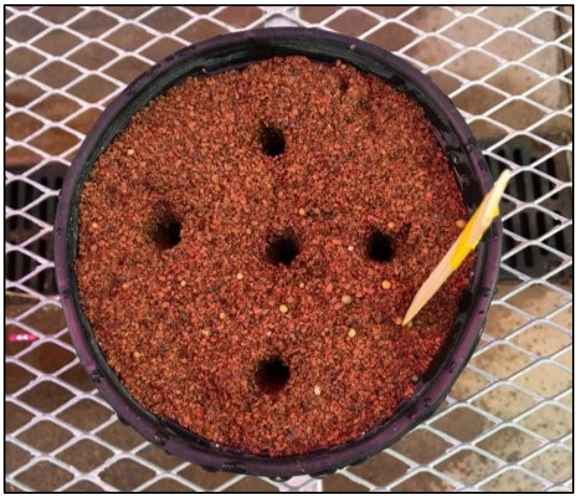

Figure 16. Sample pot for seed planting

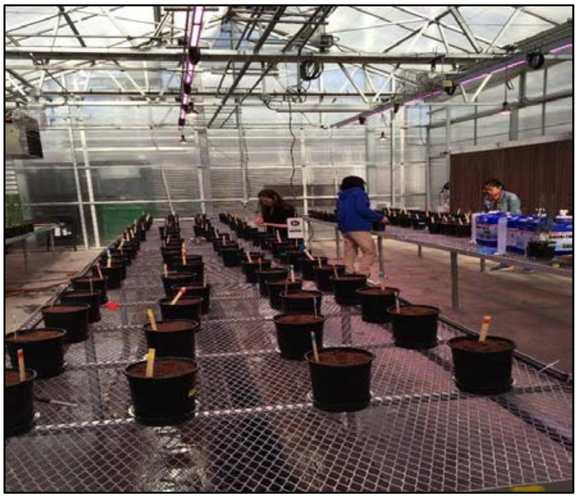

Figure 17. People were working at planting day

2.6 Irrigation Protocol

2.6.1 Initial Irrigation with Tap Water

Wheat plants required less water in the beginning stage of development. As the plants grew bigger, they needed more irrigation water for growth. During the initial watering stage, we checked surface soil moisture and decided that all plants should be watered with 100 mL (determined proper amount) of tap water three times a week (Monday, Wednesday, and Friday). Wheat growth development was monitored to determine when to begin administering PW irrigation treatments. The initial irrigation with tap water lasted for about two weeks after planting the seeds until the wheat reached Feekes Scale 3 (Figure 11, Table 12, Table 13).

Table 13. Description of initial watering stage

Stage Description	Water Treatment	Wheat Development	Day
First day of initial watering	100 mL tap water	Feekes Scale 1	Day 3
Last day of initial watering	100 mL tap water	Feekes Scale 3	Day 15

2.6.2 Water Treatment Irrigation

The diluted PW used in this study was sourced from a hydraulically fractured well in the Niobrara formation of the Colorado Denver-Julesburg Basin. PW from this well was selected because its water quality was consistent over time. Approximately 165 gallons of PW was prepared for this study, and it was stored in three 55-gallon drums in a temperature controlled cold room at 4ºC for the duration of experiment to minimize chemical changes (Sedlacko et al., 2019).

A flexible irrigation amount was used rather than watering the plants with fixed volume because the water demands of wheat vary with growth stage. In this experiment, irrigation amount was calculated based on a “80% pot water holding capacity” theory, to maintain plant health well above drought stress conditions, which is 70% water holding capacity. All pots were irrigated with around 250 mL of each water treatment type three times a week (Monday, Wednesday, and Friday) (Equation 1, Equation 2, Equation 3, Equation 4, Table 14). However, after one-week of application, it was apparent that the pots were overwatered, thus the irrigation amount was modified to 150 mL for each treatment. For TW, PW10, and SW50 treatments, wheat plants were then irrigated for seven weeks with modified volumes of water as necessary. Watering stopped when grains were filled to allow the plants to dry before harvest. For the PW50 treatment, plants were only irrigated for five weeks since most plants showed premature senescence (Table 15). Sometimes the PW50 plants were not irrigated because the surface soil was wet, but the TW plants often got extra water (300 mL) because the soil and plant looked dry. Pesticides (Mantra, NuFarm Americas Inc., Laverton, Australia) were used once at Feekes Scale 3 (Figure 11), and a sulfur lamp was frequently used overnight for fungus control.

Table 14. Water holding capacity test of Field & Fairway Soil

Replicates	Empty (g)	Filled with Soil (g)	Saturated after 24 Hours (g)
Pot #1	75.8	1575.8	2528.5
Pot #2	78.2	1578.2	2522.9
Pot #3	79.4	1579.4	2510.6
Average	77.8	1577.8	2520.7

Equation 1. Calculation of measured water holding capacity of Field & Fairway Soil:

$$2520.7\ g - 1577.8\ g = 942.9\ g$$

Equation 2. Calculation of 80% water holding capacity of Field & Fairway Soil:

$$942.9\ g \times 80\% = 754.32\ g$$

Equation 3. Calculation of weekly volume of water for irrigating one pot:

$$754.32\ g \div \frac{1\ g}{1\ mL} = 754.32\ mL$$

Equation 4. Calculation of daily volume of water for irrigating one pot:

$$754.32\ mL \div 3 = 251.44\ mL$$

Table 15. Irrigation scheme of each water treatment

Water Treatment	Water Amount	Wheat Development	Stage Duration
TW	250 mL tap water	Feekes Scale 3 to 5/6	Day 17-23
	150 mL tap water	Feekes Scale 5/6 to 11	Day 25-71
SW50	250 mL NaCl salt water	Feekes Scale 3 to 5/6	Day 17-23
	150 mL NaCl salt water	Feekes Scale 5/6 to 11	Day 25-71
PW10	250 mL 10% produced water	Feekes Scale 3 to 5/6	Day 17-23
	150 mL 10% produced water	Feekes Scale 5/6 to 11	Day 25-71
PW50	250 mL 50% produced water	Feekes Scale 3 to 5	Day 17-23
	150 mL 50% produced water	Feekes Scale 5 to 11	Day 25-59

The stock solution was prepared weekly with addition of Miracle-Grow All-Purpose Plant Food (The Scotts Company, Marysville, OH, USA), as a nutrient supplement to replenish necessary nutrients for plant growth. Moreover, the amount added were based on the given suggestion "add ½ teaspoons (1.6 grams) per 1 L water". For SW50 stock solution, NaCl was added with concentration of 17 g/L based on the TDS level of PW. For PW10 and PW50, PW used for making stock solution was obtained from the drum every week, and the water was stirred and shook thoroughly to minimize the impacts of settling.

For TW and PW50 treatments, 4.455 L of either TW or PW were mixed with 14.26 grams of dry Miracle-Grow nutrient then stored in two 20 L aquatainers separately (Equation 5, Equation 6, Equation 7, Equation 8, Equation 9, Equation 12). For SW50, same preparation as TW but 75.74 grams of NaCl was also added (Equation 10, Equation 12, Equation 13). For PW10 treatment, 0.891 L of PW and 14.26 grams of dry nutrient were mixed in a smaller 5 L aquatainer (Equation 11, Equation 12). In addition, all aquatainers were opaque to avoid photo-transformation of chemicals in water.

Equation 5. Calculation of weekly volume of water for irrigating one pot:

$$150\ mL \times 3 = 450\ mL$$

Equation 6. Calculation of weekly volume of water for irrigating one water treatment:

$$450\ mL \times 18 = 8100\ mL$$

Equation 7. Calculation of increased water volume accounts for imprecision:

$$8100\,mL \times (1 + 10\%) = 8910\,mL$$

Equation 8. Calculation of weekly volume of TW stock solution:

$$8910\,mL \times 50\% = 4455\,mL$$

Equation 9. Calculation of weekly volume of PW50 stock solution:

$$8910\,mL \times 50\% = 4455\,mL$$

Equation 10. Calculation of weekly volume of SW50 stock solution:

$$8910\,mL \times 50\% = 4455\,mL$$

Equation 11. Calculation of weekly volume of PW10 stock solution:

$$8910\,mL \times 10\% = 891\,mL$$

Equation 12. Calculation of amount of dry Miracle-Grow nutrient added to each stock solution:

$$8910\,mL \times \frac{1\,L}{1000\,mL} \times \frac{1.6\,g}{1\,L} = 14.26\,g$$

Equation 13. Calculation of amount of NaCl added to SW50 stock solution:

$$4455\,mL \times \frac{1\,L}{1000\,mL} \times \frac{17\,g}{1\,L} = 75.74\,g$$

Prior to irrigation, all plastic aquatainers were shaken thoroughly to minimize the effects of settling and precipitation. Stock solutions of TW, PW50, and SW50 treatments were diluted at 50% (Equation 14, Equation 15, Equation 16, Equation 17, Equation 18, Equation 19), and of PW10 were diluted at 90% (Equation 20, Equation 21), with TW stored in the fifth aquatainer (Table 16), then carefully hand watered by 300 mL plastic graduated cylinders. Water was evenly applied to the soil and we avoided splashing the leaves. To prevent cross contamination of pathogens, bacteria and fungi inoculated plants were irrigated lastly and separately. Also, ethanol disinfectant was applied to rinse graduated cylinders when switch of irrigating plants with different inoculation status.

Equation 14. Calculation of daily volume of TW stock solution for irrigating one pot:

$$150\ mL\ \times 50\% = 75\ mL$$

Equation 15. Calculation of daily volume of TW needed to dilute TW stock solution for irrigating one pot:

$$150\ mL\ - 75\ mL = 75\ mL$$

Equation 16. Calculation of daily volume of PW50 stock solution for irrigating one pot:

$$150\ mL\ \times 50\% = 75\ mL$$

Equation 17. Calculation of daily volume of TW needed to dilute PW50 stock solution for irrigating one pot:

$$150\ mL - 75\ mL = 75\ mL$$

Equation 18. Calculation of daily volume of SW50 stock solution for irrigating one pot:

$$150\ mL\ \times 50\% = 75\ mL$$

Equation 19. Calculation of daily volume of TW needed to dilute SW50 stock solution for irrigating one pot:

$$150\ mL - 75\ mL = 75\ mL$$

Equation 20. Calculation of daily volume of PW10 stock solution for irrigating one pot:

$$150\ mL\ \times 10\% = 15\ mL$$

Equation 21. Calculation of daily volume of TW needed to dilute PW10 stock solution for irrigating one pot:

$$150\ mL - 15\ mL = 135\ mL$$

Table 16. Description of irrigation protocol

Water Treatment	Number of Pots	Weekly Irrigation (mL)	Increased by 10% (mL)	Weekly Stock Solution (mL)	Dry Nutrient (g)	NaCl (g)	Instruction of Irrigation
TW	18	8100	8910	4455	14.26	Not available	Add 75 mL TW to 75 mL TW stock solution, mix then irrigate
SW50	18	8100	8910	4455	14.26	75.74	Add 75 mL TW to 75 mL SW50 stock solution, mix then irrigate
PW50	18	8100	8910	4455	14.26	Not available	Add 75 mL TW to 75 mL SW50 stock solution, mix then irrigate
PW10	18	8100	8910	891	14.26	Not available	Add 135 mL TW to 15 mL PW10 stock solution, mix then irrigate

2.7 Inoculation Protocol

2.7.1 Spray Inoculation

The bacterial pathogen (*Xanthomonas translucens* pv. *undulosa*) was inoculated seven weeks after planting to ensure the leaves were big enough to absorb the pathogen liquid (Table 17). Larger leaves were sprayed with the bacterial strain then marked with permanent marker and then the sprayed droplets were rubbed into the leaves. The entire wheat plant was spray inoculated with Fungal pathogen (*Zymoseptoria tritici*) eight weeks after planting when they reached heading stage (Feekes Scale 10.5, Figure 11), and all inoculated plants were covered with a mesh covering to prevent cross contamination (Figure 18). To prevent infection of other people's plants, all inoculation processes were applied in the hallway outside of the greenhouse. Also, nitrile gloves were worn during the spray inoculation of plant pathogens (Figure 19).

Table 17. Description of bacterial and fungal pathogen inoculation

Inoculum	Inoculation Method	Wheat Development	Inoculation Day
Bacterial pathogen	Spray to the largest leaf	Feekes Scale 10.5 except for PW50 plants (10.1)	48
Fungal pathogen	Spray to the entire plant	Feekes Scale 10.5	56

Figure 18. Mesh covering of inoculated wheat plants

Figure 19. Picture of spray inoculation (courtesy: Dr. Pankaj Trivedi)

2.7.2 Inoculated Leaf Sampling

The necrotic leaves were sampled three times around 1, 3, and 7 days after inoculation, and a total of 18 leaves were sampled for each pathogen inoculation. For the bacteria inoculated leaves, one marked leaf was cut from each pot, and then the sample leaf was divided into three pieces. Two plastic bags were labeled for each pot with the name of pot and sampling date, the centermost part of the leaf went in the bag that was put in the fridge for colony counts, and the remaining two pieces were flash-frozen in liquid nitrogen for future extraction of RNA and expression of pathogenesis-related genes (PR genes). For the fungi inoculated plants, the same process was performed. Scissors used for cutting were sterilized by ethanol spray when switched from sampling the bacteria to fungi leaves, clean clothes and nitrile gloves were also worn during the sampling.

2.8 Harvest Protocol

All wheat plants were harvested when they reached peak maturity. Maturity occurred when there is no more green color in the flag leaf (Feekes Scale 11, Figure 11). Wheat plants irrigated with TW, SW50, and PW10 water treatments were harvested at week 13. However, PW50 groups were harvested early at week 11 because plants showed premature senescence (Table 18).

Table 18. Description of harvest protocol

Water Treatment	Wheat Development	Harvest Day
TW	Feekes Scale 11	91
SW50	Feekes Scale 11	91
PW10	Feekes Scale 11	91
PW50	Feekes Scale 11	80

When harvesting, height of tallest tiller was measured, and number of tiller and grain head were counted. All wheat plants were then preserved in a labeled brown paper bag for further analysis, including yield weighing. Again, clean clothes and gloves were worn during the harvest to minimize contamination.

2.9 Response Evaluation

2.9.1 Water Quality and Soil Property Analysis

Chemical characterization of PW and each individual irrigation water treatment was conducted by Dr. Christopher Higgins and Erin Sedlacko from Colorado School of Mines using inductively-coupled plasma optical emission spectroscopy (ICP-OES; Optima 5300 DV, PerkinElmer, Fremont, CA) and ion chromatography (IC; ICS-900, Dionex, Sunnyvale, CA) for concentration of cations/metals and anions, respectively. Total organic carbon (TOC) and total nitrogen (TN) concentrations were measured using a carbon analyzer (Shimazu TOC-L, Columbia, MD). Alkalinity measurements were conducted by digitally titrating 1.6 N sulfuric acid into 100 mL of raw, unfiltered PW samples until the sample reached a pH of 4.5. Also, initial characterization of PW and Miracle-Grow nutrient solution (NS), as well as individual mixture of each irrigation water treatment with NS (TW + NS, SW50 + NS, PW10 + NS, PW50 + NS) were performed at the beginning of the experiment (Sedlacko et al., 2019).

Salinity is one of the main hazards with PW irrigation, thus apparent soil electrical conductivity (EC_a) was measured at day 8, 36, and 106 during the experiment (Table 12) by using a Field-scout Direct Soil EC Probe (Spectrum Technologies, Inc.).

2.9.2 Plant Physiology Measurement

Plant development was recorded with the Feekes Scale (Figure 11) throughout the irrigation period. Since plant photosynthetic efficiency plays an important role in the productivity of grain crops (Zhu, Long, & Ort, 2010), key factors that related to plant photosynthesis were measured in this experiment. Photosynthetic efficiency is mainly associated with photosystem II quantum yield (Phi2), the amount of energy that plant uses for photosynthesis. Other parameters correlated with photosynthesis include non-photochemical exciton quenching (PhiNPQ), the light energy that is dissipated as heat to regulate excess energy to reduce damage. Also, quantum yield of other unregulated non-photochemical losses (PhiNO), the remainder of energy that is absorbed but not directed towards Phi2 or PhiNPQ. Therefore, the sum of these three parameters are equal to 1.0 (Phi2 + PhiNPQ + PhiNO = 1.0) (Kramer, Johnson, Kiirats, & Edwards, 2004).

In this experiment, Phi2, PhiNPQ, and PhiNO of tallest flag leaf were recorded twice during the experiment at day 30 and 45 (Table 12), by using a non-destructive reflectance instrument namely, MultiSpeQ (PhotosynQ.org). We wanted to perform more measurements, but the wheat was already inoculated with pathogens at day 48 and 56 (Table 12), thus the third measurement was abandoned to prevent cross pathogen spread.

The MultiSpeQ is used by clamping the device around a leaf and then pressing the run button. The light sensor should not be shaded, the leaf needs to completely cover the sensor, and both the leaf and device need to be stable during the measurement (Figure 20). One measurement took around one minute, then the data were auto-populated in screen and were exported for further analysis.

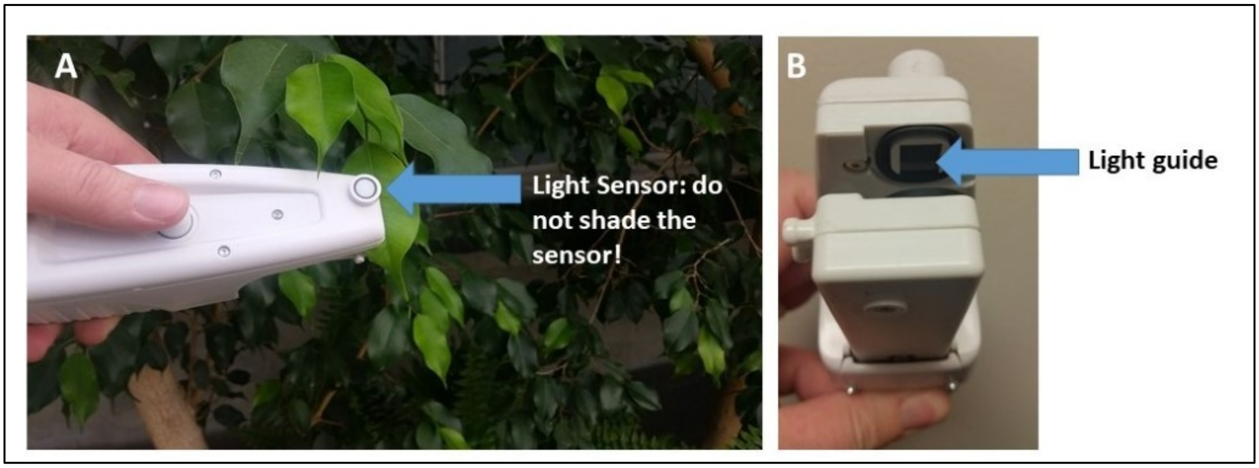

Figure 20. Guide for plant photosynthesis measurement (source: https://www.photosynq.org/)

2.9.3 Plant Yield Measurement

The grain yield of wheat is determined by the numbers of grains and individual grain weight (Slafer, 2007). Also, tiller production play key roles in grain formation in wheat plants (Maas, Lesch, Francois, & Grieve, 1994; Xie, Mayes, & Sparkes, 2016). Therefore, height of tallest tiller, and tiller and grain head number were recorded during the harvest at day 91 (Table 12). Moreover, grain yield of all wheat plants was measured in the lab by using electronic scale after harvesting at day 111 (Table 12).

2.9.4 Immune System Analysis

Hormones include salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are key factors in plant immune system response to pathogens (Robert-Seilaniantz et al., 2011). They can induce pathogenesis-related (PR) genes, which are the most common plant defense gene-encoded proteins related to pathogenic infections (Penninckx et al., 1996; Weisman et al., 2010). The PR genes have been classified into several groups based on their amino acid sequences from PR-1 to PR-14 (Muthukrishnan, Liang, Trick, & Gill, 2001), and they are induced by various signaling pathways. For example, PR-1 and PR-5 only respond to SA, while PR-4 is induced by SA, JA, and ET (Korpela, 2007). Expression of PR proteins can promote pathogen resistance of crops such as wheat, rice, and maize, specifically, PR-1 and PR-5 genes are known to help inhibit growth of bacteria and fungi in wheat (Muthukrishnan et al., 2001; Neugebauer, Bruce, Todd, Trick, & Fellers, 2018). In this experiment, PR-1 and PR-5 gene expression was analyzed from leaves inoculated with both bacterial and fungal pathogens. Also, their copy numbers were measured 1, 3, and 7 days after inoculation by running quantitative polymerase chain reaction (q-PCR).

2.10 Statistical Analysis

Post experimental statistical analyses were completed by using Statistical Package for the Social Sciences (SPSS, IBM Corporation). Mean of data set are shown with standard error (SEM), and statistical difference of means was evaluated by analysis of variance test (ANOVA) with Tukey's post-hoc method. The ANOVA p-value indicates if there is a significant difference between the means of two or more data sets, and the Tukey post-hoc p-value determines where do significant differences lie by performing a multiple pairwise comparison. Moreover, the means of Tukey separation test were listed with letters to indicate if there was a significant difference between tested groups. The p-value was considered significant when it was less than 0.05 ($p < 0.05$) at a 95% confidence level for all comparisons.

3. RESULTS

3.1 Water Characterization and Soil Electrical Conductivity

3.1.1 Water Characterization

The PW has TDS levels of 17,750 mg/L and the NaCl accounted for nearly 93% of the TDS (Table 19). We used this information to justify using 17 g/L of NaCl to prepare the stock solution of salt water control (SW50) (Equation 13). Water quality analysis data for all irrigation water types are shown in Table 20. PW contained 220 mg/L TOC, 200 mg/L Alkalinity, 31 mg/L Total Nitrogen (TN), and the pH and EC were 7.71 and > 20 dS/m, respectively. Compared to the PW, the TW contained 3.05 mg/L TOC, 31 mg/L Alkalinity, 3.14 mg/L TN, and the pH and EC are 7.47 and 0.11 dS/m, respectively (Table 20).

As expected, the water EC level of PW50 (EC_w = 18 dS/m) and SW50 (EC_w = 16 dS/m) treatments were approximately equivalent, and the EC of PW10 (EC_w = 2.4 dS/m) roughly followed trends one-fifth that of the PW50 and one-tenth that of the PW. In addition, there were various metal(loids) only present in the PW, including boron, silicon, barium, calcium, lithium, magnesium, and strontium, but they were all below limited of detection (LOD) in the NS, TW, and SW50 treatment (Table 20). The water quality analysis was focused on analysis of major ions and water quality parameters, since it was beyond the scope of this study to conduct a comprehensive analysis of all inorganic and organic species present.

Table 19. Produced water quality data collected monthly from August 2016 to October 2017. Adapted from (Sedlacko et al., 2019)

Produced Water Composition	Average
pH	6.66
Conductivity (dS/m)	28.1
TDS (mg/L)	17,750
TOC (mg/L)	220
TN (mg/L)	30.9
COD (mg/L)	2,099
F^- (mg/L)	9.73
Cl^- (mg/L)	5,996
Br^- (mg/L)	135
NO_2^- (mg/L)	21.6
NO_3^- (mg/L)	26.5
SO_4^{2-} (mg/L)	45.5
Ag (mg/L)	1.10
Al (mg/L)	1.06
As (mg/L)	12.5
B (mg/L)	16.4
Ba (mg/L)	10.0
Be (mg/L)	337
Ca (mg/L)	365
Cd (mg/L)	0.02
Co (mg/L)	0.02
Cu (mg/L)	35.1
Fe (mg/L)	21.1
K (mg/L)	37.2
Li (mg/L)	9.79
Mg (mg/L)	39.5
Mn (mg/L)	0.80
Na (mg/L)	5,996
Ni (mg/L)	0.04
Pb (mg/L)	0.15
S (mg/L)	30.3
Sb (mg/L)	0.51
Se (mg/L)	6.70
Si (mg/L)	32.8
Sr (mg/L)	44.5
Ti (mg/L)	0.12
Zn (mg/L)	0.14
Total cations (mg/L)	5,224
Total anions (mg/L)	8,359

Table 20. Water quality parameters of produced water (PW), tap water (TW), nutrient solution (NS), and all individual water treatments (TW + NS, SW50 + NS, PW10 + NS, PW50 + NS). All treatments received equal amounts of nutrient solution. Adapted from (Sedlacko et al., 2019)

Parameters	PW	TW	NS	TW + NS	SW50 + NS	PW10 + NS	PW50 + NS
pH	7.71	7.47	3.63	6.24	5.99	5.81	6.51
Conductivity (dS/m)	> 20	0.11	1.1	1.2	16	2.4	18
TOC (mg/L)	220	3.05	280	95	150	200	190
Alkalinity	200	31	98	152	21	20	111
TN (mg/L)	31	3.14	426	312	385	259	377
B (mg/L)	23.7	< 0.02	< 0.02	< 0.02	6.26	3.19	12.4
Ba (mg/L)	11.1	0.017	< 0.0003	< 0.0003	0.022	0.49	2.73
Ca (mg/L)	408	8.6	< 0.51	7.6	6.4	39.1	196
Cu (mg/L)	< 0.006	0.086	1.30	1.42	1.35	1.45	1.14
Fe (mg/L)	0.89	0.006	2.45	1.57	2.69	3.17	1.33
K (mg/L)	84.0	0.961	151	153	186	181	224
Li (mg/L)	5.87	< 0.005	< 0.005	< 0.005	< 0.005	0.57	2.48
Mg (mg/L)	47.3	0.78	0.09	1.13	0.76	4.61	21.7
Na (mg/L)	5940	1.61	5.93	12.1	1850	527	2550
Si (mg/L)	30.4	0.23	2.06	< 0.10	23.3	7.47	20.6
Sr (mg/L)	80.1	0.021	< 0.0003	0.018	0.025	5.56	31.5
Zn (mg/L)	0.222	0.0024	1.20	1.06	1.11	1.18	1.25

3.1.2 Soil Electrical Conductivity

Soil apparent electrical conductivity (EC_a) is the most commonly method for assessment of soil salinity, and measurements can be accomplished with commercial electrodes (Sudduth, Drummond, & Kitchen, 2001). We measured soil EC_a of each water treatment by using soil probes at the beginning (day 8), middle (day 36), and end (day 106) of the experiment (Table 12, Figure 21). The level of soil EC_a corresponded primarily to the concentration of NaCl in each water treatment, SW50 and PW50 had the highest level, PW10 were much lower, and TW was the lowest (Figure 21). At day 8, all water groups had low soil EC_a level since irrigation treatment has not been started yet (Table 12). At day 36, after 19 days of irrigation treatment, the soil EC_a level of SW50 and PW50 greatly increased while the PW10 treatment only increased slightly, and the TW control remained unchanged. At day 106, SW50 continued sharp rise in soil

EC_a, PW10 and TW showed a slightly increase, while PW50 exhibited a decreased soil EC_a level (Figure 21). This was unexpected since it should have an increased level of soil EC_a along with the irrigation. Possible reason for this decrease might be the interruption of irrigation, since sometimes PW50 plants did not get watered, which reduce the accumulation of salt contents in the soil.

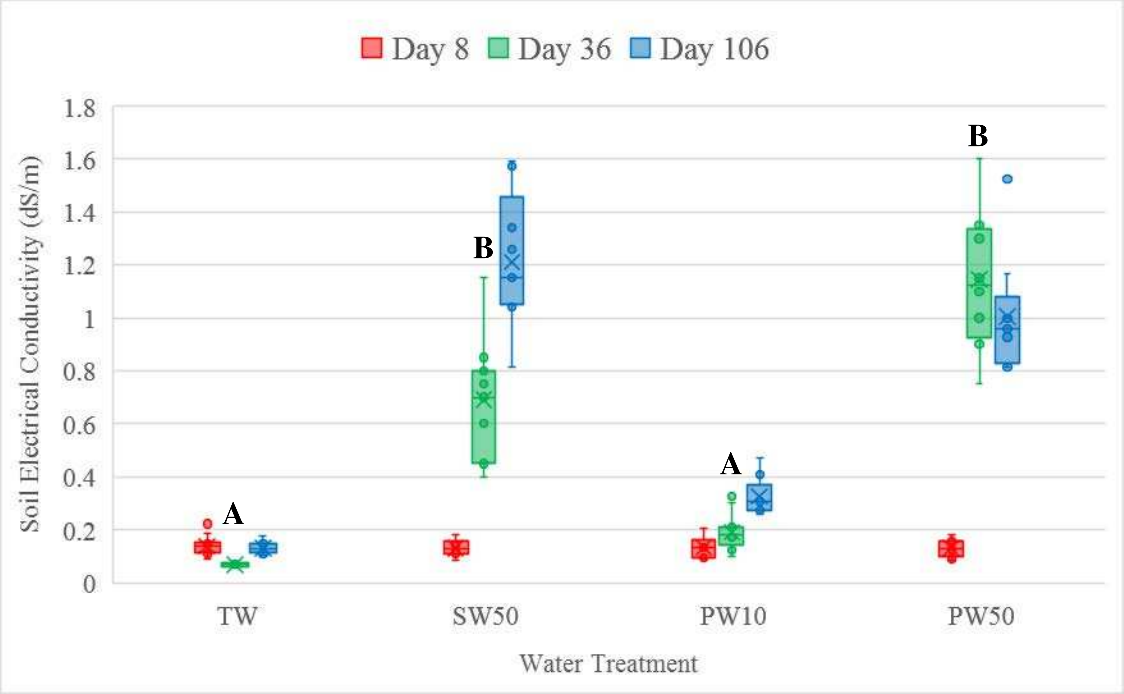

Figure 21. Soil electrical conductivity (EC) at day 8, 36, and 106 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

The statistical analysis of means was listed with letters to indicate if there were significant differences in soil EC_a between each treatment (Figure 21, ANOVA, Tukey post-hoc and mean separation). Soil irrigated with SW50 and PW50 (letter A) had significant higher EC_a level than with PW10 and TW (letter B) (Figure 21, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$), while there were no significant differences between each of two treatment themselves (Figure 21, ANOVA, Tukey post-hoc and mean separation, $p > 0.05$). The results correlated with the water EC_w level of each treatment in the initial water quality characterization, PW50 and SW50 were approximately equivalent, and were much higher than that of PW10 and TW (Table 20).

3.2 Plant Physiological Response

3.2.1 Development Stage of Wheat Plants

The development stages of the wheat plants were tracked with the Feekes Scale (Figure 11) until plants reached maturity (Figure 22). Seeds were planted at day 1 and emerged at day 6. All seedlings were initially watered with TW for two weeks before beginning irrigation with the four different treatments at day 17. Wheat plants irrigated with SW50, TW and PW10 displayed similar growth pattern (Figure 22). However, a lag in development for PW50 plants was observed at day 25 after a week of irrigation with produced water, and PW50 plants showed severe developmental delay and early senescence (Figure 22). Wheat appearances for all four water treatments were also monitored. Plants irrigated with TW looked healthy, SW50 and PW10 looked good, plants are slightly shorter but with PW50 the plants barely grew and quickly died (Figure 23).

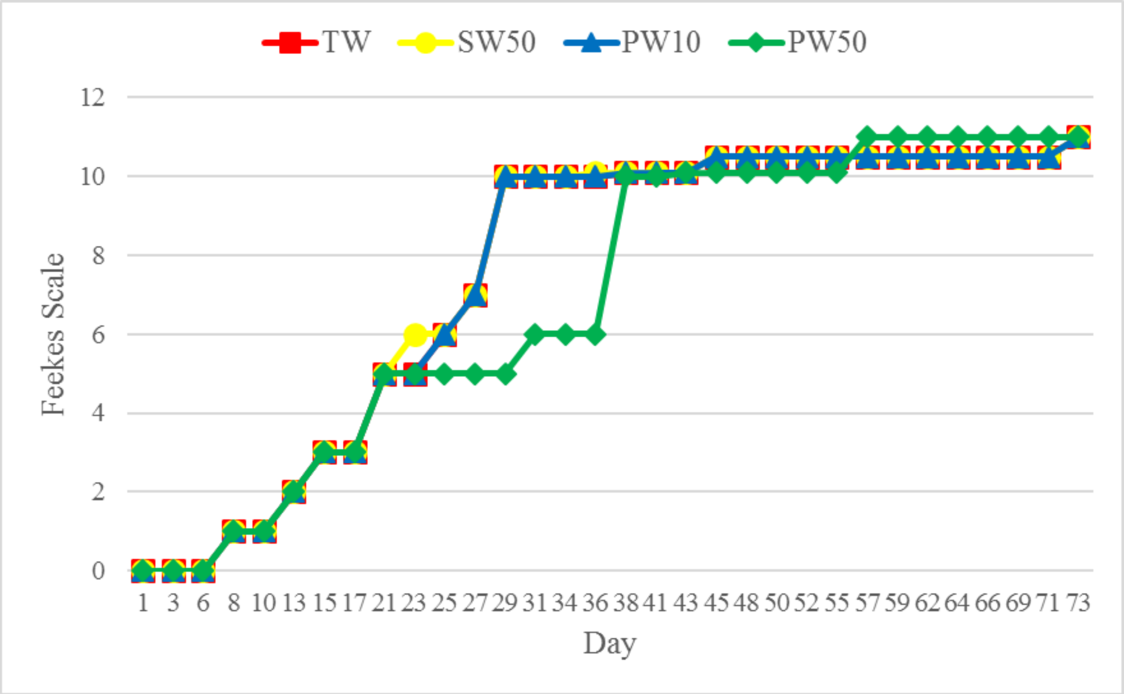

Figure 22. Feekes Scale of experimental wheat plant development over time for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

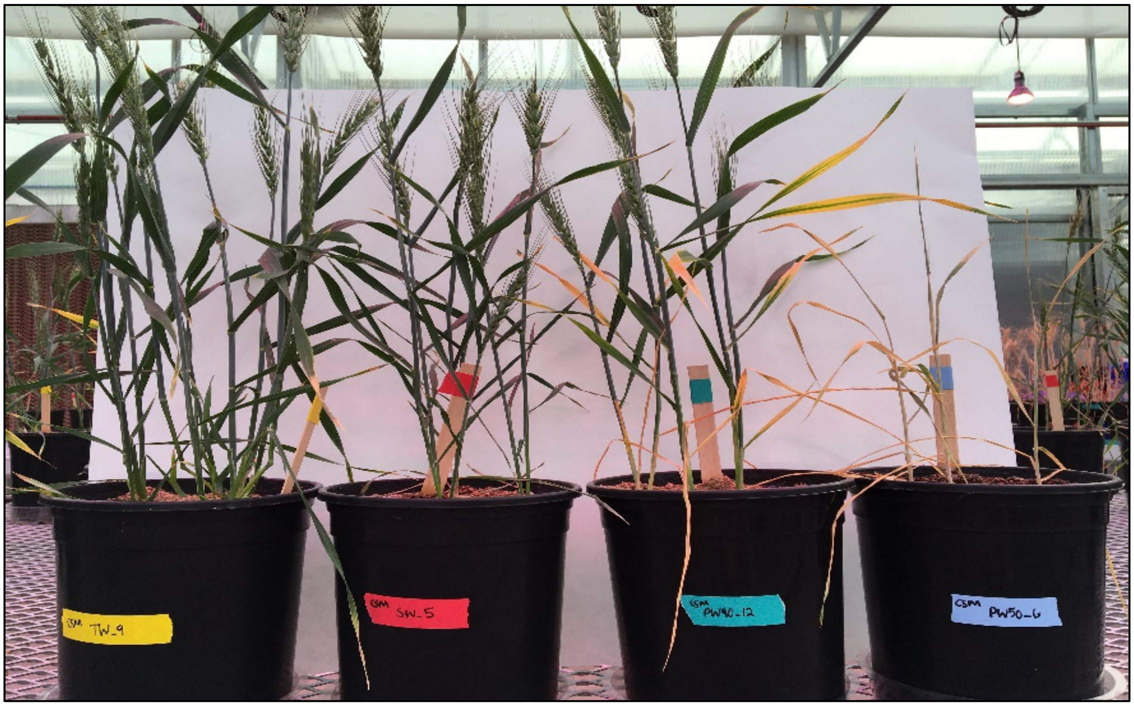

Figure 23. From left to right, wheat plants are irrigated with: Tap Water Control (TW, yellow), Salt Water Control (SW50, red), 10% Produced Water (PW10, dark green), 50% Produced Water (PW50, light blue).

3.2.2 Photosynthesis of Wheat Plants

Key parameters related to plant photosynthesis including photosystem II quantum yield (Phi2), non-photochemical exciton quenching (PhiNPQ), and quantum yield of other unregulated non-photochemical losses (PhiNO) were measured to evaluate plant physiological response to PW irrigation. All three factors were measured twice during the experiment at day 30 and day 45 (Table 12), and the sum of them are equal to 1.0 (Phi2 + PhiNPQ + PhiNO = 1.0).

The TW, SW50, and PW10 treatments remained consistent for all three parameters at both day 30 and 45 (Figure 24), and there was no variation in photosynthesis among all water treatments at day 30. However, PW50 exhibited substantial decrease in both Phi2 and PhiNO and great increase in PhiNPQ at day 45 (Figure 24). The statistical results only showed significant differences for all three parameters between PW50 (letter B) and the other three treatments (letter A) (Figure 24, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$), and no significant difference among the rest three water treatments (letter A) (Figure 24, ANOVA, Tukey post-hoc and mean separation, $p > 0.05$). The results of plant physiological response indicated that only PW50 irrigation caused significant adverse effects on photosynthesis of wheats at day 45, while neither PW10 nor SW50 contribute to negligible impacts, comparing to the TW control.

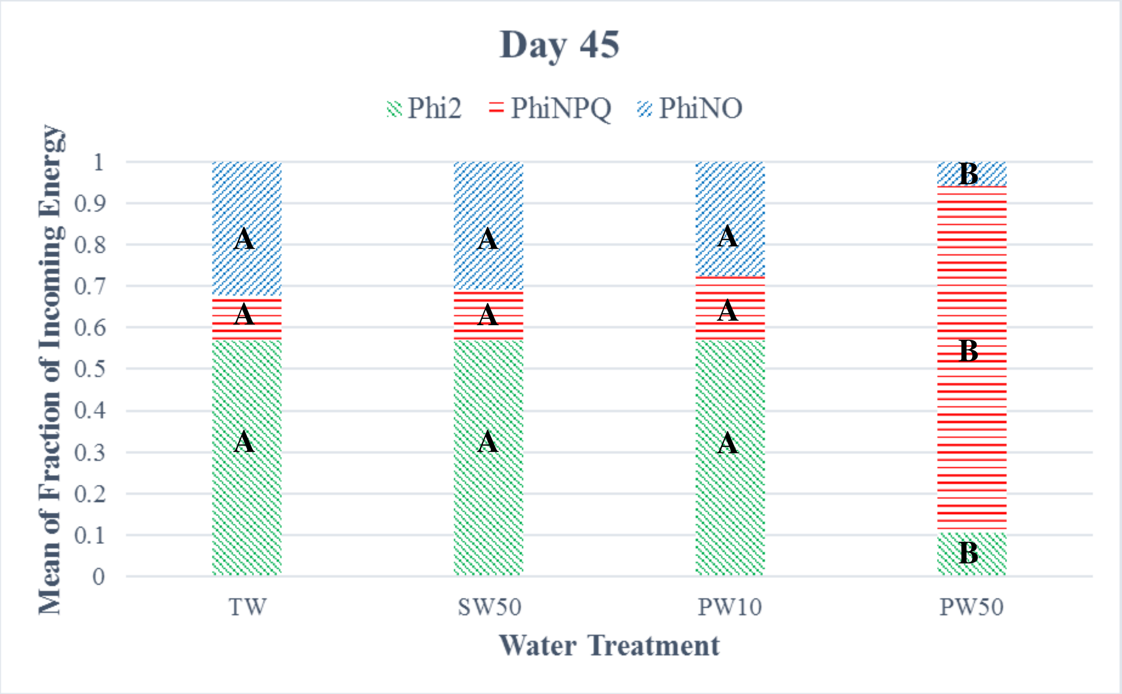

Figure 24. Mean of fraction of incoming energy at day 45 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

3.3 Plant Yield Response

3.3.1 Height of Tallest Tiller

The height of tallest tiller was significantly lower in PW50 (letter B) compared to other three water treatments (letter A) (Figure 25, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$), but no significant differences were identified between the rest of three groups themselves (Figure 25, ANOVA, Tukey post-hoc and mean separation, $p > 0.05$). The observed decrease in height of tallest tiller was not solely due to high NaCl (SW50) or other PW contaminants (e.g., organics) (PW10) but rather a combination of these two stresses (PW50) (Figure 25).

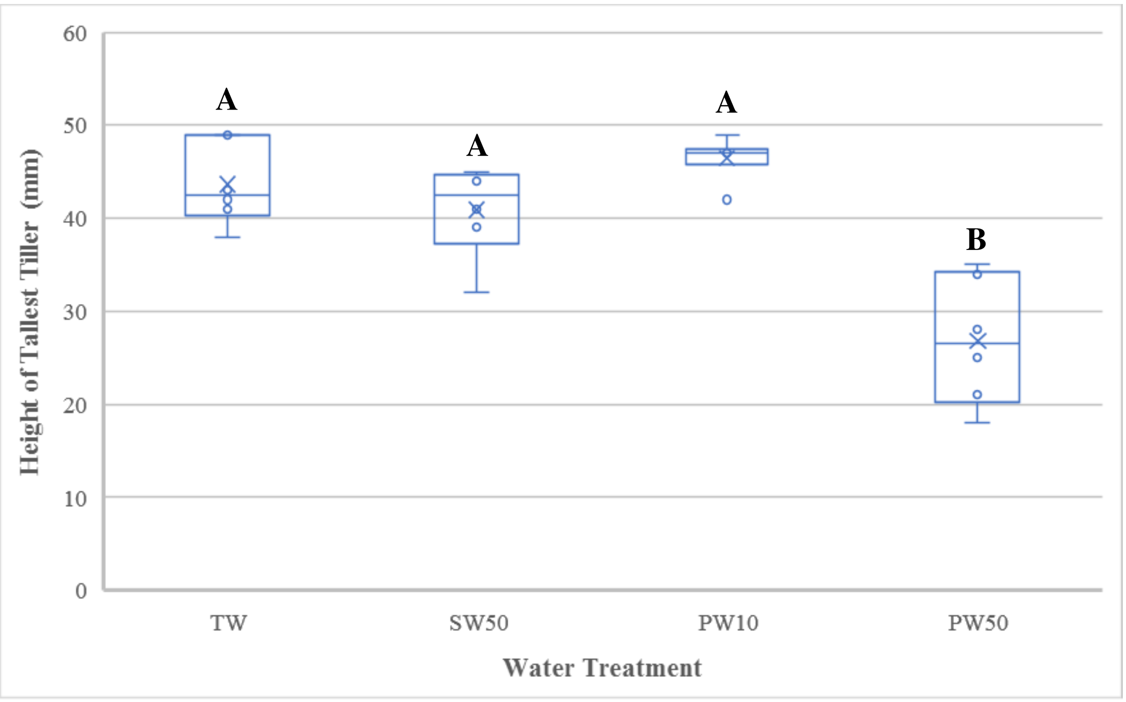

Figure 25. Height of tallest tiller of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

3.3.2 Tiller and Grain Head Number of Wheat Plants

The statistical results showed significant differences in number of tiller and grain head for most of water treatments The means of tiller number for four water treatments followed a trend of TW (letter A) > PW10 (letter B) > SW50 (letter C) > PW50 (letter D) (Figure 26, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$), but of grain head number displayed in a different way as TW (letter A) > PW10 (letter B) = SW50 (letter B) > PW50 (letter C) (Figure 27, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$). In comparison with the TW control, SW50 irrigation was more damaging than PW10 with regards to tiller production, while they contributed to the same level of effects on grain production. Again, PW50 irrigation showed significant negative impacts on both tiller and grain production (Figure 26, Figure 27).

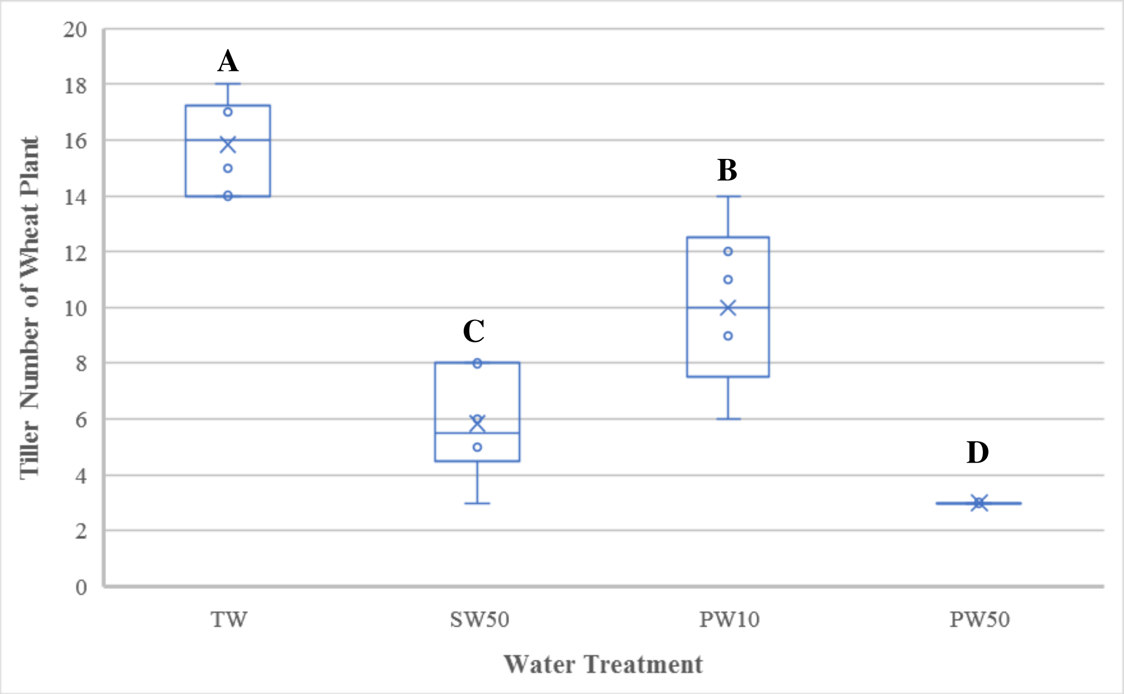

Figure 26. Tiller number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

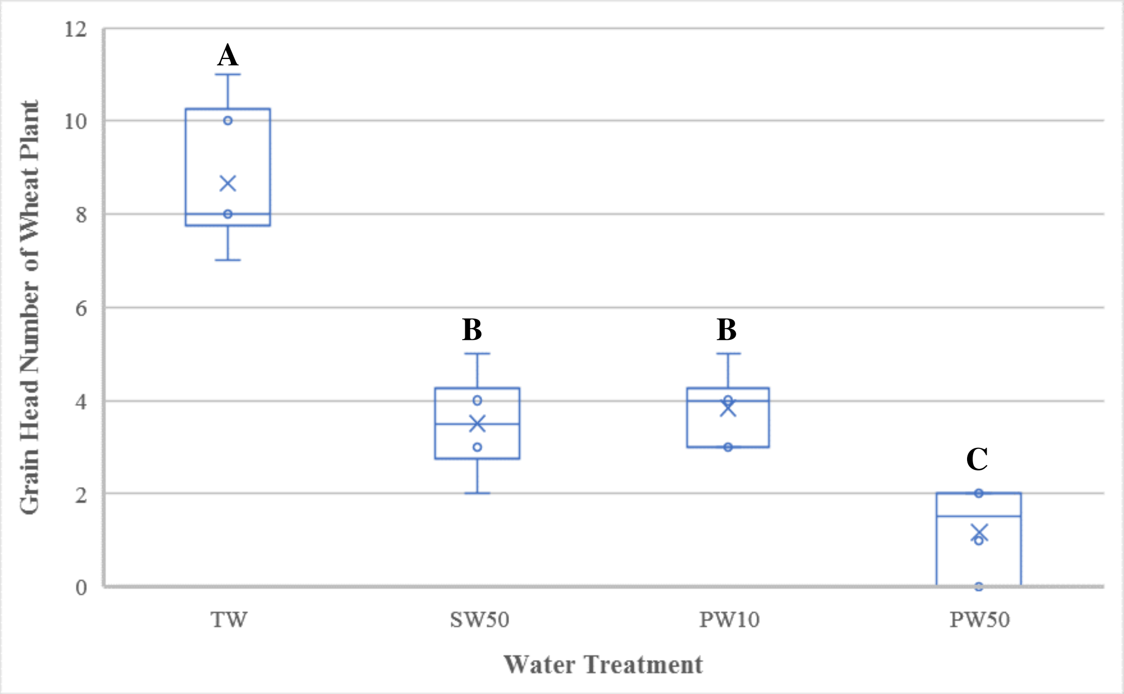

Figure 27. Grain head number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

3.3.3 Weight of Wheat Plants

The means of weight per pot for all water treatments followed a trend of TW (letter A) > SW50 (letter B) = PW10 (letter B) > PW50 (letter C) (Figure 28, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$), of weight per grain head the significant differences were only observed in PW50 (letter B) but not in the other three water treatments (letter A) (Figure 29, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$). Compared to the TW control, the SW50 and PW10 irrigation treatments contributed to the same level of stress in terms of weight per pot, but neither of them resulted in significant impacts on weight per grain head. Indeed, PW50 irrigation showed significant adverse impacts on grain yield of wheat plants (Figure 28, Figure 29).

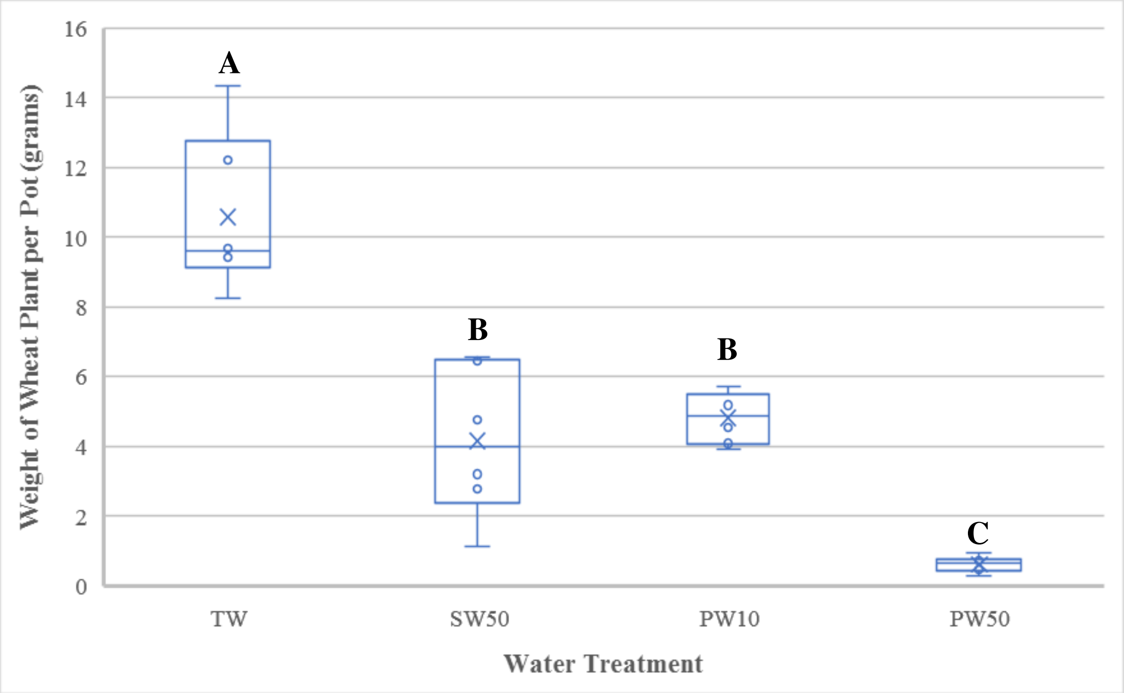

Figure 28. Weight of wheat plants per pot for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

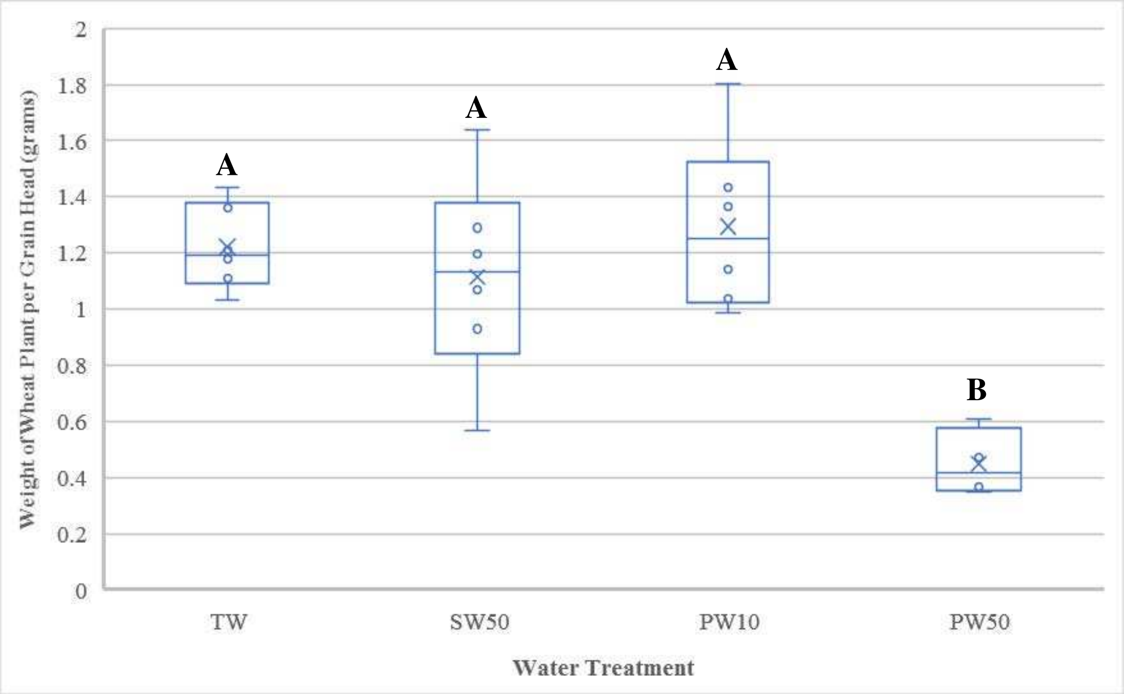

Figure 29. Weight of wheat plants per grain head for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

3.4 Immune System Response

3.4.1 PR-1 and PR-5 gene Expression of Bacteria and Fungi

The means of pathogenesis-related-1 and 5 (PR-1 & PR-5) expression of both bacteria and fungi followed a trend of TW > SW50 > PW10 > PW50 (Figure 30, Figure 31, Figure 33), except for fungi PR-1 expression (Figure 32). Moreover, only bacteria PR-5 expression changed inconsistently over time (Figure 31), while the other three gene expressions exhibited a strong temporal trend of day 7 > day 3 > day 1 (Figure 30, Figure 32, Figure 33).

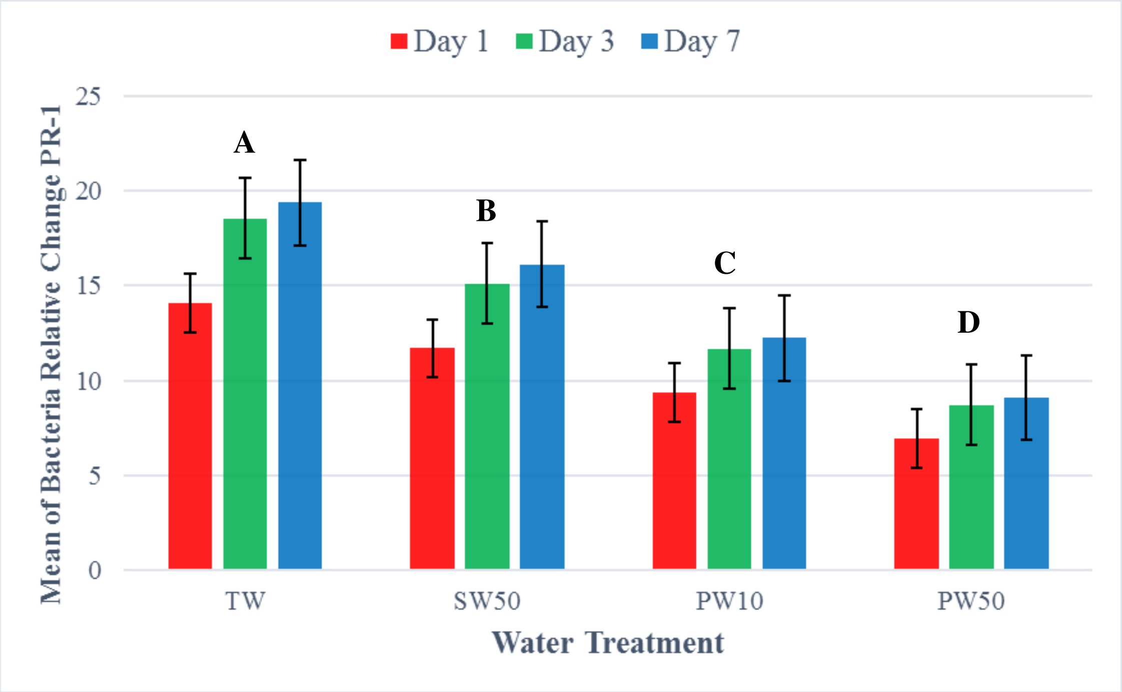

Figure 30. Mean of bacteria pathogenesis-related-1 (PR-1) gene expression at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

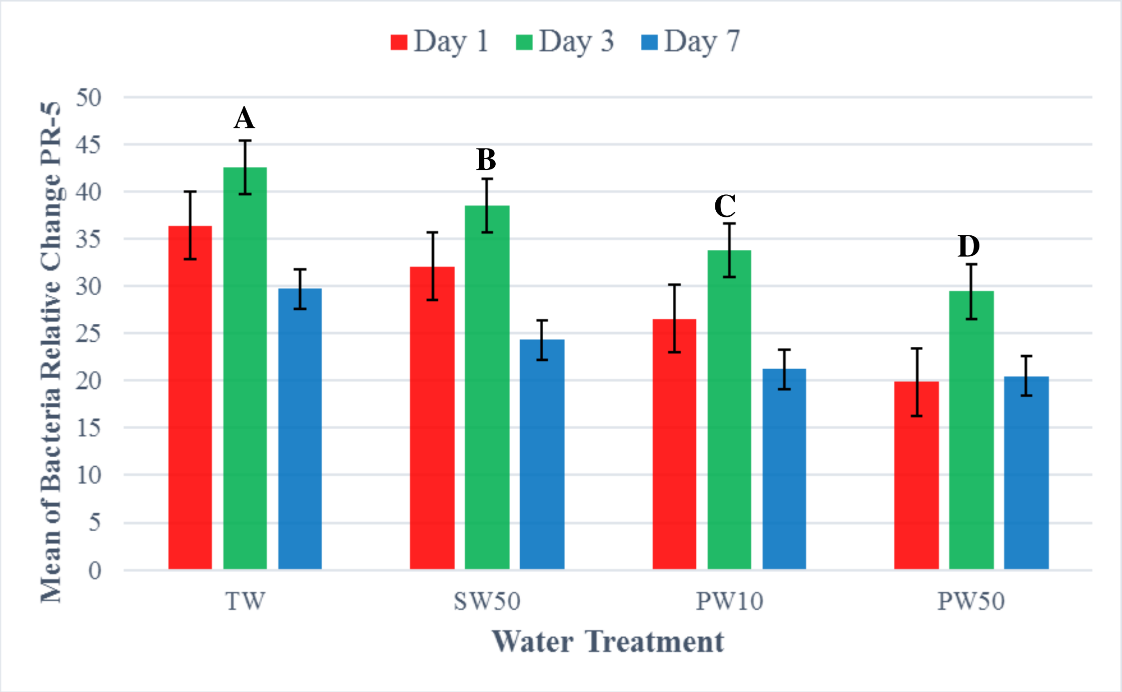

Figure 31. Mean of bacteria pathogenesis-related-5 (PR-5) gene expression at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

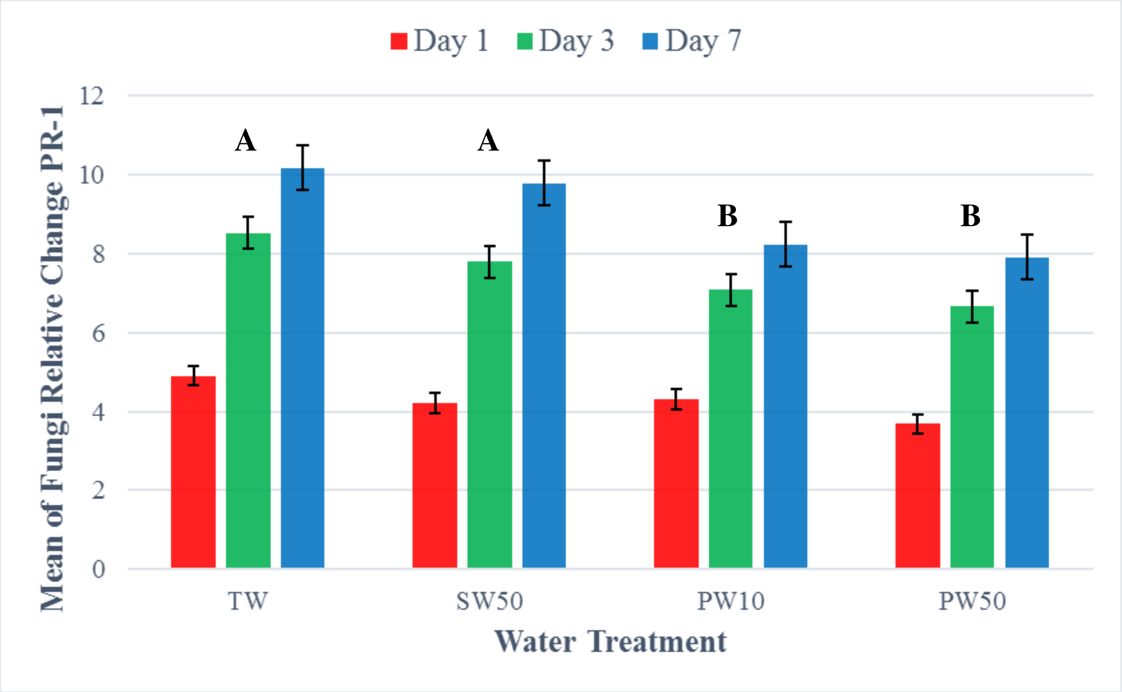

Figure 32. Mean of fungi pathogenesis-related-1 (PR-1) gene expression at day 1, 3 and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

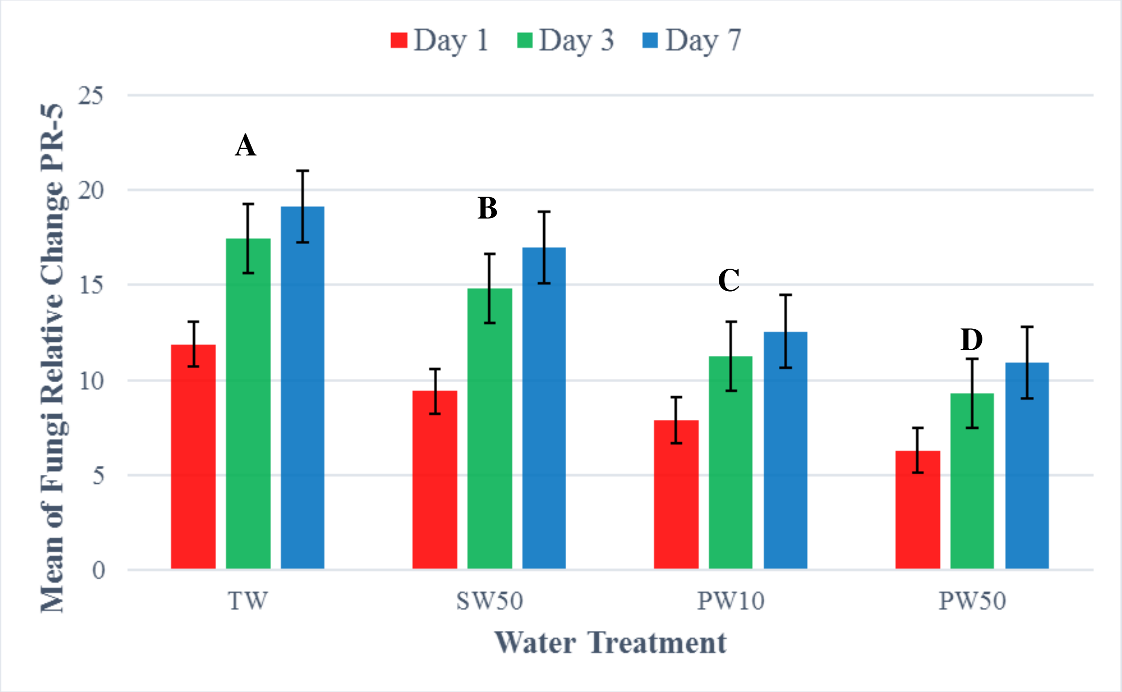

Figure 33. Mean of fungi pathogenesis-related-1 (PR-5) gene expression at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

PR-1 and PR-5 gene expression of bacteria and fungi exhibited significant differences across the water treatments. The means of expression of bacteria PR-1 and PR-5 as well as fungi PR-5 followed this trend: TW (letter A) > SW50 (letter B) > PW10 (letter C) > PW50 (letter D) (Figure 30, Figure 31, Figure 33, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$), only fungi PR-1 followed a different trend: TW (letter A) = SW50 (letter A) > PW10 (letter B) = PW50 (letter B) (Figure 32, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$). The results of PR gene expression except for PR-1 of fungi, clearly indicated that PW10 irrigation was more damaging than SW50 to plant immune system, and irrigation with PW50 resulted in the highest damage. The result of fungi PR-1 expression also showed PW10 is more damaging than SW50, but it implied that NaCl had no effect on fungi PR-1 expression since no significant differences were observed between TW and SW50 and PW10 and PW50 treatments.

3.4.2 Bacteria and Fungi Copy Numbers

Both bacteria and fungi copy numbers exhibited significant differences across water treatments, and the statistical results correlated with their PR gene expressions: PW50 (letter A) > PW10 (letter B) > SW50 (letter C) > TW (letter D) (Figure 34, Figure 35, ANOVA, Tukey post-hoc and mean separation, $p < 0.05$). The trend was not clear and copy numbers overlapped at day 1 since the pathogens just starting to grow. After several days of colonization, great differences in copy numbers among each water treatments occurred, and the trend was apparent (Figure 34, Figure 35). Interestingly, the bacteria copy number at day 3 was slightly decreased compared to day 1 for all water treatments, which was not expected and might be a result of a higher level of bacteria PR-5 expression (Figure 31, Figure 34). The results of bacteria and fungi colonization clearly indicated that PW10 irrigation cause greater damage than SW50 to plant immune system. Again, irrigation with PW50 led to the lowest level of disease resistance for wheat plants.

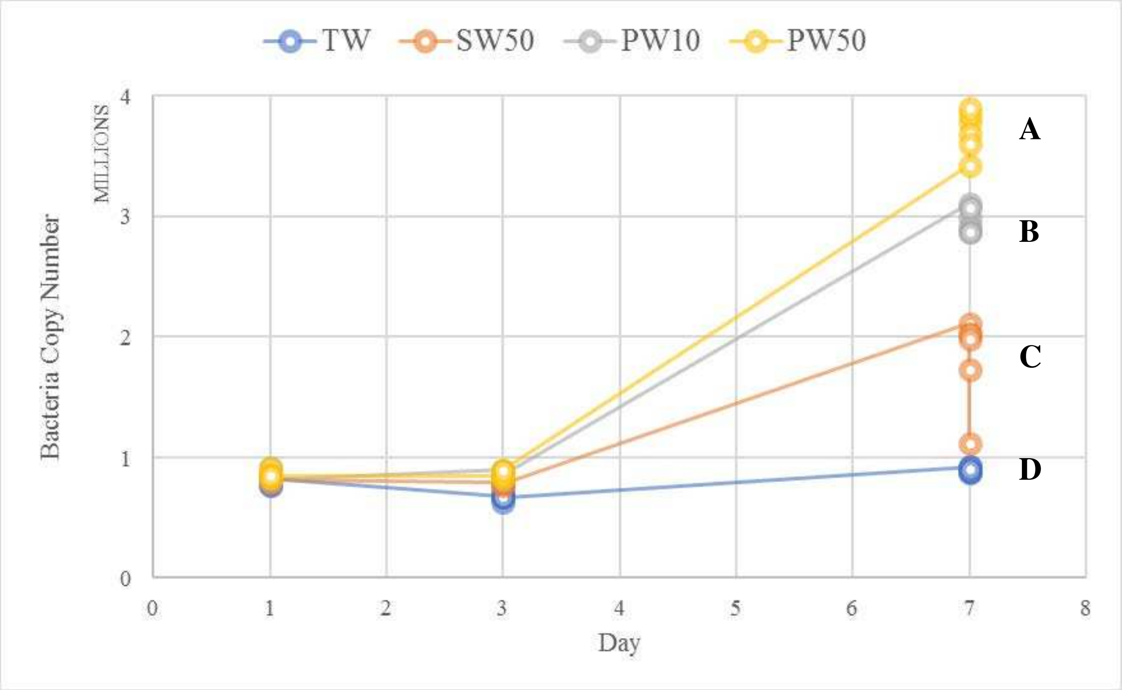

Figure 34. Bacteria copy numbers at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Figure 35. Fungi copy numbers at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

3.5 Plant Overall Response to PW Irrigation

The overall statistical results of plant photosynthesis, yield, and immune system respond to all four irrigation treatments are summarized in Table 21. Compared to the TW control, plants irrigated with PW50 showed the most impact with regard to all responses. The PW10 and SW50 had no effects on plant photosynthesis and reduced plant yield to the same extent, but apparently PW10 resulted in more inhibition than SW50 to plant immune system, despite the high salt contents in SW50 (Table 21). These findings indicate that constituents other than NaCl in PW are contributing to plant stress, and they may play a greater role in affecting plant immune function than salt stress. Again, the combination of high salt and other contaminants in the PW50 treatment had a severe impact on most plant responses and resulted in significant adverse effects on plant health.

Table 21. The overall statistical results of plant photosynthesis, yield, and immune system respond to irrigation with all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50). Letter A (dark green) > letter B (light green) > letter C (yellow) > letter D (red).

Parameters		**TW**	**SW50**	**PW10**	**PW50**
Plant photosynthesis		A	A	A	B
Plant yield	Height of tallest tiller	A	A	A	B
	Tiller number	A	C	B	D
	Grain head number	A	B	B	C
	Weight per pot	A	B	B	C
	Weight per grain head	A	A	A	B
Plant immune system	Bacteria PR-1	A	B	C	D
	Bacteria PR-5	A	B	C	D
	Fungi PR-1	A	A	B	B
	Fungi PR-5	A	B	C	D
	Bacteria copy number	D	C	B	A
	Fungi copy number	D	C	B	A

4. DISCUSSION

4.1 Evaluation of Hypothesis

We hypothesized that the PW10 and PW50 treatments were not sufficiently treated to allow for uncompromised irrigation of wheat due to residual organic constituents. The plant overall response indicated that wheat plants irrigated with PW10 and PW50 had significantly lower photosynthetic efficiency, yield, and immune system response. Therefore, our hypothesis was supported by results of this experiment.

4.2 Potential Chemicals Affecting Plant Response

Our results indicated that NaCl is not the only source of toxicity in the PW, but we cannot determine the exact contaminants of concern from the results presented here. However, we can use the chemical composition of PW to make plausible assumptions. It is likely that metal(loid)s, organic compounds, and radionuclides in PW are negatively affecting plant health. For example, irrigation with PW that has high TOC concentration resulted in significantly lower growth health and physiological characteristics of non-food biofuel plants (Pica et al., 2017). However, it is hard to tell which and how exactly these chemicals affect the plant. Further research should focus on pinpointing the specific PW chemicals that cause plant stress and how these combined stresses affect plant health.

4.3 Challenges of PW Irrigation

The results presented here indicated that contaminants present in PW other than NaCl also contribute to severe plant stress, which has hardly been discussed in the previous literature

(Sedlacko et al., 2019). Based on the results, salinity is still a big concern in terms of soil health, since SW50 and PW50 irrigation resulted in a very high level of soil EC.

For plant health, one of the challenges related to PW irrigation is salt damage, because wheat plants irrigated with SW50 and PW50 exhibited reduced plant yield, decreased photosynthetic efficiency, and declined immune function, compared to the TW control. The impact of PW chemicals (other than NaCl) on plant immune system observed in this study is novel and indicates that future studies need to include this assessment when evaluating irrigation water quality to prevent unexpected consequences. This experiment showed that plants irrigated with PW10 treatment had lower disease resistance and higher pathogen colonization than SW50, indicating that PW irrigation is more damaging than salt water to plant immune system.

In addition, plant bioaccumulation and translocation of PW toxic chemicals may also result in negative health consequences for food consumption (Chaîneau et al., 2010; Samsøe-Petersen et al., 2002; Tao et al., 2009). However, that was not studied as a part of this project. Most plant uptake studies imply that further human health risk assessment of consuming wastewater irrigated food crops is necessary (Blaine et al., 2014; Goldstein et al., 2014; Malchi et al., 2014; Shenker et al., 2011; Wu et al., 2014), since human exposure to xenobiotics via ingestion of wastewater irrigated produce has already been demonstrated (Paltiel et al., 2016). To ensure environmental sustainability and food security, the abovementioned challenges has to be addressed when considering reuse PW for irrigation.

4.4 Implications for Future Research

Reusing PW for food crop irrigation has become an attractive option to fulfill water deficits and water management requirements. To make irrigation reuse of PW meaningful, low environmental hazards and high-quality crops must be guaranteed. Therefore, comprehensive risk assessments of PW irrigation with respect to soil, plant, and human health are imperative for future decision making.

Pinpointing the specific PW chemicals that cause plant stress and investigating combined stresses affecting plant health are extremely important next steps for this research. For future studies, more control groups not only salt (NaCl) but also metal(loid)s, radionuclides, and organics, would need to be conducted to narrow down the list of possible toxic chemicals.

Additionally, to broaden knowledge in this field, other widely grown crops (e.g., rice and corn) and common plant pathogens (e.g., virus) as well as different types of soils (e.g., clay and silt) should also be selected and tested. Because the composition of chemicals in PW vary widely with time and geographic location (C. E. Clark & Veil, 2009; K. B. Gregory et al., 2011; King, 2012), it would be helpful to have a comprehensive characterization of PW before the experiment to better determine the chemicals for each control group.

PW can be treated to meet regulatory guidelines by using a treatment train including both pretreatment (primary treatment) and advanced treatment (secondary & tertiary treatment) (Fakhru’l-Razi et al., 2009; Munirasu, Haija, & Banat, 2016). Biological and electrochemical technologies are cost-effective and environmentally friendly techniques for PW pretreatment, and membrane technology is one of the finest methods for advanced treatment of PW today (Fakhru’l-Razi et al., 2009; Igunnu & Chen, 2014). For example, a combination of electrocoagulation and reverse osmosis (EC-RO) are shown high efficiency of PW contaminants removal (Zhao, Huang, Cheng, Wang, & Fu, 2014). Although PW is commonly regarded as a toxic waste, it can be beneficial to humans if properly treated (Fakhru’l-Razi et al., 2009; Igunnu & Chen, 2014).

5. CONCLUSION

Although the evaluation of PW reuse for agricultural irrigation is an emerging topic of study with numerous future research possibilities, the impact of PW chemicals on the plant immune system has never been studied previously and thus many questions remain unanswered. The findings of this study clearly indicated that constituents other than NaCl in PW are playing an important role in affecting plant immune function. To the best of our knowledge, this is the first study evaluating the impact of PW irrigation on plant immune system. To broaden our knowledge in this field, future research should focus on identifying the specific chemicals in PW that affect plant health and assessing the potential human health consequences of consuming PW irrigated food crops. Additionally, different types of soil and crops should also be investigated to elucidate their role in controlling the impact of PW irrigation on plant immune function.

The introduction chapter illustrates the feasibility of reusing treated PW for food crop irrigation. The results of this study indicated that diluting PW at a ratio of 1:1 and 1:9 with TW is not sufficient to produce an economically viable and health crop, since great decline in yields, photosynthetic efficiency, and immune system response were observed. Therefore, further treatment is required to remove unwanted chemicals and to meet certain minimum standards of water quality for irrigation reuse.

Again, to fully assess the impacts of treated PW irrigation on plant health and determine the chemicals that need to be eliminated for successful crop production, experiments need to be designed that consider a variety of crop species, soil types, pathogens, and irrigation water controls. As shown in this study, having only a salt (NaCl) control is not sufficient to help pinpointing all the chemicals in PW that affect plant health. Therefore, more water controls with targeted chemicals must be considered. Reuse of treated PW for agricultural irrigation is not an easy task, due to the complexity of PW matrix and stringent quality requirements of irrigation water. However, as long as the unwanted chemicals in PW that impede irrigation reuse are identified, specific treatments can be developed and applied to remove these constituents, and likely allow for sustainable reuse of treated PW for agricultural usage.

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APPENDIX

A.1 Support Information for Introduction

Table A1. Global water withdrawal by sectors around 2010. Six regions include World, Africa, America, Asia, Europe, and Oceania. Three sectors are municipal, industrial, and agricultural. (source: http://www.fao.org/nr/water/aquastat/tables/WorldData-Withdrawal_eng.pdf)

Region	Agricultural (%)	Industrial (%)	Municipal (%)
World	69	19	12
Africa	81	4	15
America	48	37	14
Asia	81	10	9
Europe	25	54	21
Oceania	65	15	20

Table A2. Estimated use of water in the United States in 2000, 2005, 2010, and 2015 (Dieter et al., 2018; Hutson et al., 2004; Kenny et al., 2009; Maupin et al., 2014)

Category	Water Withdrawals (million gallons per day)			
	Year 2000	Year 2005	Year 2010	Year 2015
Public Supply	43,300	44,200	42,000	39,000
Domestic	3,590	3,830	3,600	3,260
Irrigation	137,000	128,000	115,000	118,000
Livestock	1,760	2,140	2,000	2,000
Aquaculture	3,700	8,780	9,420	7,550
Industrial	19,700	18,200	15,900	14,800
Mining	3,490	4,020	5,320	4,000
Thermoelectric Power	195,000	201,000	161,000	133,000

Table A3. Estimated irrigation water uses of western contiguous United States in 2000, 2005, 2010, and 2015 (Dieter et al., 2018; Hutson et al., 2004; Kenny et al., 2009; Maupin et al., 2014)

State	Irrigation Water Withdrawals (million gallons per day)			
	Year 2000	Year 2005	Year 2010	Year 2015
Arizona	5,400	4,810	4,570	4,530
California	30,500	24,400	23,100	19,000
Colorado	11,400	12,300	9,710	9,000
Idaho	17,100	16,600	14,000	15,300
Montana	7,950	9,670	7,160	9,450
Nevada	2,110	1,500	1,570	2,070
New Mexico	2,860	2,810	2,700	2,370
Oregon	6,080	5,710	5,260	5,160

Utah	3,860	4,000	3,220	3,030
Washington	3,040	3,520	3,150	2,520
Wyoming	4,500	3,990	4,370	7,790
Western U.S.	94,800	89,310	78,810	80,220
Total U.S.	137,000	128,000	115,000	118,000

Table A4. Estimated produced water generation in the United States in 2012 (Veil, 2015)

State	Produced Water Generation (million gallons per day)
Alabama	12.26
Arizona	0.01
Arkansas	21.26
California	353.55
Colorado	41.21
Florida	7.20
Illinois	11.40
Indiana	6.62
Kansas	122.01
Kentucky	2.26
Louisiana	106.67
Michigan	13.45
Mississippi	26.59
Missouri	0.24
Montana	21.02
Nebraska	6.74
Nevada	0.67
New Mexico	89.22
New York	0.06
North Dakota	33.48
Ohio	0.64
Oklahoma	267.37
Pennsylvania	3.92
South Dakota	0.61
Tennessee	0.17
Texas	855.02
Utah	19.20
Virginia	0.37
West Virginia	1.58
Wyoming	250.46

Table A5. Estimated irrigation water use in the United States in 2015 (Dieter et al., 2018)

State	Irrigation Water Use (million gallons per day)
Alabama	223
Alaska	1.52
Arizona	4,530
Arkansas	11,600
California	19,000
Colorado	9,000
Connecticut	11.3
Delaware	113
Florida	2,450
Georgia	738
Hawaii	385
Idaho	5,300
Illinois	234
Indiana	133
Iowa	35
Kansas	2,680
Kentucky	39.6
Louisiana	1,050
Maine	18.9
Maryland	64.1
Massachusetts	139
Michigan	332
Minnesota	276
Mississippi	1,770
Missouri	1,370
Montana	9,450
Nebraska	6,090
Nevada	2,070
New Hampshire	5.2
New Jersey	93.9
New Mexico	2,370
New York	53.5
North Carolina	325
North Dakota	233
Ohio	55
Oklahoma	931
Oregon	5,160
Pennsylvania	34.3
Rhode Island	4.25
South Carolina	126
South Dakota	211

Tennessee	63.8
Texas	5,490
Utah	3,030
Vermont	3.11
Virginia	51.7
Washington	2,520
West Virginia	4.15
Wisconsin	460
Wyoming	7,790

Table A6. U.S. potential for reusing produced water to meet irrigation needs

State	Daily Produced Water Generation as a Percent of Daily Irrigation Water Use
Alabama	5.50
Arizona	0.00
Arkansas	0.18
California	1.86
Colorado	0.46
Florida	0.29
Illinois	4.87
Indiana	4.98
Kansas	4.55
Kentucky	5.72
Louisiana	10.16
Michigan	4.05
Mississippi	1.50
Missouri	0.02
Montana	0.22
Nebraska	0.11
Nevada	0.03
New Mexico	3.76
New York	0.11
North Dakota	14.37
Ohio	1.16
Oklahoma	28.72
Pennsylvania	11.43
South Dakota	0.29
Tennessee	0.27
Texas	15.57
Utah	0.63
Virginia	0.72
West Virginia	38.16
Wyoming	3.22

Table A7. Estimated produced water generation in Colorado in 2018 (source: https://cogcc.state.co.us/data.html#/cogis)

County	Produced Water Generation (million gallons per day)
Adams County	0.2070
Arapahoe County	0.0828
Archuleta County	0.0731
Baca County	0.1731
Bent County	0.0001
Boulder County	0.0029
Broomfield County	0.0022
Cheyenne County	0.9567
Delta County	0.0028
Dolores County	0.0369
Elbert County	0.0037
Fremont County	0.0000
Garfield County	3.2408
Gunnison County	0.0511
Huerfano County	0.0005
Jackson County	0.3207
Kiowa County	0.2040
Kit Carson County	0.0001
La Plata County	2.2788
Larimer County	0.6436
Las Animas County	3.8065
Lincoln County	0.2765
Logan County	0.4454
Mesa County	1.0558
Moffat County	0.9763
Montezuma County	0.2037
Morgan County	0.3097
Phillips County	0.0202
Prowers County	0.0006
Rio Blanco County	12.3670
Routt County	0.0002
San Miguel County	0.0036
Sedgwick County	0.0001
Washington County	2.3127
Weld County	8.6266
Yuma County	0.0820

Table A8. Estimated irrigation water use in Colorado in 2015 (Dieter et al., 2018)

County	Irrigation Water Use (million gallons per day)
Adams County	87
Alamosa County	137
Arapahoe County	3.56
Archuleta County	42
Baca County	125
Bent County	168
Boulder County	104
Broomfield County	1.38
Chaffee County	115
Cheyenne County	29.4
Clear Creek County	0
Conejos County	294
Costilla County	92.8
Crowley County	11.9
Custer County	34.8
Delta County	341
Denver County	1.53
Dolores County	4.16
Douglas County	11.7
Eagle County	132
EI Paso County	8.58
Elbert County	15.1
Fremont County	95.5
Garfield County	234
Gilpin County	0
Grand County	183
Gunnison County	521
Hinsdale County	20.4
Huerfano County	38.1
Jackson County	340
Jefferson County	22.3
Kiowa County	1.71
Kit Carson County	111
La Plata County	322
Lake County	14
Larimer County	119
Las Animas County	21.1
Lincoln County	4.54
Logan County	143
Mesa County	757
Mineral County	7.02

Moffat County	121
Montezuma County	185
Montrose County	672
Morgan County	123
Otero County	349
Ouray County	94.3
Park County	33.6
Phillips County	73.6
Pitkin County	117
Prowers County	110
Pueblo County	266
Rio Blanco County	136
Rio Grande County	557
Routt County	172
Saguache County	366
San Juan County	0
San Miguel County	47.1
Sedgwick County	73.6
Summit County	50.1
Teller County	2.48
Washington County	46.2
Weld County	366
Yuma County	323

Table A9. Colorado potential for reusing produced water to meet irrigation needs

County	Daily Produced Water Generation as a Percent of Daily Irrigation Water Use
Adams County	0.2379
Arapahoe County	2.3254
Archuleta County	0.1741
Baca County	0.1385
Bent County	0.0001
Boulder County	0.0028
Broomfield County	0.1596
Cheyenne County	3.2541
Delta County	0.0008
Dolores County	0.8870
Elbert County	0.0242
Fremont County	0.0000
Garfield County	1.3850
Gunnison County	0.0098
Huerfano County	0.0014
Jackson County	0.0943
Kiowa County	11.9274
Kit Carson County	0.0001
La Plata County	0.7077
Larimer County	0.5408
Las Animas County	18.0404
Lincoln County	6.0907
Logan County	0.3115
Mesa County	0.1395
Moffat County	0.8069
Montezuma County	0.1101
Morgan County	0.2518
Phillips County	0.0275
Prowers County	0.0005
Rio Blanco County	9.0934
Routt County	0.0001
San Miguel County	0.0075
Sedgwick County	0.0001
Washington County	5.0059
Weld County	2.3570
Yuma County	0.0254

A.2 Support Information for Experimental Method

Table A10. Field & Fairway Soil profile

Soil Parameters		**Description**
Materials		A calcined, non-swelling illite and non-crystalline opal CT mineral
Porosity		Total 74%, with 39% capillary and 35% non-capillary
pH Range		5.5 ± 1
Cation Exchange Capacity		33.6 mEq/100 g
Stability		Sulfate soundness testing (ASTM C-88) and static degradation test not to exceed 4% loss over 20 years
Bulk Density		36 ± 2 lb/ft^3
Color Range		Reddish/Tan
Packaging		50 pounds valve bags, 2,000 pounds super sacks, bulk dump truck loads
Sieve Analysis	+ 10 MESH	0.1%
	- 10 + 20 MESH	47.0%
	- 20 + 50 MESH	52.2%
	- 50 MESH	0.7%
Chemical Description	SiO_2	74.00%
	Al_2O_3	11.00%
	Fe_2O_3	5.00%
	Others	5.00%

Table A11. Random number and position of each pot

Label of Pot	Random Number	Random Position
TW_b_1	0.006404436	1
SW50_b_6	0.008153098	2
PW10_f_6	0.052988025	3
PW50_f_1	0.059947816	4
SW50_b_1	0.060742998	5
PW10_6	0.081632232	6
PW10_b_4	0.091173345	7
SW50_3	0.111134176	8
TW_1	0.122825904	9
PW10_b_5	0.126837628	10
PW10_b_1	0.134108361	11
PW10_b_2	0.134111089	12
SW50_1	0.150123598	13
PW10_4	0.151235143	14
TW_2	0.214420169	15
PW50_b_3	0.225013233	16
TW_f_5	0.227757895	17
TW_f_1	0.251923922	18
PW50_b_2	0.260883827	19
TW_b_4	0.299495574	20
SW50_b_5	0.323641675	21
PW50_b_4	0.331254470	22
SW50_f_1	0.339353155	23
TW_f_2	0.340718721	24
SW50_5	0.349124498	25
TW_4	0.363301741	26
PW10_f_3	0.371533514	27
PW50_f_2	0.404761519	28
PW10_b_3	0.406384785	29
SW50_4	0.423741249	30
TW_f_3	0.466209708	31
SW50_f_2	0.468898321	32
SW50_b_2	0.474261097	33
SW50_f_6	0.488000088	34
PW10_3	0.504647777	35
TW_3	0.536285428	36
PW50_b_6	0.545383913	37
TW_f_6	0.553239155	38
SW50_f_5	0.554064055	39
PW50_f_3	0.573044214	40
TW_b_3	0.575959760	41

PW10_f_4	0.581867156	42
SW50_f_3	0.589720131	43
TW_b_6	0.594484028	44
SW50_b_4	0.607609343	45
PW50_6	0.629080883	46
PW50_b_1	0.642131196	47
PW50_5	0.644274197	48
PW50_f_4	0.648068294	49
PW50_f_5	0.649635886	50
TW_b_5	0.658863742	51
PW10_b_6	0.662370812	52
PW10_2	0.665166149	53
PW50_f_6	0.681404715	54
SW50_2	0.699880070	55
SW50_f_4	0.770432952	56
PW10_f_1	0.800118485	57
PW10_5	0.809716734	58
PW50_2	0.814139739	59
TW_b_2	0.826446544	60
PW10_1	0.832241269	61
PW50_1	0.850525679	62
PW50_b_5	0.854756557	63
TW_5	0.857729207	64
TW_f_4	0.883116975	65
SW50_6	0.891977081	66
PW10_f_5	0.918877035	67
TW_6	0.925461128	68
PW50_3	0.926708848	69
SW50_b_3	0.931775928	70
PW50_4	0.940841176	71
PW10_f_2	0.982752305	72

A.3 Support Information for Results

Table A12. Summary descriptive statistics of soil electrical conductivity (EC) at day 8, 36, and 106 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Water Treatment	Mean	Standard Deviation	N		
				Tested	Missing	Total
Day 8	TW	0.138	0.035	13	5	18
	SW50	0.130	0.032	7	11	18
	PW10	0.134	0.041	7	11	18
	PW50	0.130	0.032	9	9	18
	Total	0.134	0.034	36	36	72
Day 36	TW	0.069	0.009	4	14	18
	SW50	0.691	0.215	11	7	18
	PW10	0.192	0.066	12	6	18
	PW50	1.144	0.270	8	10	18
	Total	0.552	0.438	35	37	72
Day 106	TW	0.132	0.020	15	3	18
	SW50	1.210	0.258	9	9	18
	PW10	0.327	0.069	9	9	18
	PW50	1.004	0.225	9	9	18
	Total	0.592	0.488	42	30	72
Total	TW	0.126	0.034	32	22	54
	SW50	0.719	0.464	27	27	54
	PW10	0.221	0.098	28	26	54
	PW50	0.745	0.498	26	28	54
	Total	0.434	0.434	113	103	216

Table A13. ANOVA results of soil electrical conductivity (EC) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water

Constituent	ANOVA p-value
Water Treatment	0.000

Table A14. Tukey pairwise comparison of soil electrical conductivity (EC) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.056
	PW50	0.000
SW50	TW	0.000
	PW10	0.000
	PW50	0.911
PW10	TW	0.056
	SW50	0.000
	PW50	0.000
PW50	TW	0.000
	SW50	0.911
	PW10	0.000

Table A15. Tukey mean separation test of soil electrical conductivity (EC) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	0.12638	A
SW50	0.71863	B
PW10	0.22079	A
PW50	0.74454	B

Table A16. Soil electrical conductivity (EC) at day 8, 36, and 106 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	**Soil Electrical Conductivity (deciSiemens per meter)**		
	Day 8	**Day 36**	**Day 106**
TW	0.091	Not available	0.135
TW	0.223	Not available	0.153
TW	0.120	Not available	0.128
TW	Not available	0.055	0.175
TW	Not available	0.070	0.105
TW	0.157	Not available	0.148
TW	0.110	Not available	0.108
TW	0.137	Not available	0.124
TW	0.115	Not available	0.149
TW	0.148	Not available	0.114
TW	Not available	0.75	0.116
TW	0.185	Not available	0.147
TW	Not available	Not available	Not available
TW	Not available	0.75	0.135
TW	0.137	Not available	Not available
TW	0.136	Not available	0.127
TW	0.109	Not available	Not available
TW	0.123	Not available	0.114
SW50	Not available	0.450	Not available
SW50	0.128	Not available	1.342
SW50	Not available	0.700	Not available
SW50	0.084	Not available	Not available
SW50	0.180	Not available	1.060
SW50	Not available	0.750	1.593
SW50	Not available	1.150	0.813
SW50	0.111	Not available	Not available
SW50	0.143	Not available	Not available
SW50	Not available	0.400	1.040
SW50	Not available	0.850	1.572
SW50	Not available	0.600	Not available
SW50	0.111	Not available	1.153
SW50	Not available	0.450	1.060
SW50	Not available	0.750	Not available
SW50	Not available	0.800	Not available
SW50	0.156	Not available	Not available
SW50	Not available	0.700	1.257
PW10	Not available	0.325	0.323
PW10	Not available	0.125	Not available
PW10	Not available	0.210	0.273
PW10	0.088	Not available	Not available

PW10	Not available	0.155	Not available
PW10	0.093	Not available	0.303
PW10	Not available	0.140	Not available
PW10	0.134	Not available	Not available
PW10	Not available	0.175	0.330
PW10	0.115	Not available	Not available
PW10	Not available	0.100	0.257
PW10	0.164	Not available	0.470
PW10	0.204	Not available	0.307
PW10	0.141	0.190	Not available
PW10	Not available	0.300	Not available
PW10	Not available	0.170	0.273
PW10	Not available	0.210	0.407
PW10	Not available	0.200	Not available
PW50	0.107	Not available	Not available
PW50	Not available	1.100	Not available
PW50	Not available	1.600	Not available
PW50	Not available	Not available	0.997
PW50	Not available	1.150	1.523
PW50	0.138	Not available	0.960
PW50	0.159	Not available	Not available
PW50	Not available	0.900	0.813
PW50	Not available	1.000	0.997
PW50	0.090	Not available	0.840
PW50	0.180	Not available	Not available
PW50	Not available	1.350	Not available
PW50	0.123	Not available	Not available
PW50	0.085	Not available	0.813
PW50	Not available	1.300	Not available
PW50	0.159	Not available	Not available
PW50	Not available	0.750	1.167
PW50	0.130	Not available	0.927

Table A17. Feekes Scale of experimental wheat plant development over time for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Feekes Scale of Plant Development			
	TW	SW50	PW10	PW50
1	0	0	0	0
3	0	0	0	0
6	1	1	1	1
8	1	1	1	1
10	1	1	1	1
13	2	2	2	2
15	3	3	3	3
17	3	3	3	3
21	5	5	5	5
23	5	6	5	5
25	6	6	5	5
27	7	7	6	5
29	10	10	10	5
31	10	10	10	6
34	10	10	10	6
36	10	10.1	10	6
38	10.1	10.1	10.1	10
41	10.1	10.1	10.1	10.1
43	10.1	10.1	10.1	10.1
45	10.5	10.5	10.5	10.1
48	10.5	10.5	10.5	10.1
50	10.5	10.5	10.5	10.1
52	10.5	10.5	10.5	10.1
55	10.5	10.5	10.5	10.1
57	10.5	10.5	10.5	11
59	10.5	10.5	10.5	11
62	10.5	10.5	10.5	11
64	10.5	10.5	10.5	11
66	10.5	10.5	10.5	11
69	10.5	10.5	10.5	11
71	10.5	10.5	10.5	11
73	11	11	11	11

Table A18. Summary descriptive statistics of photosystem II quantum yield (Phi2) at day 30 and 45 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Water Treatment	Mean	Standard Deviation	N		
				Tested	Missing	Total
Day 30	TW	0.534	0.062	6	0	6
	SW50	0.525	0.048	6	0	6
	PW10	0.556	0.047	6	0	6
	PW50	0.541	0.065	6	0	6
	Total	0.539	0.053	24	0	24
Day 45	TW	0.567	0.078	6	0	6
	SW50	0.569	0.061	6	0	6
	PW10	0.568	0.022	6	0	6
	PW50	0.105	0.059	6	0	6
	Total	0.452	0.212	24	0	24
Total	TW	0.550	0.069	12	0	12
	SW50	0.547	0.057	12	0	12
	PW10	0.562	0.035	12	0	12
	PW50	0.323	0.235	12	0	12
	Total	0.496	0.159	48	0	48

Table A19. Summary descriptive statistics of non-photochemical exciton quenching (PhiNPQ) at day 30 and 45 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Water Treatment	Mean	Standard Deviation	N		
				Tested	Missing	Total
Day 30	TW	0.132	0.012	6	0	6
	SW50	0.138	0.017	6	0	6
	PW10	0.139	0.028	6	0	6
	PW50	0.133	0.052	6	0	6
	Total	0.135	0.029	24	0	24
Day 45	TW	0.109	0.012	6	0	6
	SW50	0.123	0.018	6	0	6
	PW10	0.155	0.036	6	0	6
	PW50	0.836	0.088	6	0	6
	Total	0.306	0.317	24	0	24
Total	TW	0.121	0.017	12	0	12
	SW50	0.130	0.018	12	0	12
	PW10	0.147	0.032	12	0	12
	PW50	0.485	0.374	12	0	12
	Total	0.221	0.239	48	0	48

Table A20. Summary descriptive statistics of quantum yield of unregulated non-photochemical losses (PhiNO) at day 30 and 45 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Water Treatment	Mean	Standard Deviation	N		
				Tested	Missing	Total
Day 30	TW	0.335	0.057	6	0	6
	SW50	0.338	0.040	6	0	6
	PW10	0.305	0.038	6	0	6
	PW50	0.326	0.045	6	0	6
	Total	0.326	0.045	24	0	24
Day 45	TW	0.324	0.066	6	0	6
	SW50	0.308	0.048	6	0	6
	PW10	0.277	0.026	6	0	6
	PW50	0.059	0.031	6	0	6
	Total	0.242	0.117	24	0	24
Total	TW	0.329	0.059	12	0	12
	SW50	0.323	0.045	12	0	12
	PW10	0.291	0.034	12	0	12
	PW50	0.192	0.144	12	0	12
	Total	0.284	0.098	48	0	48

Table A21. Mean of fraction of incoming energy for all four water treatments at day 30: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Phi2	PhiNPQ	PhiNO
TW	0.534 ± 0.049	0.132 ± 0.010	0.335 ± 0.046
SW50	0.525 ± 0.038	0.138 ± 0.014	0.338 ± 0.032
PW10	0.556 ± 0.037	0.139 ± 0.022	0.305 ± 0.030
PW50	0.541 ± 0.052	0.133 ± 0.041	0.326 ± 0.036

Table A22. Mean of fraction of incoming energy for all four water treatments at day 45: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Phi2	PhiNPQ	PhiNO
TW	0.567 ± 0.062	0.109 ± 0.010	0.324 ± 0.052
SW50	0.569 ± 0.049	0.123 ± 0.014	0.308 ± 0.038
PW10	0.568 ± 0.018	0.155 ± 0.029	0.277 ± 0.021
PW50	0.105 ± 0.047	0.836 ± 0.071	0.059 ± 0.024

Table A23. ANOVA results of photosystem II quantum yield (Phi2), non-photochemical exciton quenching (PhiNPQ), and quantum yield of unregulated non-photochemical losses (PhiNO) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	Variable	ANOVA p-value
Water Treatment	Phi2	0.000
	PhiNPQ	0.000
	PhiNO	0.000

Table A24. Tukey pairwise comparison of photosystem II quantum yield (Phi2) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.999
	PW10	0.955
	PW50	0.000
SW50	TW	0.999
	PW10	0.907
	PW50	0.000
PW10	TW	0.955
	SW50	0.907
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A25. Tukey mean separation test of photosystem II quantum yield (Phi2) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	0.55033	A
SW50	0.54667	A
PW10	0.56233	A
PW50	0.32300	B

Table A26. Tukey pairwise comparison of non-photochemical exciton quenching (PhiNPQ) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.934
	PW10	0.402
	PW50	0.000
SW50	TW	0.934
	PW10	0.758
	PW50	0.000
PW10	TW	0.402
	SW50	0.758
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A27. Tukey mean separation test of non-photochemical exciton quenching (PhiNPQ) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	0.12050	A
SW50	0.13042	A
PW10	0.14692	A
PW50	0.48458	B

Table A28. Tukey pairwise comparison of quantum yield of unregulated non-photochemical losses (PhiNO) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.987
	PW10	0.182
	PW50	0.000
SW50	TW	0.987
	PW10	0.323
	PW50	0.000
PW10	TW	0.182
	SW50	0.323
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A29. Tukey mean separation test of quantum yield of unregulated non-photochemical losses (PhiNO) for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	0.32925	A
SW50	0.32300	A
PW10	0.29092	A
PW50	0.19233	B

Table A30. Photosystem II quantum yield (Phi2) at day 30 and 45 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Photosystem II Quantum Yield (Phi2)	
	Day 30	Day 45
TW	0.513	0.647
TW	0.571	0.571
TW	0.477	0.610
TW	0.605	0.451
TW	0.453	0.497
TW	0.582	0.627
SW50	0.435	0.557
SW50	0.544	0.558
SW50	0.552	0.466
SW50	0.538	0.634
SW50	0.512	0.570
SW50	0.567	0.627
PW10	0.535	0.551
PW10	0.545	0.548
PW10	0.520	0.595
PW10	0.518	0.591
PW10	0.581	0.547
PW10	0.639	0.578
PW50	0.600	0.010
PW50	0.522	0.108
PW50	0.463	0.075
PW50	0.566	0.149
PW50	0.620	0.109
PW50	0.475	0.179

Table A31. Non-photochemical exciton quenching (PhiNPQ) at day 30 and 45 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Non-photochemical Exciton Quenching (PhiNPQ)	
	Day 30	Day 45
TW	0.143	0.096
TW	0.134	0.110
TW	0.135	0.098
TW	0.135	0.125
TW	0.136	0.123
TW	0.108	0.103
SW50	0.164	0.129
SW50	0.135	0.135
SW50	0.147	0.140
SW50	0.115	0.090
SW50	0.126	0.127
SW50	0.138	0.119
PW10	0.169	0.212
PW10	0.152	0.148
PW10	0.147	0.117
PW10	0.119	0.155
PW10	0.154	0.177
PW10	0.093	0.120
PW50	0.118	0.982
PW50	0.104	0.812
PW50	0.150	0.888
PW50	0.109	0.771
PW50	0.087	0.830
PW50	0.229	0.735

Table A32. Quantum yield of unregulated non-photochemical losses (PhiNO) at day 30 and 45 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	**Quantum Yield of Unregulated Non-photochemical Losses (PhiNO)**	
	Day 30	**Day 45**
TW	0.344	0.257
TW	0.295	0.319
TW	0.388	0.292
TW	0.260	0.424
TW	0.411	0.380
TW	0.310	0.271
SW50	0.401	0.314
SW50	0.322	0.307
SW50	0.301	0.394
SW50	0.347	0.276
SW50	0.362	0.303
SW50	0.295	0.254
PW10	0.297	0.237
PW10	0.303	0.304
PW10	0.333	0.288
PW10	0.363	0.255
PW10	0.265	0.276
PW10	0.269	0.301
PW50	0.282	0.008
PW50	0.374	0.080
PW50	0.387	0.037
PW50	0.324	0.079
PW50	0.293	0.062
PW50	0.296	0.086

Table A33. Summary descriptive statistics of height of tallest tiller for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Standard Deviation	N		
			Tested	Missing	Total
TW	43.67	4.457	6	0	6
SW50	40.92	4.944	6	0	6
PW10	46.50	2.345	6	0	6
PW50	26.83	6.853	6	0	6
Total	39.48	8.985	24	0	24

Table A34. ANOVA results of height of tallest tiller for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A35. Tukey pairwise comparison of height of tallest tiller for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.921
	PW10	0.606
	PW50	0.000
SW50	TW	0.921
	PW10	0.301
	PW50	0.000
PW10	TW	0.606
	SW50	0.301
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A36. Tukey mean separation test of height of tallest tiller for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	42.471	A
SW50	41.375	A
PW10	44.389	A
PW50	26.000	B

Table A37. Height of tallest tiller for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Height of Tallest Tiller (mm)
TW	49
TW	42
TW	41
TW	49
TW	38
TW	43
SW50	44
SW50	32
SW50	41
SW50	44.5
SW50	45
SW50	39
PW10	47
PW10	47
PW10	42
PW10	47
PW10	47
PW10	49
PW50	34
PW50	25
PW50	28
PW50	18
PW50	21
PW50	35

Table A38. Summary descriptive statistics of tiller number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Standard Deviation	N		
			Tested	Missing	Total
TW	15.83	1.722	6	0	6
SW50	5.830	1.941	6	0	6
PW10	10.00	2.898	6	0	6
PW50	3.000	0.000	6	0	6
Total	8.670	5.256	24	0	24

Table A39. ANOVA results of tiller number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A40. Tukey pairwise comparison of tiller number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.000
	PW50	0.000
SW50	TW	0.000
	PW10	0.042
	PW50	0.000
PW10	TW	0.000
	SW50	0.042
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A41. Tukey mean separation test of tiller number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	15.65	A
SW50	6.83	C
PW10	8.72	B
PW50	3.00	D

Table A42. Summary descriptive statistics of grain head number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Standard Deviation	N		
			Tested	Missing	Total
TW	8.670	1.506	6	0	6
SW50	3.500	1.049	6	0	6
PW10	3.830	0.753	6	0	6
PW50	1.170	0.983	6	0	6
Total	4.290	2.971	24	0	24

Table A43. ANOVA results of grain head number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A44. Tukey pairwise comparison of grain head number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.000
	PW50	0.000
SW50	TW	0.000
	PW10	0.812
	PW50	0.000
PW10	TW	0.000
	SW50	0.812
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A45. Tukey mean separation test of grain head number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	9.18	A
SW50	3.44	B
PW10	3.78	B
PW50	1.17	C

Table A46. Tiller number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Tiller Number of Wheat Plants
TW	14
TW	17
TW	14
TW	17
TW	15
TW	18
SW50	8
SW50	5
SW50	3
SW50	6
SW50	8
SW50	5
PW10	12
PW10	14
PW10	6
PW10	8
PW10	9
PW10	11
PW50	3
PW50	3
PW50	3
PW50	3
PW50	3
PW50	3

Table A47. Grain head number of wheat plants for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Grain Head Number of Wheat Plants
TW	8
TW	11
TW	8
TW	7
TW	8
TW	10
SW50	4
SW50	3
SW50	2
SW50	4
SW50	5
SW50	3
PW10	5
PW10	4
PW10	4
PW10	3
PW10	3
PW10	4
PW50	2
PW50	0
PW50	0
PW50	1
PW50	2
PW50	2

Table A48. Summary descriptive statistics of weight of wheat plants per pot for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Standard Deviation	N		
			Tested	Missing	Total
TW	10.57	2.265	6	0	6
SW50	4.153	2.161	6	0	6
PW10	4.820	0.733	6	0	6
PW50	0.625	0.223	6	0	6
Total	5.043	3.942	24	0	24

Table A49. ANOVA results of weight of wheat plants per pot for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A50. Tukey pairwise comparison of weight of wheat plants per pot for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.000
	PW50	0.000
SW50	TW	0.000
	PW10	1.000
	PW50	0.000
PW10	TW	0.000
	SW50	1.000
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A51. Tukey mean separation test of weight of wheat plants per pot for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	10.3429	A
SW50	4.2456	B
PW10	4.2844	B
PW50	0.5894	C

Table A52. Summary descriptive statistics of weight of wheat plants per grain head for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Standard Deviation	N		
			Tested	Missing	Total
TW	1.221	0.152	6	0	6
SW50	1.115	0.361	6	0	6
PW10	1.294	0.306	6	0	6
PW50	0.449	0.120	4	2	6
Total	1.071	0.395	22	2	24

Table A53. ANOVA results of weight of wheat plants per grain head for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A54. Tukey pairwise comparison of weight of wheat plants per grain head for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.691
	PW10	0.998
	PW50	0.000
SW50	TW	0.691
	PW10	0.788
	PW50	0.000
PW10	TW	0.998
	SW50	0.788
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A55. Tukey mean separation test of weight of wheat plants per grain head for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	1.137	A
SW50	1.226	A
PW10	1.152	A
PW50	0.467	B

Table A56. Weight of wheat plants per pot for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Weight of Wheat Plant per Pot (g)
TW	9.67
TW	12.2
TW	9.43
TW	9.52
TW	8.24
TW	14.4
SW50	6.56
SW50	3.21
SW50	1.13
SW50	4.78
SW50	6.45
SW50	2.79
PW10	5.19
PW10	5.73
PW10	3.94
PW10	4.09
PW10	5.41
PW10	4.56
PW50	0.73
PW50	0.29
PW50	0.48
PW50	0.61
PW50	0.70
PW50	0.94

Table A57. Weight of wheat plants per grain head for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Weight of Wheat Plants per Grain Head (g)
TW	1.21
TW	1.11
TW	1.18
TW	1.36
TW	1.03
TW	1.44
SW50	1.64
SW50	1.07
SW50	0.57
SW50	1.20
SW50	1.29
SW50	0.93
PW10	1.04
PW10	1.43
PW10	0.99
PW10	1.36
PW10	1.80
PW10	1.14
PW50	0.37
PW50	Not available
PW50	Not available
PW50	0.61
PW50	0.35
PW50	0.47

Table A58. Summary descriptive statistics of bacteria pathogenesis-related-1 (PR-1) expression at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Water Treatment	Mean	Standard Deviation	N		
				Tested	Missing	Total
Day 1	TW	14.10	0.907	9	0	9
	SW50	11.69	0.668	9	0	9
	PW10	9.351	1.050	9	0	9
	PW50	6.914	0.867	9	0	9
	Total	10.51	2.837	36	0	36
Day 3	TW	18.55	0.506	9	0	9
	SW50	15.10	0.598	9	0	9
	PW10	11.68	1.017	9	0	9
	PW50	8.703	0.485	9	0	9
	Total	13.51	3.798	36	0	36
Day 7	TW	19.38	0.758	9	0	9
	SW50	16.14	0.323	9	0	9
	PW10	12.25	0.936	9	0	9
	PW50	9.108	0.720	9	0	9
	Total	14.22	3.997	36	0	36
Total	TW	17.34	2.469	27	0	27
	SW50	14.31	2.005	27	0	27
	PW10	11.09	1.598	27	0	27
	PW50	8.242	1.186	27	0	27
	Total	12.75	3.896	108	0	108

Table A59. Summary descriptive statistics of bacteria pathogenesis-related-5 (PR-5) expression at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Water Treatment	Mean	Standard Deviation	N		
				Tested	Missing	Total
Day 1	TW	36.41	1.346	9	0	9
	SW50	32.05	0.985	9	0	9
	PW10	26.57	1.493	9	0	9
	PW50	19.88	1.882	9	0	9
	Total	28.73	6.423	36	0	36
Day 3	TW	42.54	2.008	9	0	9
	SW50	38.49	1.842	9	0	9
	PW10	33.75	2.724	9	0	9
	PW50	29.42	3.211	9	0	9
	Total	36.05	5.548	36	0	36
Day 7	TW	29.72	1.912	9	0	9
	SW50	24.32	2.522	9	0	9
	PW10	21.20	1.699	9	0	9
	PW50	20.50	1.422	9	0	9
	Total	23.94	4.121	36	0	36
Total	TW	36.22	5.604	27	0	27
	SW50	31.62	6.176	27	0	27
	PW10	27.17	5.593	27	0	27
	PW50	23.27	4.959	27	0	27
	Total	29.57	7.360	108	0	108

Table A60. Summary descriptive statistics of fungi pathogenesis-related-1 (PR-1) expression at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	**Water Treatment**	**Mean**	**Standard Deviation**	**N**		
				Tested	**Missing**	**Total**
Day 1	TW	4.903	0.821	9	0	9
	SW50	4.220	0.829	9	0	9
	PW10	4.311	0.799	9	0	9
	PW50	3.689	0.546	9	0	9
	Total	4.281	0.846	36	0	36
Day 3	TW	8.522	1.377	9	0	9
	SW50	7.800	0.577	9	0	9
	PW10	7.089	0.586	9	0	9
	PW50	6.667	0.831	9	0	9
	Total	7.519	1.122	36	0	36
Day 7	TW	10.18	1.304	9	0	9
	SW50	9.789	1.818	9	0	9
	PW10	8.233	0.700	9	0	9
	PW50	7.911	0.672	9	0	9
	Total	9.028	1.527	36	0	36
Total	TW	7.868	2.520	27	0	27
	SW50	7.270	2.616	27	0	27
	PW10	6.544	1.808	27	0	27
	PW50	6.089	1.924	27	0	27
	Total	6.943	2.317	108	0	108

Table A61. Summary descriptive statistics of fungi pathogenesis-related-5 (PR-5) expression at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	**Water Treatment**	**Mean**	**Standard Deviation**	**N**		
				Tested	**Missing**	**Total**
Day 1	TW	11.87	0.892	9	0	9
	SW50	9.406	0.844	9	0	9
	PW10	7.888	1.154	9	0	9
	PW50	6.299	1.067	9	0	9
	Total	8.866	2.291	36	0	36
Day 3	TW	17.44	1.229	9	0	9
	SW50	14.83	1.003	9	0	9
	PW10	11.25	1.456	9	0	9
	PW50	9.300	0.923	9	0	9
	Total	13.20	3.384	36	0	36
Day 7	TW	19.14	1.146	9	0	9
	SW50	16.99	1.408	9	0	9
	PW10	12.57	1.015	9	0	9
	PW50	10.92	1.527	9	0	9
	Total	14.91	3.570	36	0	36
Total	TW	16.15	3.335	27	0	27
	SW50	13.74	3.422	27	0	27
	PW10	10.57	2.325	27	0	27
	PW50	8.840	2.267	27	0	27
	Total	12.33	4.019	108	0	108

Table A62. ANOVA results of bacteria pathogenesis-related-1 (PR-1) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A63. Tukey pairwise comparison of bacteria pathogenesis-related-1 (PR-1) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.000
	PW50	0.000
SW50	TW	0.000
	PW10	0.000
	PW50	0.000
PW10	TW	0.000
	SW50	0.000
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A64. Tukey mean separation test of bacteria pathogenesis-related-1 (PR-1) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	17.3411	A
SW50	14.3119	B
PW10	11.0907	C
PW50	8.24190	D

Table A65. ANOVA results of bacteria pathogenesis-related-5 (PR-5) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A66. Tukey pairwise comparison of bacteria pathogenesis-related-5 (PR-5) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.000
	PW50	0.000
SW50	TW	0.000
	PW10	0.000
	PW50	0.000
PW10	TW	0.000
	SW50	0.000
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A67. Tukey mean separation test of bacteria pathogenesis-related-5 (PR-5) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	36.2211	A
SW50	31.6219	B
PW10	27.1748	C
PW50	23.2685	D

Table A68. ANOVA results of fungi pathogenesis-related-1 (PR-1) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A69. Tukey pairwise comparison of fungi pathogenesis-related-1 (PR-1) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.119
	PW10	0.000
	PW50	0.000
SW50	TW	0.119
	PW10	0.000
	PW50	0.000
PW10	TW	0.000
	SW50	0.038
	PW50	0.325
PW50	TW	0.000
	SW50	0.000
	PW10	0.325

Table A70. Tukey mean separation test of fungi pathogenesis-related-1 (PR-1) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	7.8678	A
SW50	7.2696	A
PW10	6.5444	B
PW50	6.0889	B

Table A71. ANOVA results of fungi pathogenesis-related-5 (PR-5) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A72. Tukey pairwise comparison of fungi pathogenesis-related-5 (PR-5) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.000
	PW50	0.000
SW50	TW	0.000
	PW10	0.000
	PW50	0.000
PW10	TW	0.000
	SW50	0.000
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A73. Tukey mean separation test of fungi pathogenesis-related-5 (PR-5) expression for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	16.1515	A
SW50	13.7426	B
PW10	10.5670	C
PW50	8.84000	D

Table A74. Bacteria relative change pathogenesis-related-1 (PR-1) at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Bacteria Relative Change Pathogenesis-related Genes-1			
	TW	SW50	PW10	PW50
1	14.2	12.1	10.1	6.12
1	12.3	11.2	11.1	7.12
1	13.5	12.1	7.98	6.22
1	15.2	10.2	10.1	8.34
1	15.0	12.2	8.78	6.21
1	13.5	11.2	8.78	6.67
1	14.5	12.0	10.1	6.45
1	14.0	12.0	8.92	8.34
1	14.8	12.1	8.24	6.76
3	18.2	15.2	11.3	8.30
3	19.3	15.3	12.3	7.80
3	18.9	14.2	13.4	9.12
3	17.9	15.9	10.2	9.23
3	19.2	15.3	10.2	8.99
3	18.5	14.3	11.6	8.91
3	18.3	15.3	12.0	9.00
3	18.7	14.6	12.0	8.76
3	18.0	15.8	12.0	8.22
7	19.8	16.3	11.0	10.1
7	20.0	16.8	12.0	9.12
7	19.3	16.2	14.2	8.12
7	18.3	16.3	11.5	7.89
7	19.1	16.0	12.2	9.11
7	20.2	15.7	11.9	9.20
7	19.9	15.9	12.1	9.90
7	19.7	15.9	13.1	9.22
7	18.1	16.2	12.3	9.30

Table A75. Mean of bacteria relative change pathogenesis-related-1 (PR-1) at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	TW	SW50	PW10	PW50
1	14.10 ± 0.592	11.69 ± 0.436	9.351 ± 0.686	6.914 ± 0.567
3	18.55 ± 0.331	15.10 ± 0.391	11.68 ± 0.665	8.703 ± 0.317
7	19.38 ± 0.495	16.14 ± 0.211	12.25 ± 0.611	9.108 ± 0.471

Table A 76. Bacteria relative change pathogenesis-related-5 (PR-5) at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	**Bacteria Relative Change Pathogenesis-related Genes-5**			
	TW	**SW50**	**PW10**	**PW50**
1	36.4	32.5	25.2	20.1
1	37.9	33.4	28.2	24.2
1	35.8	33.2	28.3	19.1
1	37.8	32.2	25.3	21.1
1	37.8	30.3	27.2	18.2
1	34.6	31.2	26.7	19.5
1	35.6	31.6	28.2	17.9
1	34.6	32.5	25.3	19.2
1	37.2	31.6	24.6	19.7
3	41.2	40.3	32.2	36.3
3	40.0	41.2	30.2	32.2
3	43.3	38.7	31.4	29.3
3	42.2	37.9	35.2	29.3
3	41.5	38.2	32.2	25.6
3	46.2	37.9	34.4	26.2
3	41.1	36.4	38.8	28.1
3	42.3	40.2	36.5	29.1
3	45.1	35.6	32.9	28.6
7	30.2	22.5	22.2	20.2
7	30.3	25.2	18.6	21.1
7	34.1	22.1	21.8	20.3
7	30.1	19.9	18.2	18.9
7	28.3	23.4	21.3	18.6
7	28.9	26.2	23.2	19.2
7	29.6	25.2	22.3	22.3
7	27.7	27.7	21.1	21.5
7	28.2	26.6	22.1	22.4

Table A77. Mean of bacteria relative change pathogenesis-related-5 (PR-5) at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	**TW**	**SW50**	**PW10**	**PW50**
1	36.41 ± 0.879	32.05 ± 0.644	26.57 ± 0.975	19.88 ± 1.229
3	42.54 ± 1.312	38.49 ± 1.204	33.75 ± 1.780	29.42 ± 2.098
7	29.72 ± 1.249	24.32 ± 1.648	21.20 ± 1.110	20.50 ± 0.9293

Table A78. Fungi relative change pathogenesis-related-1 (PR-1) at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Fungi Relative Change Pathogenesis-related Genes-1			
	TW	SW50	PW10	PW50
1	5.2	4.2	3.1	3.2
1	4.6	3.3	3.9	3.9
1	5.1	3.5	3.6	3.3
1	6.1	5.2	4.5	3.9
1	5.0	4.2	5.9	3.1
1	5.0	3.9	4.2	4.5
1	3.0	3.2	4.9	4.2
1	5.0	5.3	4.2	4.1
1	5.1	5.1	4.5	3.0
3	8.1	7.4	6.7	6.2
3	6.4	8.1	7.2	5.8
3	6.9	7.9	7.8	5.7
3	7.6	8.3	6.9	7.9
3	8.9	8.9	6.6	7.1
3	9.2	7.5	6.1	6.2
3	10.6	7.2	7.1	6.2
3	9.8	7.8	7.5	7.1
3	9.2	7.1	7.9	7.8
7	8.9	8.9	8.3	7.9
7	10.1	11.2	7.8	8.1
7	11.2	13.1	8.1	7.2
7	7.9	10.8	7.2	9.0
7	9.1	7.8	7.9	6.8
7	11.2	8.5	7.6	7.9
7	11.4	7.4	8.9	7.5
7	10.2	10.3	9.2	8.4
7	11.6	10.1	9.1	8.4

Table A79. Mean of fungi relative change pathogenesis-related-1 (PR-1) at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	TW	SW50	PW10	PW50
1	4.903 ± 0.536	4.220 ± 0.542	4.311 ± 0.522	3.689 ± 0.357
3	8.522 ± 0.900	7.800 ± 0.377	7.089 ± 0.383	6.667 ± 0.543
7	10.18 ± 0.852	9.789 ± 1.188	8.233 ± 0.457	7.911 ± 0.439

Table A80. Fungi relative change pathogenesis-related-5 (PR-5) at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Fungi Relative Change Pathogenesis-related Genes-5			
	TW	SW50	PW10	PW50
1	12.1	9.90	8.20	6.45
1	11.2	8.22	6.20	7.23
1	12.2	10.2	5.90	7.21
1	11.1	10.1	8.11	6.63
1	12.2	9.12	7.80	4.56
1	13.3	8.60	8.23	7.40
1	12.2	10.3	8.12	6.23
1	10.2	8.34	9.23	6.42
1	12.2	9.80	9.20	4.56
3	16.2	13.2	11.2	10.4
3	15.6	14.1	12.3	8.90
3	18.8	13.9	14.1	10.2
3	17.2	14.7	9.90	7.90
3	18.0	15.2	10.1	9.20
3	19.2	15.7	10.5	9.40
3	16.5	14.6	11.2	8.10
3	18.2	16.2	9.60	9.20
3	17.3	15.9	12.3	10.4
7	18.2	17.2	14.1	10.3
7	19.2	15.6	12.2	9.60
7	20.3	16.4	11.6	10.1
7	19.2	18.2	12.3	12.1
7	18.4	16.4	13.2	8.99
7	21.2	15.9	11.3	10.1
7	19.6	15.4	14.1	10.8
7	18.7	19.5	12.0	13.1
7	17.4	18.3	12.3	13.2

Table A81. Mean of fungi relative change pathogenesis-related-5 (PR-5) at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	TW	SW50	PW10	PW50
1	11.87 ± 0.583	9.406 ± 0.551	7.888 ± 0.754	6.299 ± 0.697
3	17.44 ± 0.803	14.83 ± 0.655	11.25 ± 0.951	9.300 ± 0.603
7	19.14 ± 0.749	16.99 ± 0.920	12.57 ± 0.663	10.92 ± 0.998

Table A82. Summary descriptive statistics of bacteria copy number at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Water Treatment	Mean	Standard Deviation	N		
				Tested	Missing	Total
Day 1	TW	792,038	22,205	6	0	6
	SW50	803,766	14,807	6	0	6
	PW10	838,649	38,272	6	0	6
	PW50	854,918	27,386	6	0	6
	Total	822,343	36,223	24	0	24
Day 3	TW	675,356	29,137	6	0	6
	SW50	783,723	7,822	6	0	6
	PW10	851,474	24,878	6	0	6
	PW50	863,438	26,511	6	0	6
	Total	793,498	79,398	24	0	24
Day 7	TW	893,691	22,276	6	0	6
	SW50	1,829,216	372,087	6	0	6
	PW10	2,968,073	101,719	6	0	6
	PW50	3,704,261	172,967	6	0	6
	Total	2,348,810	1,114,032	24	0	24
Total	TW	787,028	94.690	18	0	18
	SW50	1,138,902	541,439	18	0	18
	PW10	1,552,732	1,031,599	18	0	18
	PW50	1,807,539	1,383,411	18	0	18
	Total	1,321,550	969,385	72	0	72

Table A83. Summary descriptive statistics of fungi copy number at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	**Water Treatment**	**Mean**	**Standard Deviation**	**N**		
				Tested	**Missing**	**Total**
Day 1	TW	38,964	4,700	6	0	6
	SW50	44,386	12,155	6	0	6
	PW10	39,355	14,116	6	0	6
	PW50	31,387	1,593	6	0	6
	Total	38,523	10,162	24	0	24
Day 3	TW	86,993	8,163	6	0	6
	SW50	108,976	10,456	6	0	6
	PW10	167,218	17,553	6	0	6
	PW50	199,438	1,667	6	0	6
	Total	140,656	46,950	24	0	24
Day 7	TW	204,679	6,310	6	0	6
	SW50	293,987	29,860	6	0	6
	PW10	420,880	31,072	6	0	6
	PW50	485,282	51,112	6	0	6
	Total	351,207	115,720	24	0	24
Total	TW	110,212	71,898	18	0	18
	SW50	149,116	110,386	18	0	18
	PW10	209,151	164,473	18	0	18
	PW50	238,702	194,789	18	0	18
	Total	176,795	149,240	72	0	72

Table A84. ANOVA results of bacteria copy number for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A85. Tukey pairwise comparison of bacteria copy number for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.000
	PW50	0.000
SW50	TW	0.000
	PW10	0.000
	PW50	0.000
PW10	TW	0.000
	SW50	0.000
	PW50	0.000
PW50	TW	0.000
	SW50	0.000
	PW10	0.000

Table A86. Tukey mean separation test of bacteria copy number for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	787,028	A
SW50	1,138,902	B
PW10	1,552,732	C
PW50	1,807,539	D

Table A87. ANOVA results of fungi copy number for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Constituent	ANOVA p-value
Water Treatment	0.000

Table A88. Tukey pairwise comparison of fungi copy number for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment		Tukey p-value
TW	SW50	0.000
	PW10	0.000
	PW50	0.000
SW50	TW	0.000
	PW10	0.000
	PW50	0.000
PW10	TW	0.000
	SW50	0.000
	PW50	0.001
PW50	TW	0.000
	SW50	0.000
	PW10	0.001

Table A89. Tukey mean separation test of fungi copy number for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Water Treatment	Mean	Tukey Grouping
TW	110,212	A
SW50	149,116	B
PW10	209,151	C
PW50	238,702	D

Table A90. Bacteria copy numbers at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Bacteria Copy Number			
	TW	SW50	PW10	PW50
1	762,327	823,434	912,323	908,234
1	812,222	810,293	832,899	845,601
1	782,982	782,343	842,723	856,000
1	781,232	792,323	812,343	843,822
1	790,232	801,876	823,233	830,222
1	823,232	812,329	808,375	845,628
3	678,283	792,323	892,322	843,000
3	720,122	783,434	823,434	832,600
3	629,034	790,123	860,232	892,765
3	672,343	769,923	840,234	874,566
3	682,342	783,433	832,466	845,366
3	670,012	783,102	860,155	892,333
7	918,823	2,109,098	3,102,323	3,423,400
7	920,122	2,023,623	2,982,388	3,782,322
7	878,982	2,018,923	2,910,654	3,843,984
7	867,222	1,982,323	2,866,754	3,889,456
7	879,011	1,723,033	3,072,888	3,684,034
7	897,983	1,118,293	2,873,432	3,602,367

Table A91. Fungi copy numbers at day 1, 3, and 7 for all four water treatments: Tap Water Control (TW), Salt Water Control (SW50), 10% Produced Water (PW10), and 50% Produced Water (PW50)

Day	Fungi Copy Number			
	TW	SW50	PW10	PW50
1	34,323	65,323	65,233	30,909
1	43,435	45,634	34,102	30,978
1	39,823	34,546	23,943	30,898
1	36,645	45,234	43,526	34,545
1	45,323	45,345	34,763	30,989
1	34,234	30,232	34,563	30,003
3	87,834	93,222	156,233	198,923
3	98,934	100,932	153,234	201,222
3	78,734	112,019	198,832	197,233
3	89,888	112,123	156,343	200,012
3	76,734	123,237	162,345	201,232
3	89,834	112,323	176,323	198,008
7	198,239	236,776	382,309	434,543
7	212,221	288,989	398,083	467,234
7	198,234	301,232	410,234	429,993
7	203,423	310,293	420,011	498,923
7	203,746	320,195	462,320	560,763
7	212,212	306,434	452,323	520,234