

Safety and Technical Feasibility of Sustainable Reuse of Shale Gas Flowback and Produced Water after Advanced Treatment Aimed at Wheat Irrigation

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1 Date: Mar 12, 2022

2 Grains Metal Toxicity and Transcriptomic Analysis of  
3 Wheat Irrigated with Treated Shale Gas Flowback and  
4 Produced Water

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23   **ABSTRACT:**

24   Flowback and produced water (FPW) from shale gas extraction is proposed to be rationally  
25   reused for agricultural irrigation. The effects of FPW on the germination period, macroscopic  
26   growth, element enrichment, and grain gene expression of wheat were investigated upon  
27   dilution and advanced membrane treatment of the liquid stream. Compared to tap water,  
28   irrigation with treated FPW shortened the germination time, slightly improved the seed vigor  
29   index, and ensured germination rate. On the other hand, the biomass and grain yield of  
30   mature wheat irrigated with treated FPW and with FPW diluted to 5% groups decreased  
31   compared to tests deploying tap water. After a whole growth cycle of wheat cultivated with  
32   three kinds of irrigation water, the enrichment content of several heavy metals in soil was  
33   within the prescribed risk control value. Higher concentrations of nutrients, such as K, Ca,  
34   and Mg were enriched in mature wheat tissue irrigated with treated FPW. A total of 1973  
35   differentially expression genes were mainly related to binding, catalytic activity, cellular  
36   process, metabolic process, and cell part, more than half of which were up-regulated. These  
37   findings provide critical guidance for agricultural application of shale gas wastewater reuse  
38   from the perspective of plant uptake, environmental safety, and health risks.

39   **Keywords:** *shale gas wastewater, agricultural irrigation, wheat, heavy metal, transcriptomic*  
40   *analysis*

41   **Synopsis:** Treated shale gas flowback and produced water is deployed to irrigate wheat,  
42   providing critical guidance for rational and safe reuse of this stream.

43

## 44 INTRODUCTION

45 Rapid increase in global production of shale gas, an emerging resource of unconventional  
46 fossil fuels, comes with risks of local environmental pollution and health-related concerns.<sup>1-4</sup>  
47 The expansion of energy production from shale or unconventional plays results in massive  
48 consumption of freshwater for hydraulic fractures and in the co-production and potentially  
49 improper disposal of large volumes of high-salinity flowback and produced water (FPW).  
50 This trend has brought about environmental and economic challenges, exacerbated by water  
51 competition between different water-intensive sectors, and has caused health problems  
52 especially in densely populated areas and where water resources are scarce.<sup>5-9</sup> Management  
53 options for shale gas wastewater typically include injection of FPW into subsurface  
54 formations, which may however stimulate tight shale formations inducing earthquakes, and  
55 beneficial reuse strategies, such as for dust suppression, industrial power generation, and  
56 irrigation.<sup>10-12</sup>

57 FPW is composed by the natural formation water extracted from underground oil & gas  
58 resources, so-called produced water, and by the injection fluid utilized during the hydraulic  
59 fracturing process, so-called flowback water. Therefore, FPW usually contains high  
60 concentrations of total dissolved solids (TDS), organic matter (dissolved organic carbon;  
61 DOC), metals, hydrocarbons and other volatile compounds, some synthetic organic chemical  
62 additives, as well as some naturally occurring radioactive materials that may be present in the  
63 formation.<sup>13-16</sup> In recent years, the option of applying FPW directly or indirectly for  
64 agricultural irrigation has become a focus of growing discussion.<sup>17-19</sup> Small amounts of  
65 organic matter and micronutrient elements in municipal sewage and industrial wastewaters, at

66 times also including rare earth elements, have been shown to promote the growth of some  
67 algae strains or higher plants under certain circumstances. On the other hand, the salinity,  
68 alkalinity, and ionic composition of oilfield produced water may significantly hinder its reuse  
69 in agriculture, since these characteristics can cause nutrient imbalances, as well as osmotic  
70 and specific ion stress in plant cells.<sup>23-2520, 21</sup> Methods of reusing produced water with  
71 minimal TDS treatment or upon dilution for farmland irrigation were applied in Kern County  
72 (California) a few years ago, but the complexity of FPW composition has gradually aroused  
73 public concern.<sup>22</sup>

74 The available literature suggests that the concentration of TDS and DOC of irrigation  
75 water should be lower than 3500 mg/L and 5 mg/L, respectively, to maintain normal plant  
76 growth and biomass accumulation, and that the use of water with substance contents above the  
77 current guidelines to irrigate crops may lead to yield reduction and germination problems.<sup>26,</sup>  
78 <sup>27</sup> Previous studies on the irrigation-oriented reuse of oil & gas wastewater without treatment  
79 or upon simple dilution have shown that this approach has potential significant side effects on  
80 plant growth, yield, gene expression, and soil ecology. Applying different percentages of PW  
81 to irrigate wheat has been observed to lead to a decline in yield and a negative effect on  
82 physiological parameters, even when the wastewater was diluted by as much as 90% with tap  
83 water, also inducing an adverse impact on soil health and microbial diversity.<sup>17, 20, 27</sup> In  
84 addition, irrigating wheat with diluted PW can inhibit the expression of some  
85 disease-resistant genes in the crop.<sup>28</sup> Other studies reported that simulated produced water  
86 used to irrigate non-food biofuel crops resulted in significantly lower growth and worse  
87 physiological characteristics of the crops due to high PW salinity and excess organic

88 carbon.<sup>21</sup>

89 This study investigates the effects on the physiological and biochemical characteristics of  
90 wheat of the use of effluents from a shale gas wastewater treatment plant for irrigation. Also,  
91 differences in wheat grain gene expression as well as accumulation levels of various nutrient  
92 and toxic elements are evaluated. This study aims at providing a better understanding of the  
93 potential side-effects of FPW reuse for irrigation purposes to support improvements in this  
94 reuse strategy and promote water saving strategies.

## 95 MATERIALS AND METHODS

96 **Preparation of water, soil, and seeds.** The untreated shale gas flowback and produced  
97 water was obtained from a shale gas mining station in the Sichuan Basin. The treated stream  
98 was instead collected from China's first shale gas wastewater treatment plant located in  
99 Fuling, Chongqing. As shown in **Figure S1**, the treatment plant consists of a multi-stage  
100 pretreatment coupled with two-stage reverse osmosis filtration, for a daily processing  
101 capacity of 2400 m<sup>3</sup>. The treated effluent meets the first level standard of the Integrated  
102 Wastewater Discharge Standard (GB8978-1996).<sup>29</sup> The soil was excavated from the campus  
103 land of Sichuan University (Chengdu, China), then thoroughly mixed and crushed into  
104 smaller particles before the experiments. Wheat was selected as the crop of interest for this  
105 study.<sup>30</sup> The spring wheat (*Triticum aestivum*, Jinchun 6) seeds were purchased from Fu Yi  
106 Chun Seed Co., Ltd (HeJian, China), and stored in the dark and under dry conditions until  
107 use.

108 **Germination test of wheat seeds.** Wheat seeds were cultivated with three different kinds  
109 of irrigation water sources: a tap water (TW) control, treated flowback and produced water

(TFPW), and diluted untreated flowback and produced water (FPW5, equivalent to 95% FPW + 5% TW). Each culture group contained 30 wheat seeds, individually weighing  $0.045 \pm 0.005$  g, and was equally divided and transferred to five sterile petri dishes comprising a qualitative filter paper and 15 mL of corresponding water, which were both replaced every 24 h. All petri dishes were exposed to natural light at room temperature ( $\sim 20^\circ\text{C}$ )<sup>31</sup> for six days, and the number of wheat roots and sprouts were recorded daily to compare the germination rates between the control and the experimental groups. Seedlings height and root length were measured with a millimeter ruler. Seed vigor index (SVI) was calculated as described previously.<sup>32</sup>

**Greenhouse trial and harvesting.** After the 6-day incubation in petri dishes described above, six wheat seedlings were picked for each group, and the transplanted into an open plastic container, two-thirds of which were filled with the soil. The transplantation depth of all seedlings was about 3 ~ 4 cm, and the above-ground height was between 4 and 5 cm. All seedlings were cultured for 65 days and irrigated with the corresponding water every two days. Each container consumed approximately 300 mL of corresponding water at a time. The wheat growth of each treatment group was recorded every six days. Upon harvesting, all wheat seedlings were rinsed with tap water, washed with ultrapure water (ULUPURE, Chengdu, China) three times, then dried with an electric thermostatic drying oven (DHG-9070A, Shanghai) at  $60^\circ\text{C}$  until constant weight was achieved. The above-ground biomass, under-ground biomass, and grain yield of each plant were thus determined.

**Analysis for the irrigation water quality.** The TDS and conductivity were determined by an Ultrameter II 6PFC portable multifunctional meter (Myron L Company, Carlsbad, CA,

USA). The turbidity and UV absorbance were measured by a turbidimeter (2100Q, Hach Company, Loveland, CO, U.S.A.) and a UV–Vis spectrophotometry (Orion AquaMate 8000, Thermo Fisher Scientific, Inc., MA, USA), respectively. Samples were filtered through 0.45 µm polyethersulfone membrane filters and analyzed for TOC and TN by a TOC analyzer (TOC-L CPH, Shimadzu, Kyoto, Japan). In addition, quantitative analyses of ions and elements were performed with ion chromatography (Dionex ICS-1100, Thermo Fisher Scientific, Inc., MA, USA) and an inductively coupled plasma mass spectrometer (ICP-MS, NexION 1000G PerkinElmer, Inc., MA, USA).

**Soil and wheat tissues digestion and quantitative analysis.** Samples of broken soil (not used to cultivate plants) and rhizosphere soil (extracted from the vicinity of mature wheat roots in each treatment group) were collected and classified as raw soil, TFPW soil, TW soil, and FPW5 soil. These samples were mashed, then passed through a 100-mesh nylon sieve, and finally placed in the electric thermostatic drying oven until constant weight was reached. A total of 100 mg dry soil samples were thus placed in a polytetrafluoroethylene (PTFE) tube, mixed with 6 mL of nitric acid and 1 mL of hydrogen peroxide, and digested with a microwave dissolver (MDS-6G, SINEO, Shanghai, China). Then, samples of the resulting solutions were diluted to a volume of 50 mL using ultrapure water. Similarly, mature wheat tissues (root, stem, leaf, and grain) intended for quantitative analysis of macro and trace elements were also washed, dried, digested, and diluted. After digestion and dilution, all samples were filtered using a 0.22 µm PTFE filter and quantitatively assayed for elemental composition. For homogeneity and expedient comparison, the elements content in soil and plant tissue samples is presented as mass ratio (mg/kg).

**Transcriptome sequencing of wheat grains.** Before wheat matured completely, less than 0.1 g of fresh grains in each group was taken out, washed with DEPC water (RNase-free), frozen directly in liquid nitrogen for 30 min, and stored at  $-80^{\circ}\text{C}$  for RNA extraction. The total RNA was extracted from wheat grains using TRIzol® Reagent. Afterward, the transcriptome sequencing process was executed with the Illumina NovaSeq 6000 sequencer (Majorbio Bio-pharm Technology Co., Ltd, Shanghai, China). Details including RNA extraction, sequencing, read mapping, DEGs (differentially expressed genes), and functional enrichment are summarized in **Text S1 (SI)**. Raw sequencing data were stored in NCBI database under bioproject number PRJNA813217, with biosample numbers SAMN26455859, SAMN26455866, and SAMN26455867.

## **RESULTS AND DISCUSSION**

**Characterization of irrigation water.** The detailed water quality indicators of the three irrigation water sources are summarized in **Table 1**. The TFPW was within the limits set by China and by FAO standards with respect to maximum salinity and elements toxicity, while the other two waters did not fully respect the limits. In particular, strontium (Sr) in TW exceeded the limit (0.295 mg/L), being slightly higher than the concentration of 0.2 mg/L recommended by FAO guidelines. With respect to FPW5, several parameters, including TDS and As, were orders of magnitude higher compared to those in TFPW and TW. The relatively low concentration of heavy metals in TFPW might minimize accumulation of these elements in plants and soil. However, the contents of DOC, TN, Ag, and Sn in TFPW were the highest among the three types of irrigation water. The presence of these substances and that of specific organic compounds, such as benzenes and polycyclic aromatic hydrocarbons (PAHs),

may lead to detrimental effects on the crops, which requires further research.<sup>33, 34</sup>

**Effects on seeds germination and phenotypic analysis.** Results on the physiological conditions of wheat in different treatment groups during germination stages are summarized in **Figure 1**, including germination number, seedling height, root length, and seed vigor index. After 6-day germination culture, the germination number of wheat seeds exposed to TFPW was comparable to that of the TW control group, whereas the germination number was lower for FPW5 irrigation (**Figure 1A**). In detail, the germination rates of TFPW and TW groups exceeded 85%, while the FPW5 was only 70%. Therefore, irrigation with TFPW slightly shortened the germination period. Application of TFPW practically maintained the same seedling height and seed vigor observed for TW. On the contrary, application of FPW5 remarkably decreased ( $p < 0.05$ ) both seedling height and seed vigor index by roughly 20 and 45% (**Figures 1B and 1D**). **Figure 1C** presents the results obtained for the root length related to root numbers in the seed germination test. Even within the same irrigation group, the number and length of taproots were diverse. When considering samples with the same number of roots, the relationship between the average root length of the three groups was: TFPW>TW>FPW5. In summary, the use of treated shale gas wastewater was suitable in the germination stage of wheat seeds, consistent with previously reported leafy vegetables cultivation.<sup>31, 35</sup>

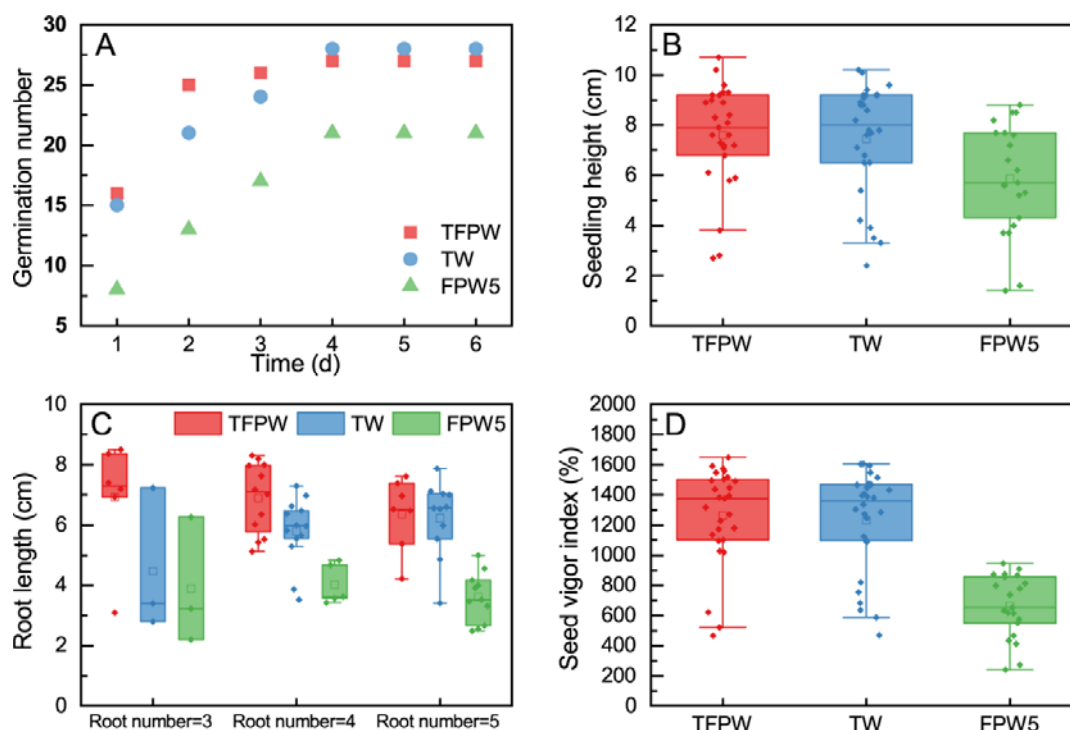
**Table 1.** Water quality of three irrigation waters (TFPW, TW, FPW5); national and international guidelines for irrigation.

Indicator	TFPW	TW	FPW5	Water quality for irrigation (China) <sup>36</sup>	Water quality for irrigation (UN) <sup>38</sup>
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Turbidity	0.20	0.23	0.67	/	/
Conductivity ( $\mu\text{S}/\text{cm}$ )	49	336	3224	/	3000
DOC (mg/L)	2.90	0.85	1.18	/	/
TN (mg/L)	5.136	0.854	3.456	/	/
UV <sub>254</sub> ( $\text{cm}^{-1}$ )	0.006	0.010	0.013	/	/
TDS (mg/L)	22	160	1660	1000	2000
Na (mg/L)	6.220	4.920	534.785	/	920
Ca (mg/L)	0.464	50.293	105.204	/	400
Mg (mg/L)	0.029	11.486	17.301	/	60
K (mg/L)	0.310	3.090	12.630	/	2
Ba (mg/L)	0.091	0.252	4.573	/	/
Sr (mg/L)	0.097	0.295	7.687	/	0.2
Fe ( $\mu\text{g}/\text{L}$ )	21.91	494.86	756.93	/	5000
Mn ( $\mu\text{g}/\text{L}$ )	0.053	0.704	37.705	300	200
Cu ( $\mu\text{g}/\text{L}$ )	0.609	0.663	9.493	500	200
Zn ( $\mu\text{g}/\text{L}$ )	57.55	404.15	393.70	2000	2000
Mo ( $\mu\text{g}/\text{L}$ )	0.5	1.3	1.7	500	10
Ni ( $\mu\text{g}/\text{L}$ )	0.088	4.750	6.100	100	200
Cr ( $\mu\text{g}/\text{L}$ )	10.45	11.05	11.15	100	100
Se ( $\mu\text{g}/\text{L}$ )	0.15	0.52	5.26	20	20
As ( $\mu\text{g}/\text{L}$ )	0.009	0.396	2.705	50	100
Pb ( $\mu\text{g}/\text{L}$ )	0.010	0.15	0.200	200	5000
Cd ( $\mu\text{g}/\text{L}$ )	NA	NA	0.005	10	10
Ag ( $\mu\text{g}/\text{L}$ )	0.008	0.004	0.005	/	/
Al ( $\mu\text{g}/\text{L}$ )	7.165	101.494	109.04	/	5000
Co ( $\mu\text{g}/\text{L}$ )	NA	0.061	0.089	1000	50
Sb ( $\mu\text{g}/\text{L}$ )	0.40	0.50	2.75	/	/
Sn ( $\mu\text{g}/\text{L}$ )	0.125	0.039	0.085	/	/
V ( $\mu\text{g}/\text{L}$ )	0.040	0.408	16.078	100	100
F <sup>-</sup> (mg/L)	0.039	0.172	0.444	2	1
Cl <sup>-</sup> (mg/L)	8.501	30.871	870.265	350	350
Br <sup>-</sup> (mg/L)	0.105	3.480	5.670	/	/
NO <sub>3</sub> <sup>-</sup> (mg/L)	0.145	5.330	7.160	/	10
SO <sub>4</sub> <sup>2-</sup> (mg/L)	0.584	44.803	48.209	/	960

197 Note: NA, not available.

198



**Figure 1.** Germination performance of wheat seeds in different irrigation groups: treated flowback and produced water (TFPW, red), tap water control (TW, blue), diluted FPW (FPW5, green). (A) Germination number; (B) seedlings height on the sixth day; (C) root length for samepls in which the number of seedling roots were 3, 4, and 5; (D) seed vigor index.

**Effects on wheat growth and harvesting.** After being transplanted, the wheat seedlings were cultivated for 65 days and then harvested (**Figures S2 and 2A**). The average wheat above-ground height of the three irrigatioj groups changed with time (**Figure 2B**). Irrigation with FPW5 slightly increased the above-ground height of wheat in the first 20 days, compared with the other two groups, but the wheat treated with TW grew faster at a later stage. The results suggest that the growth of wheat in soil followed an opposite trend compared to germination in the petri dishes, with respect to the use of the three irrigation

213 waters.

214 Irrigation with reused wastewater showed some stress effects. Compared to the control,

215 TFPW irrigation significantly ( $p < 0.05$ ) increased the root to shoot biomass ratio (R:S ratio).

216 Additionally, irrigation with TFPW and FPW5 significantly ( $p < 0.05$ ) decreased wheat total

217 biomass by approximately 23 % and 10 %, respectively (**Figure 2C**). The results were in line

218 with the reports suggesting that higher concentration of organic matter in the irrigation water

219 leads to smaller biomass.<sup>21</sup> As presented in **Figure 2D**, the total yield per spike in TFPW and

220 FPW5 groups decreased markedly ( $p < 0.05$ ) by 34 % and 17 %; the grain weight reduced

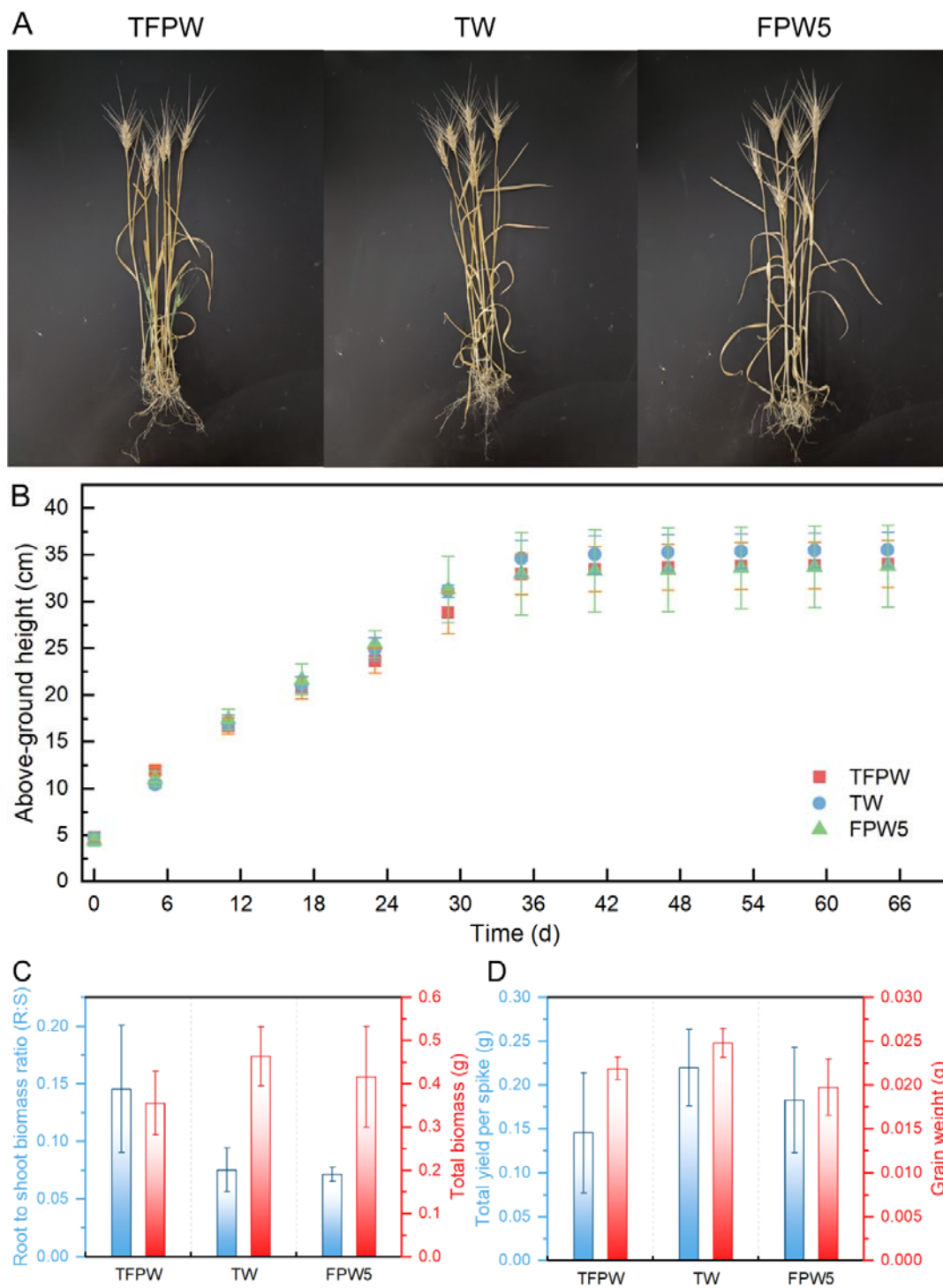
221 markedly by 12 % for TFPW irrigation ( $p < 0.05$ ) and 20 % for FPW5 irrigation ( $p < 0.05$ ).

222 Among the three irrigation waters, the TOC concentration of TFPW was the highest and

223 equal to 2.9 mg/L, which was however lower than the maximum value of 5 mg/L suggested

224 in the literature.<sup>21</sup>

225



**Figure 2.** (A) Representative images of mature wheat irrigated with different waters. (B) Above-ground height of wheat irrigated with different waters in soil. (C) Wheat total biomass and root-shoot ratio. (D) Total wheat yield per spike and average grain weight.

230

231     **Effect on elements accumulation in soil.** As displayed in **Figure S3**, fifteen elements in  
232 the soil, including crop nutrient elements, heavy metal elements, metalloid elements, were  
233 measured at the beginning and at the end of the irrigation experiment, with the relative  
234 standard deviations (RSDs) presented in **Table S1**. Compared to the raw soil, the contents of  
235 most elements in TFPW-irrigated, TW-irrigated, and FPW5-irrigated soil increased to a  
236 certain extent. The accumulation of zinc (Zn), copper (Cu), manganese (Mn), cobalt (Co),  
237 nickel (Ni), strontium (Sr), and vanadium (V) in FPW5 soil was much higher than that of the  
238 other two groups. On the contrary, molybdenum (Mo), and barium (Ba) accumulated more in  
239 TFPW soil and TW soil. The accumulation of heavy metals in soil may not only adversely  
240 affects soil biota through microbial processes and soil-microbe interactions, but also harm  
241 human health through the food chain.<sup>39, 40</sup> Combined with soil environmental quality  
242 standards summarized in **Table S3**, the content of several heavy metals in the soil of each  
243 irrigation group were lower than the recommended risk control value, indicating that the  
244 irrigation reuse of properly diluted or treated shale gas wastewater within a wheat life cycle  
245 might not cause adverse effects on the soil environment. Nevertheless, diluted and treated  
246 effluents contributed to addition of chemical components to the soil, which may accumulate  
247 over time.

248     **Effect on elements accumulation in wheat tissues.** Nutrient elements and toxic heavy  
249 metals in plant tissues were measured to provide insights into the translocation and  
250 accumulation effects.<sup>40-42</sup> The content of seven nutrient elements and eight toxic elements in  
251 wheat root, stem, leaf, and grain are displayed in **Figures 3 and 4**, respectively, with the

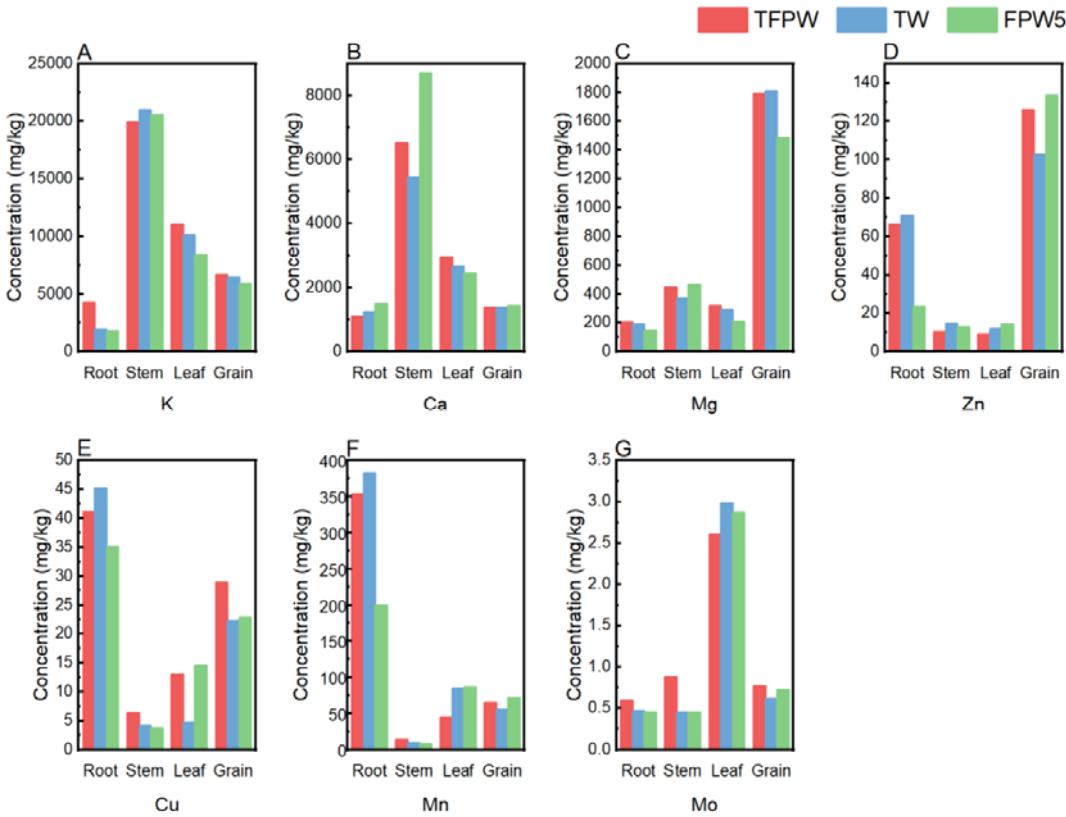
relative standard deviations (RSDs) presented in **Table S2**. Obviously, the accumulation of these elements in wheat leaves was higher than in grains (except for Mg, Zn, Cu, and Mn), indicating that the transport of these elements in wheat affected leaf tissues rather than continuing to reach grains, regardless of the type of irrigation water.<sup>20, 43</sup> Except for Mn and Mo, the concentration trends of each element in the experimental groups for different tissues of the plant was consistent. Interestingly, although FPW5 contained higher concentration of substances and salinity, the content of several elements within specific tissues of wheat cultivated with FPW5 were not the highest among the three groups. In fact, the key factor affecting plant osmotic stress may not be the overall salinity gradient itself, but may be related to ion composition and ratios.<sup>27, 44</sup>

In detail, compared with the TW group, the wheat in the other two groups absorbed and stored more Ca, since Ca could facilitate plant resistance to stress.<sup>45</sup> The K content in the wheat cultivated with TFPW increased significantly, and even the content in the roots of the wheat was twice that of the other groups, suggesting that TFPW promoted wheat uptake of K from soil. As shown in **Figure 3C**, compared with TW and FPW5, the Mg content associated with photosynthesis and carbohydrate synthesis accumulation was relatively higher in wheat leaves irrigated by TFPW. K, Ca and Mg exist in significant amounts in various tissues of plants and play a wide range of roles, including but not limited to regulating cell permeability, activating enzyme, regulating product transport, and participating in cell structure composition. Results in **Figure 3** also indicate that the content of Zn, Cu, Mn, and Mo in various tissues of wheat were smaller than that of K, Ca, and Mg by several orders of magnitude. Their presence in trace amounts is essential for plant growth and grain yield, but

274 toxic when exceeding certain threshold levels.<sup>20, 46</sup> For instance, the presence of high  
275 concentrations of Zn and Cu in plants can reduce metabolic activity, generate reactive oxygen  
276 species, and induce oxidative damage.<sup>46</sup> Similarly, several toxic heavy metals and metalloids  
277 listed in **Figure 4** were classified as non-essential elements for plant growth and might cause  
278 serious acute and chronic health hazards to plants and humans. Pb and Cr are also toxic  
279 and/or carcinogenic to humans, and can cause damage to the nervous system and to the  
280 immune system, causing a variety of diseases similar to excessive As.<sup>40, 47, 48</sup>  
281 The actual content of these toxic elements in the grains of wheat are a concern for food safety.  
282 As shown in **Figure 4B**, the accumulation of As in the roots of wheat was much higher than  
283 that in the shoots (stem, leaf, grain), which was consistent with what described in a previous  
284 study.<sup>49</sup> No significant differences were observed between the content of As in wheat grains  
285 irrigated by different waters. This study found that only Cr and Pb exceeded the maximum  
286 values of 1 mg/kg for Cr and 0.2 mg/kg for Pb recommended by China, while the other  
287 metals met the requirements.<sup>50</sup> Specifically, the Pb contents in wheat grains irrigated with  
288 TFPW and FPW5 were 0.217 mg/kg and 0.226 mg/kg, respectively, slightly above the limit  
289 and approximately half that of the TW group. The concentrations of Cr in the three groups of  
290 wheat grains were not significantly different (7-10 mg/kg), but were all considerably higher  
291 than the limit. Previous studies showed that plants cultivated under controlled indoor  
292 conditions were not only polluted by heavy metals from anthropogenic sources, but also more  
293 sensitive to heavy metal pollution than open field crops.<sup>40, 51</sup> In addition, the transport of  
294 many heavy metals in plants were regulated by the same transporter, which led to a  
295 competitive relationship in heavy metal accumulation. For example, the translocation and

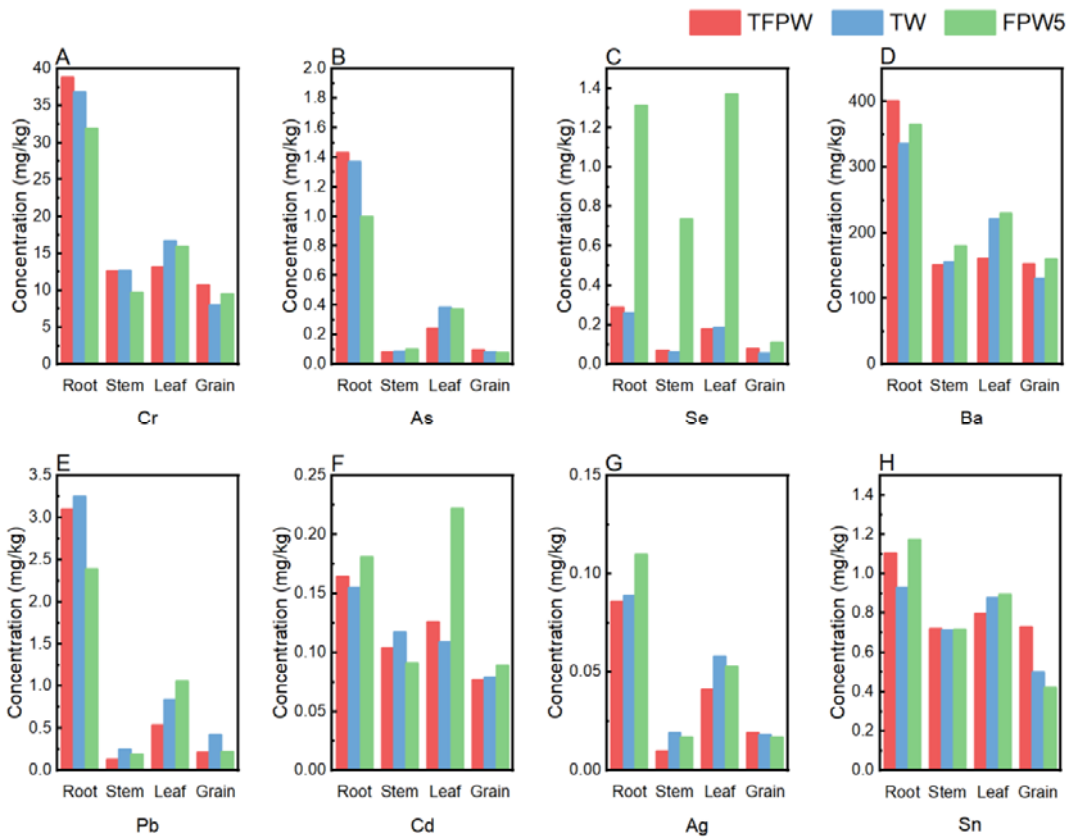
redistribution of Cd and Zn were regulated by plant cadmium resistance proteins.<sup>52</sup>

From the results, it could be concluded that the type of irrigation water was not the main contributor to the grain element contents exceeding the limits. Studies indicate that the uptake of any specific element or compounds by plants depend on several variables, including but not limited to plant species, water quality, soil quality, physicochemical properties of the element or compound, and plant physiology.<sup>53, 54</sup> The differences in the content of elements absorbed by plant tissues in each group not only reflect the competition and complexation between the components of irrigation water, but also the comprehensive results of plants responding to various stress conditions.



**Figure 3.** Concentrations of K (A), Ca (B), Mg (C), Zn (D), Cu (E), Mn (F), and Mo (G) in wheat tissues, including root, stem, leaf, and grain in wheat cultivated for 65 days applying

different irrigation waters.



**Figure 4.** Concentrations of Cr (A), As (B), Se (C), Ba (D), Pb (E), Cd (F), Ag (G) and Sn (H) in wheat tissue including root, stem, leaf, and grain in wheat cultivated for 65 days applying different irrigation waters.

**Effect on transcriptome sequencing in wheat grains.** To better understand the effect of irrigation with shale gas wastewater on wheat grain genetic and physiological basis traits, grains at grain filling stage were used for transcriptome analysis based on the RNA-seq technology.<sup>30, 55</sup> The RNA bands were clear and free of impurities, such as pigments, proteins, and sugars, based on the results reported in **Table S4**. High-quality mapped reads for

transcript assembly and expression calculation were obtained, and their derivation distribution and detailed sequencing data are presented in **Tables S5 and S6**, respectively. In this experiment, the total number of known expressed genes in wheat grains irrigated with TFPW, TW, FPW5 was 71570, 70278, and 67836, respectively, with a large amount of co-expressed genes (62788) identified in all detective samples (**Figure S4**).

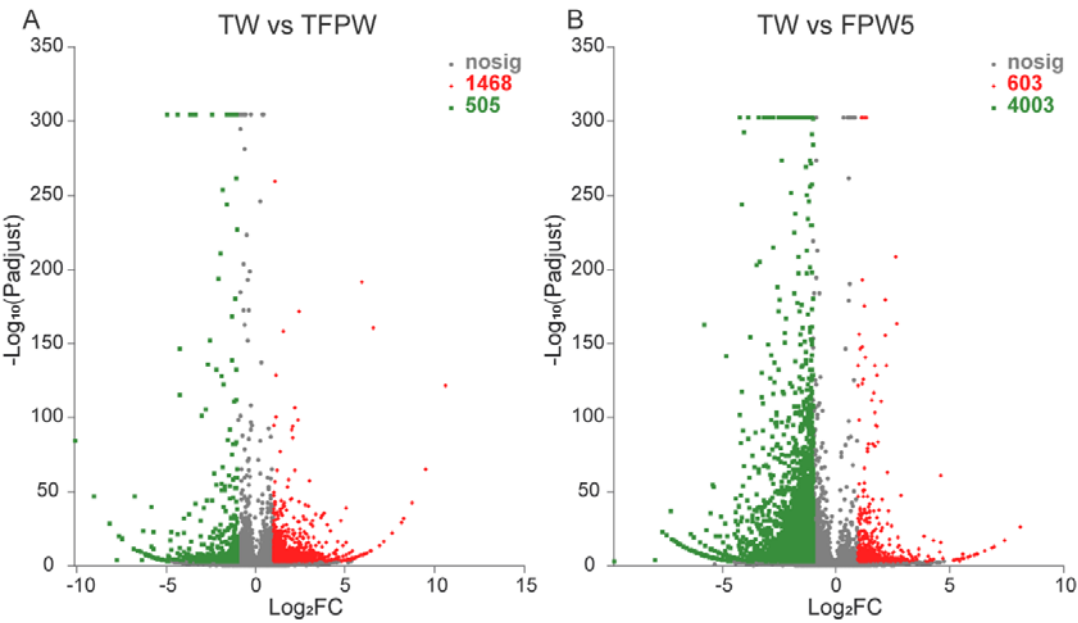
Results indicate that a total of 1973 genes were differentially expressed when comparing the use of TW and TFPW, of which the expression of 1468 genes was up-regulated and that of 505 was down-regulated when the irrigation water was TFPW (**Figure 5A**). In addition, irrigation with FPW5 led to 4606 genes differentially expressed compared with TW control. Among these DEGs, 4003 genes were down-regulated and 603 were up-regulated in TW with respect to FPW5 (**Figure 5B**). The emergence of up-regulated and down-regulated genes positively and negatively affect the physiological and developmental characteristics of wheat at grain filling stage, respectively, which in turn determined the size and number of mature grains.<sup>56</sup>

The aforementioned DEGs in TW vs. TFPW and TW vs. FPW5 were analyzed for functional and biological information using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation, respectively. GO annotation analysis showed that the DEGs could be annotated into three categories, namely, biological processes, molecular functions, and cellular components, with the top 20 enriched GO subcategories terms displayed in **Figure 6**. A high percentage of intersection DEGs related to binding, catalytic activity, cellular process, metabolic process, and cell part were induced by TFPW irrigation water in wheat grains (**Figures 6A**). However, in the intersection group of TW vs. FPW5, cell

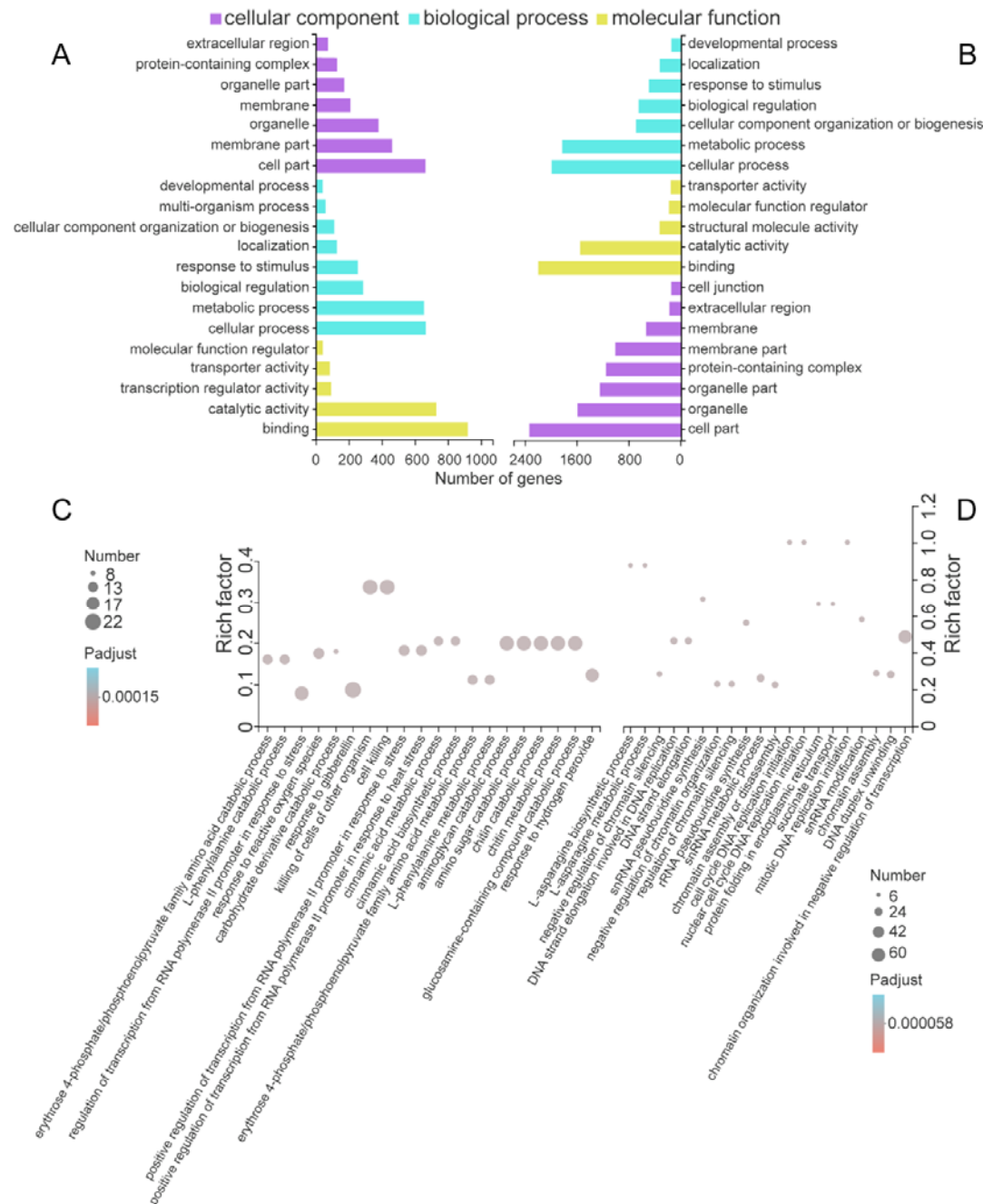
343 part, binding, cellular process, metabolic process, organelle, and catalytic activity apparently  
 344 ranked as the top six terms (**Figures 6B**). The detailed regulated genes annotated by the  
 345 KEGG database were classified into five pathways: metabolism, genetic information  
 346 processing, environmental information processing, cellular processes, and organismal  
 347 systems. In the intersection group of TW vs. TFPW, only the number of up-regulated DEGs  
 348 annotated to carbohydrate metabolism pathways was not higher than that of down-regulated  
 349 DEGs (**Tables S7**). Interestingly, the complete opposite trend was observed in TW vs. FPW5  
 350 (**Tables S8**).

351 Furthermore, combined with enrichment analysis, we explored the effect of TFPW and FPW5  
 352 on major gene functions and metabolic pathways in wheat grain compared to irrigation with  
 353 TW. **Figure 6C** shows that, apart from the highest enrichment degree of functional genes that  
 354 determined cells death (0.34), the genes related to glucosamine-containing compound  
 355 catabolic process, chitin catabolic process, aminoglycan catabolic process, and cinnamic acid  
 356 biosynthetic and metabolic process possessed high rich factor (0.2-0.22) among the top 20  
 357 ranked GO terms of DEGs. In addition, carbohydrate derivative catabolic process, cell killing,  
 358 glucosamine-containing compound catabolic process, response to reactive oxygen species  
 359 were more abundant functional groups in comparisons. **Figure S5A** presents the top 10  
 360 ranked KEGG pathways of enrichment, of which the highest enrichment was MAPK  
 361 signaling pathway-plant, followed by amino sugar and nucleotide sugar metabolism, and  
 362 protein processing in endoplasmic reticulum. Moreover, a large number of DEGs were  
 363 involved in the phenylpropanoid biosynthesis and phenylalanine metabolism pathways, and  
 364 starch and sucrose metabolism, which play an important role in plant growth, development

and response to stress. As presented in **Figures 6D** and **S5B**, significantly different from TW vs. TFPW analysis, a large number of DEGs related to DNA replication and ribosome pathways appeared in TW vs. FPW5 analysis. Genes related to uptake of heavy metals by plant roots and transport of heavy metals from roots to shoots might be regulated by irrigation water.



**Figure 5.** Volcano plots of differences in gene expression among (A) TW vs. TFPW and (B) TW vs. FPW5 analysis. Gray dots were not considered as significantly differentially expressed. Red (up-regulation) and green (down-regulation) dots indicate DEGs ( $|\log_2(\text{Fold Change})| > 1$ ,  $\text{P}_{\text{adj}}(\text{FDR}) < 0.05$ ).



**Figure 6.** Function annotation and enrichment analysis of the intersection differentially expressed genes (DEGs) of wheat grain byased on gene ontology (GO) databases. (A, B): sub-categories of Gene Ontology (GO) terms of the DEGs in TW vs TFPW and TW vs FPW5 analysis, respectively. (C, D): bubble diagram of top 20 ranked GO terms of DEGs in TW vs. TFPW and TW vs. FPW5 analysis, respectively.

383

384 In summary, compared with tap water, TFPW inhibited the total biomass and yield of mature  
385 wheat, promoted the accumulation of nutrient elements, and induced the up-regulation of  
386 more than half of the differentially expressed genes related to binding, catalytic activity,  
387 cellular process, metabolic process. With the continuous use of irrigation water, the content of  
388 many elements in the soil increased slightly. Notably, including the TW control, the abnormal  
389 accumulation of heavy metals in wheat grains might pose potential health risks to the  
390 environment and humans, which require further field studies. In short, the present study  
391 provides practical insights into the macro and micro effects of crop growth, toxicological  
392 characteristics and gene transcriptional expression differences of shale gas flowback and  
393 produced water reuse in farmland irrigation and is useful toward efforts on the proper  
394 management as well as optimal treatment of shale gas wastewater.

395

## 396 **ASSOCIATED CONTENT**

### 397 **Supporting Information**

398 The supporting information is available free of charge.

399

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404

## 405 **Notes**

The authors declare no competing financial interests.

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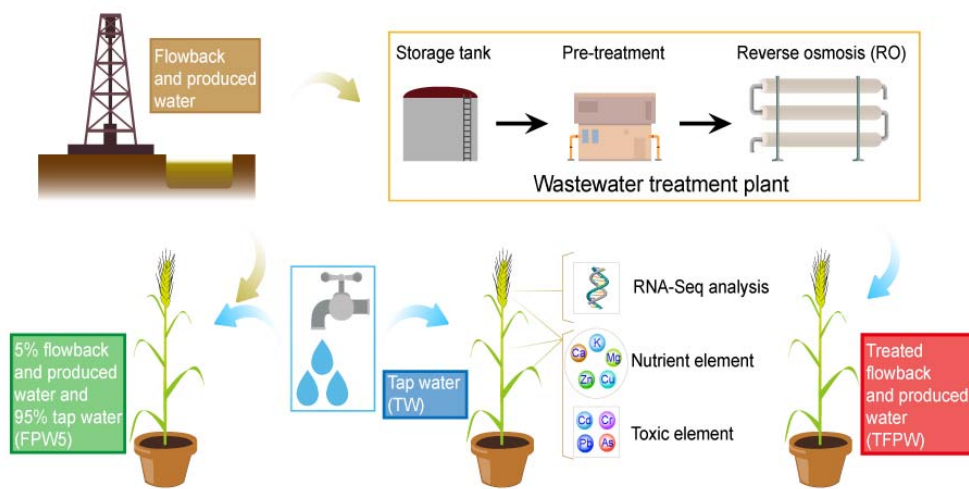
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