

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

*Original*

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

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22

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>23-25,20, 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

110 (TFPW), and diluted untreated flowback and produced water (FPW5, equivalent to 95% FPW  
111 + 5% TW). Each culture group contained 30 wheat seeds, individually weighing  $0.045 \pm 0.005$   
112 g, and was equally divided and transferred to five sterile petri dishes comprising a qualitative  
113 filter paper and 15 mL of corresponding water, which were both replaced every 24 h. All petri  
114 dishes were exposed to natural light at room temperature ( $\sim 20\text{ }^{\circ}\text{C}$ )<sup>31</sup> for six days, and the  
115 number of wheat roots and sprouts were recorded daily to compare the germination rates  
116 between the control and the experimental groups. Seedlings height and root length were  
117 measured with a millimeter ruler. Seed vigor index (SVI) was calculated as described  
118 previously.<sup>32</sup>

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

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



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

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

154 **Transcriptome sequencing of wheat grains.** Before wheat matured completely, less than  
155 0.1 g of fresh grains in each group was taken out, washed with DEPC water (RNase-free),  
156 frozen directly in liquid nitrogen for 30 min, and stored at  $-80^{\circ}\text{C}$  for RNA extraction. The  
157 total RNA was extracted from wheat grains using TRIzol® Reagent. Afterward, the  
158 transcriptome sequencing process was executed with the Illumina NovaSeq 6000 sequencer  
159 (Majorbio Bio-pharm Technology Co., Ltd, Shanghai, China). Details including RNA  
160 extraction, sequencing, read mapping, DEGs (differentially expressed genes), and functional  
161 enrichment are summarized in **Text S1 (SI)**. Raw sequencing data were stored in NCBI  
162 database under bioproject number PRJNA813217, with biosample numbers SAMN26455859,  
163 SAMN26455866, and SAMN26455867.

## 164 **RESULTS AND DISCUSSION**

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

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

177 **Effects on seeds germination and phenotypic analysis.** Results on the physiological  
178 conditions of wheat in different treatment groups during germination stages are summarized  
179 in **Figure 1**, including germination number, seedling height, root length, and seed vigor index.  
180 After 6-day germination culture, the germination number of wheat seeds exposed to TFPW  
181 was comparable to that of the TW control group, whereas the germination number was lower  
182 for FPW5 irrigation (**Figure 1A**). In detail, the germination rates of TFPW and TW groups  
183 exceeded 85%, while the FPW5 was only 70%. Therefore, irrigation with TFPW slightly  
184 shortened the germination period. Application of TFPW practically maintained the same  
185 seedling height and seed vigor observed for TW. On the contrary, application of FPW5  
186 remarkably decreased ( $p < 0.05$ ) both seedling height and seed vigor index by roughly 20 and  
187 45% (**Figures 1B and 1D**). **Figure 1C** presents the results obtained for the root length related  
188 to root numbers in the seed germination test. Even within the same irrigation group, the  
189 number and length of taproots were diverse. When considering samples with the same  
190 number of roots, the relationship between the average root length of the three groups was:  
191 TFPW>TW>FPW5. In summary, the use of treated shale gas wastewater was suitable in the  
192 germination stage of wheat seeds, consistent with previously reported leafy vegetables  
193 cultivation.<sup>31, 35</sup>

194

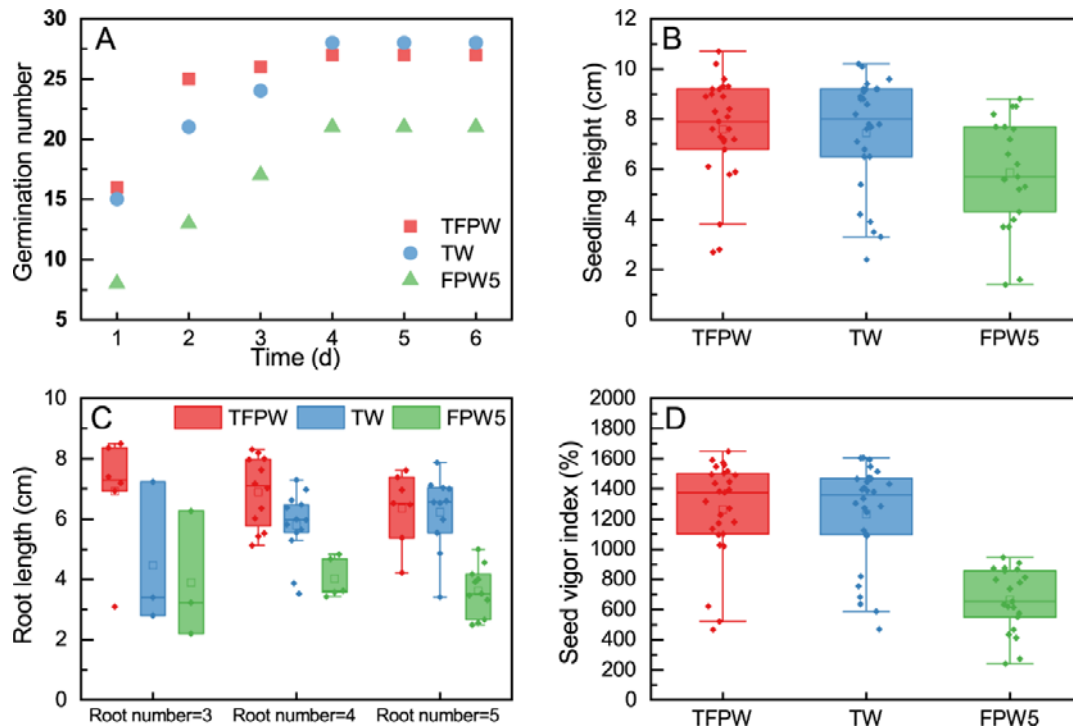
195 **Table 1.** Water quality of three irrigation waters (TFPW, TW, FPW5); national and  
196 international guidelines for irrigation.

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



199

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

205

206 **Effects on wheat growth and harvesting.** After being transplanted, the wheat seedlings  
 207 were cultivated for 65 days and then harvested (**Figures S2 and 2A**). The average wheat  
 208 above-ground height of the three irrigatioj groups changed with time (**Figure 2B**). Irrigation  
 209 with FPW5 slightly increased the above-ground height of wheat in the first 20 days,  
 210 compared with the other two groups, but the wheat treated with TW grew faster at a later  
 211 stage. The results suggest that the growth of wheat in soil followed an opposite trend  
 212 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

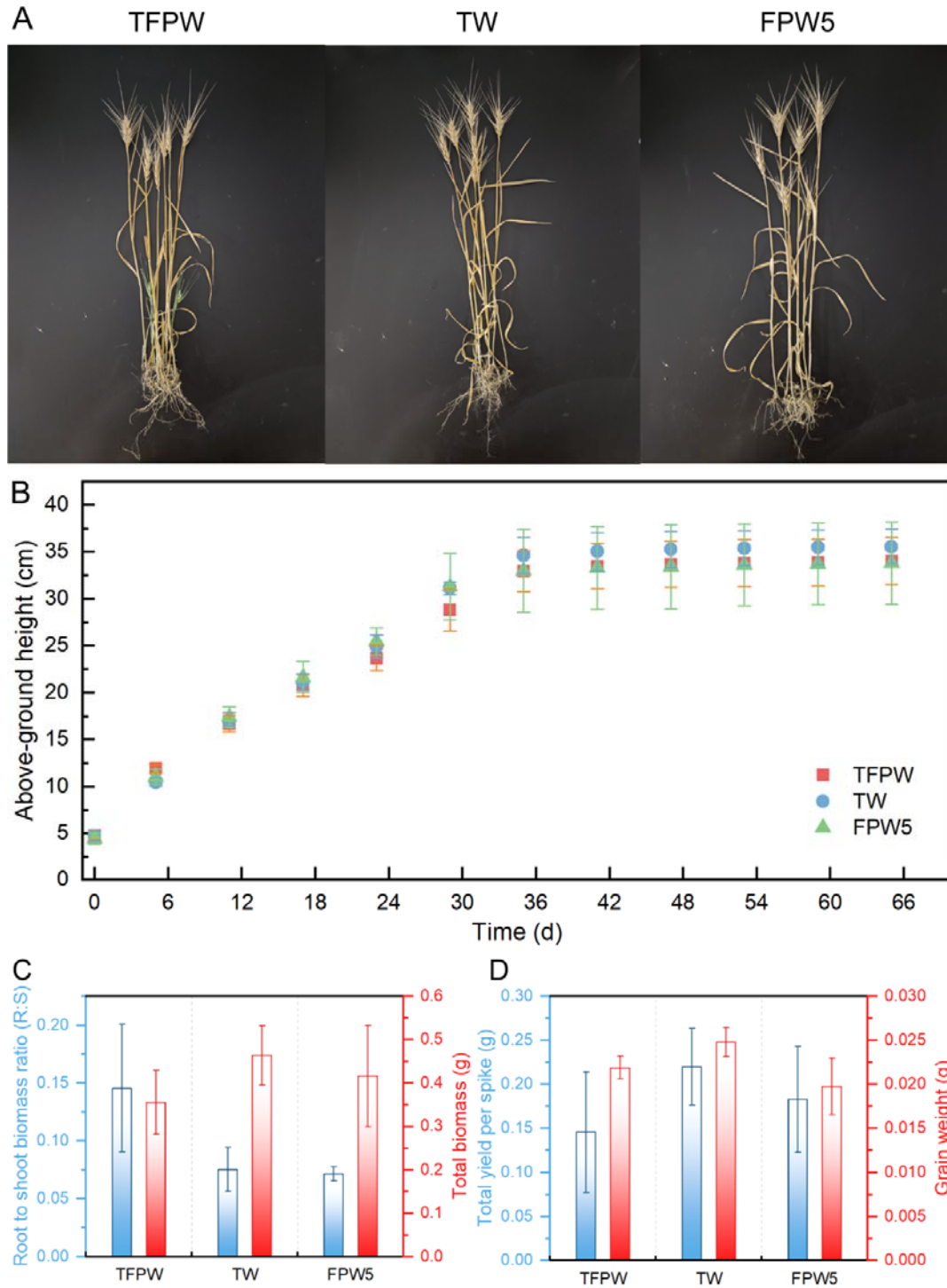
221 markedly by 12 % for TFPW irrigation ( $p < 0.05$ ) and 20 % for FPW5 irrigation ( $p < 0.05$ ).

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



226

227 **Figure 2.** (A) Representative images of mature wheat irrigated with different waters. (B)

228 Above-ground height of wheat irrigated with different waters in soil. (C) Wheat total biomass

229 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



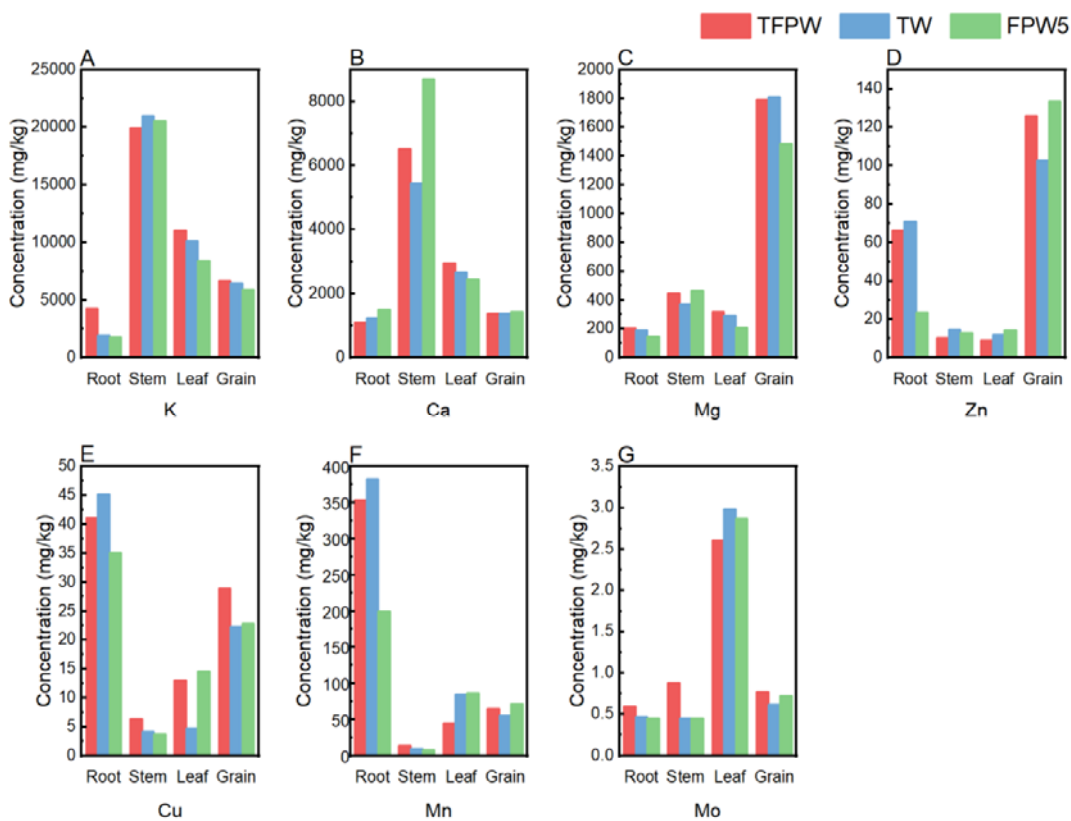
252 relative standard deviations (RSDs) presented in **Table S2**. Obviously, the accumulation of  
253 these elements in wheat leaves was higher than in grains (except for Mg, Zn, Cu, and Mn),  
254 indicating that the transport of these elements in wheat affected leaf tissues rather than  
255 continuing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

262 In detail, compared with the TW group, the wheat in the other two groups absorbed and  
263 stored more Ca, since Ca could facilitate plant resistance to stress.<sup>45</sup> The K content in the  
264 wheat cultivated with TFPW increased significantly, and even the content in the roots of the  
265 wheat was twice that of the other groups, suggesting that TFPW promoted wheat uptake of K  
266 from soil. As shown in **Figure 3C**, compared with TW and FPW5, the Mg content associated  
267 with photosynthesis and carbohydrate synthesis accumulation was relatively higher in wheat  
268 leaves irrigated by TFPW. K, Ca and Mg exist in significant amounts in various tissues of  
269 plants and play a wide range of roles, including but not limited to regulating cell permeability,  
270 activating enzyme, regulating product transport, and participating in cell structure  
271 composition. Results in **Figure 3** also indicate that the content of Zn, Cu, Mn, and Mo in  
272 various tissues of wheat were smaller than that of K, Ca, and Mg by several orders of  
273 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

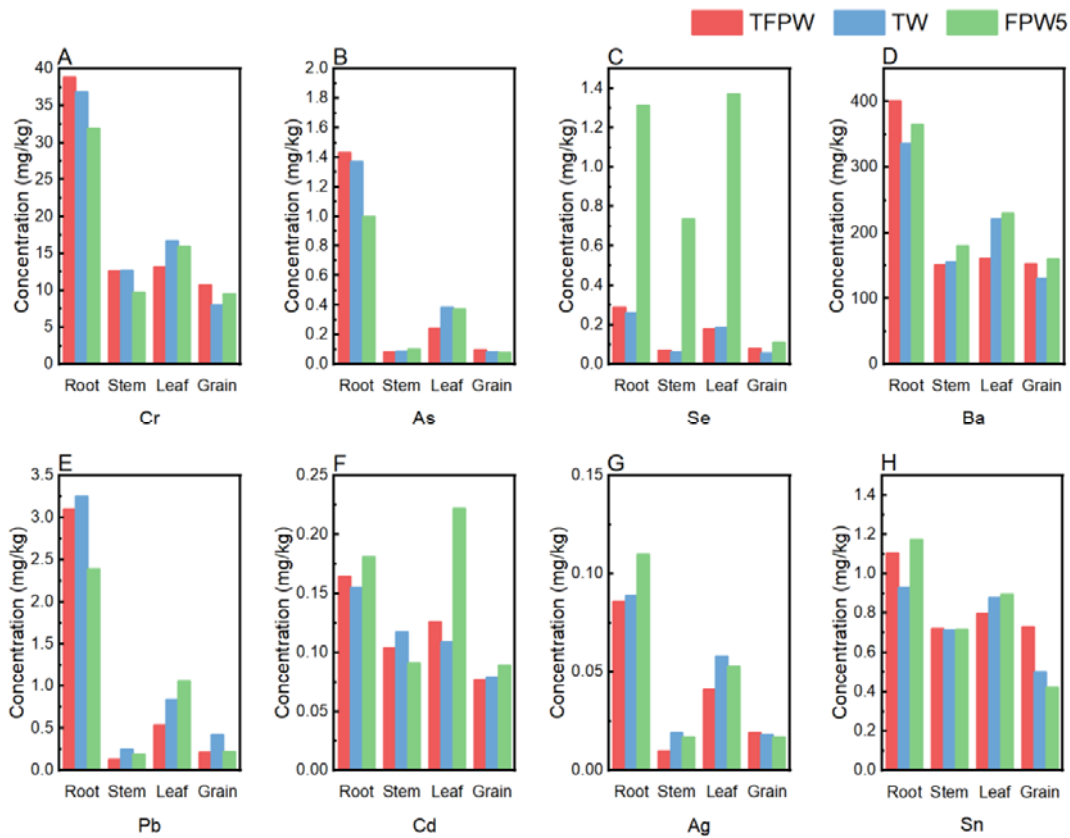
296 redistribution of Cd and Zn were regulated by plant cadmium resistance proteins.<sup>52</sup>  
 297 From the results, it could be concluded that the type of irrigation water was not the main  
 298 contributor to the grain element contents exceeding the limits. Studies indicate that the uptake  
 299 of any specific element or compounds by plants depend on several variables, including but  
 300 not limited to plant species, water quality, soil quality, physicochemical properties of the  
 301 element or compound, and plant physiology.<sup>53, 54</sup> The differences in the content of elements  
 302 absorbed by plant tissues in each group not only reflect the competition and complexation  
 303 between the components of irrigation water, but also the comprehensive results of plants  
 304 responding to various stress conditions.  
 305



306  
 307 **Figure 3.** Concentrations of K (A), Ca (B), Mg (C), Zn (D), Cu (E), Mn (F), and Mo (G) in  
 308 wheat tissues, including root, stem, leaf, and grain in wheat cultivated for 65 days applying

309 different irrigation waters.

310



311

312 **Figure 4.** Concentrations of Cr (A), As (B), Se (C), Ba (D), Pb (E), Cd (F), Ag (G) and Sn (H)

313 in wheat tissue including root, stem, leaf, and grain in wheat cultivated for 65 days applying

314 different irrigation waters.

315

316 **Effect on transcriptome sequencing in wheat grains.** To better understand the effect of

317 irrigation with shale gas wastewater on wheat grain genetic and physiological basis traits,

318 grains at grain filling stage were used for transcriptome analysis based on the RNA-seq

319 technology.<sup>30,55</sup> The RNA bands were clear and free of impurities, such as pigments, proteins,

320 and sugars, based on the results reported in **Table S4**. High-quality mapped reads for

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

326 Results indicate that a total of 1973 genes were differentially expressed when comparing the  
327 use of TW and TFPW, of which the expression of 1468 genes was up-regulated and that of  
328 505 was down-regulated when the irrigation water was TFPW (**Figure 5A**). In addition,  
329 irrigation with FPW5 led to 4606 genes differentially expressed compared with TW control.  
330 Among these DEGs, 4003 genes were down-regulated and 603 were up-regulated in TW with  
331 respect to FPW5 (**Figure 5B**). The emergence of up-regulated and down-regulated genes  
332 positively and negatively affect the physiological and developmental characteristics of wheat  
333 at grain filling stage, respectively, which in turn determined the size and number of mature  
334 grains.<sup>56</sup>

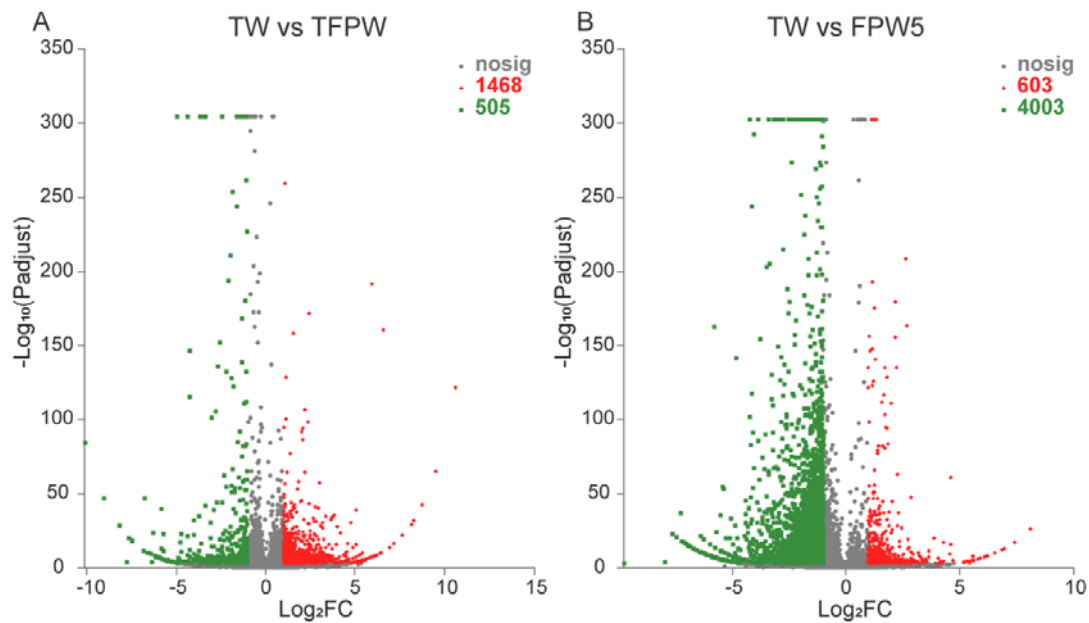
335 The aforementioned DEGs in TW vs. TFPW and TW vs. FPW5 were analyzed for functional  
336 and biological information using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes  
337 and Genomes (KEGG) annotation, respectively. GO annotation analysis showed that the  
338 DEGs could be annotated into three categories, namely, biological processes, molecular  
339 functions, and cellular components, with the top 20 enriched GO subcategories terms  
340 displayed in **Figure 6**. A high percentage of intersection DEGs related to binding, catalytic  
341 activity, cellular process, metabolic process, and cell part were induced by TFPW irrigation  
342 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

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

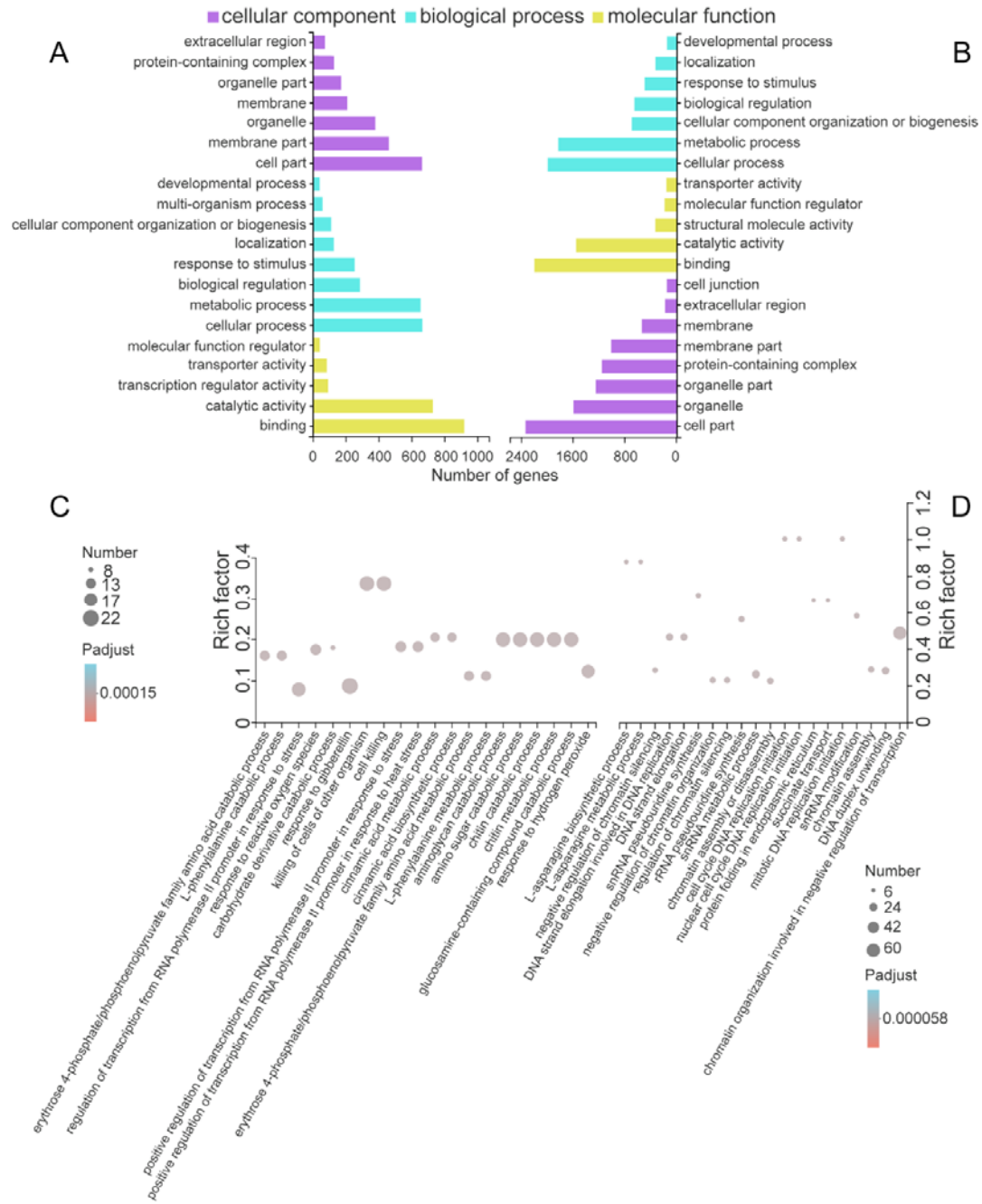
370



371

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

376



377

378 **Figure 6.** Function annotation and enrichment analysis of the intersection differentially

379 expressed genes (DEGs) of wheat grain byased on gene ontology (GO) databases. (A, B):

380 sub-categories of Gene Ontology (GO) terms of the DEGs in TW vs TFPW and TW vs FPW5

381 analysis, respectively. (C, D): bubble diagram of top 20 ranked GO terms of DEGs in TW vs.

382 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

## 400 **AUTHOR INFORMATION**

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404

## 405 **Notes**

406 The authors declare no competing financial interests.

407

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412

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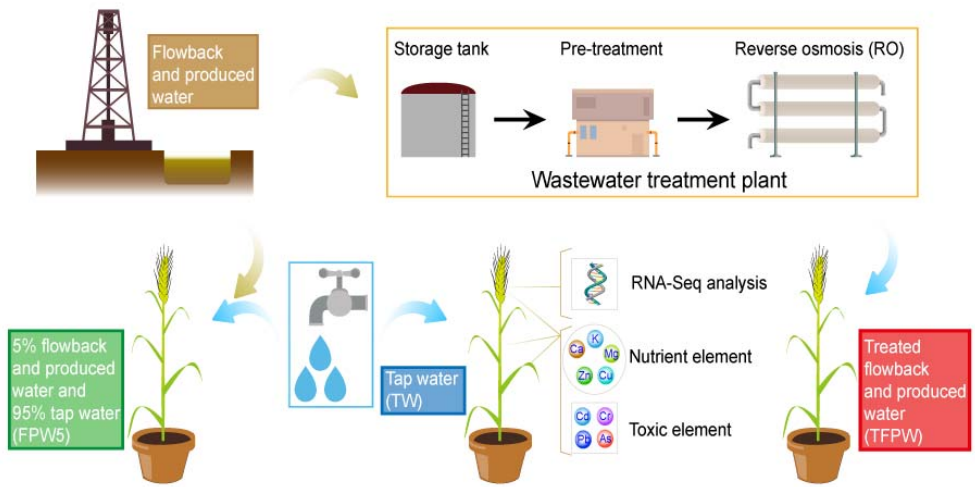
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589 **Table of Contents:**



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