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

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**ABSTRACT**：

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

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

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

## INTRODUCTION

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

FPW is composed by the natural formation water extracted from underground oil & gas resources, so-called produced water, and by the injection fluid utilized during the hydraulic fracturing process, so-called flowback water. Therefore, FPW usually contains high concentrations of total dissolved solids (TDS), organic matter (dissolved organic carbon; DOC), metals, hydrocarbons and other volatile compounds, some synthetic organic chemical additives, as well as some naturally occurring radioactive materials that may be present in the formation.[13-16] In recent years, the option of applying FPW directly or indirectly for agricultural irrigation has become a focus of growing discussion.[17-19] Small amounts of organic matter and micronutrient elements in municipal sewage and industrial wastewaters, at times also including rare earth elements, have been shown to promote the growth of some algae strains or higher plants under certain circumstances. On the other hand, the salinity, alkalinity, and ionic composition of oilfield produced water may significantly hinder its reuse in agriculture, since these characteristics can cause nutrient imbalances, as well as osmotic and specific ion stress in plant cells. [23-2520, 21] Methods of reusing produced water with minimal TDS treatment or upon dilution for farmland irrigation were applied in Kern County (California) a few years ago, but the complexity of FPW composition has gradually aroused public concern.[22]

The available literature suggests that the concentration of TDS and DOC of irrigation water should be lower than 3500 mg/L and 5 mg/L, respectively, to maintain normal plant growth and biomass accumulation, and that the use of water with substance conents above the current guidelines to irrigate crops may lead to yield reduction and germination problems.[26, 27] Previous studies on the irrigation-oriented reuse of oil & gas wastewater without treatment or upon simple dilution have shown that this approach has potential significant side effects on plant growth, yield, gene expression, and soil ecology. Applying different percentages of PW to irrigate wheat has been observed to lead to a decline in yield and a negative effect on physiological parameters, even when the wastewater was diluted by as much as 90% with tap water, also inducing an adverse impact on soil health and microbial diversity.[17, 20, 27] In addition, irrigating wheat with diluted PW can inhibit the expression of some disease-resistant genes in the crop.[28] Other studies reported that simulated produced water used to irrigate non-food biofuel crops resulted in significantly lower growth and worse physiological characteristics of the crops due to high PW salinity and excess organic carbon.[21]

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

## MATERIALS AND METHODS

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

**Germination test of wheat seeds.** Wheat seeds were cultivated with three different kinds 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±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 (~20 °C)[31] 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.[32]

**Greenhouse trial and harvesting.** After the 6-day incubation in petri dishes described above, six wheat seedlings were picked for ech 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 ℃ until constant weight was achieved. The above-ground biomass, under-ground biomass, and grain yield of each plant were thus determined.

**Analysis fo 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 °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.[33, 34]

**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.[31, 35]

**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)[36,] | Water quality for irrigation (UN)[38] |
|---|---|---|---|---|---|

| | | | | 37 | |
|---|---|---|---|---|---|
| Turbidity | 0.20 | 0.23 | 0.67 | / | / |
| Conductivity (μs/cm) | 49 | 336 | 3224 | / | 3000 |
| DOC (mg/L) | 2.90 | 0.85 | 1.18 | / | / |
| TN (mg/L) | 5.136 | 0.854 | 3.456 | / | / |
| $UV_{254}$ ($cm^{-1}$) | 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 (μg/L) | 21.91 | 494.86 | 756.93 | / | 5000 |
| Mn (μg/L) | 0.053 | 0.704 | 37.705 | 300 | 200 |
| Cu (μg/L) | 0.609 | 0.663 | 9.493 | 500 | 200 |
| Zn (μg/L) | 57.55 | 404.15 | 393.70 | 2000 | 2000 |
| Mo (μg/L) | 0.5 | 1.3 | 1.7 | 500 | 10 |
| Ni (μg/L) | 0.088 | 4.750 | 6.100 | 100 | 200 |
| Cr (μg/L) | 10.45 | 11.05 | 11.15 | 100 | 100 |
| Se (μg/L) | 0.15 | 0.52 | 5.26 | 20 | 20 |
| As (μg/L) | 0.009 | 0.396 | 2.705 | 50 | 100 |
| Pb (μg/L) | 0.010 | 0.15 | 0.200 | 200 | 5000 |
| Cd (μg/L) | NA | NA | 0.005 | 10 | 10 |
| Ag (μg/L) | 0.008 | 0.004 | 0.005 | / | / |
| Al (μg/L) | 7.165 | 101.494 | 109.04 | / | 5000 |
| Co (μg/L) | NA | 0.061 | 0.089 | 1000 | 50 |
| Sb (μg/L) | 0.40 | 0.50 | 2.75 | / | / |
| Sn (μg/L) | 0.125 | 0.039 | 0.085 | / | / |
| V (μg/L) | 0.040 | 0.408 | 16.078 | 100 | 100 |
| $F^-$ (mg/L) | 0.039 | 0.172 | 0.444 | 2 | 1 |
| $Cl^-$ (mg/L) | 8.501 | 30.871 | 870.265 | 350 | 350 |
| $Br^-$ (mg/L) | 0.105 | 3.480 | 5.670 | / | / |
| $NO_3^-$ (mg/L) | 0.145 | 5.330 | 7.160 | / | 10 |
| $SO_4^{2-}$ (mg/L) | 0.584 | 44.803 | 48.209 | / | 960 |

Note: NA, not available.

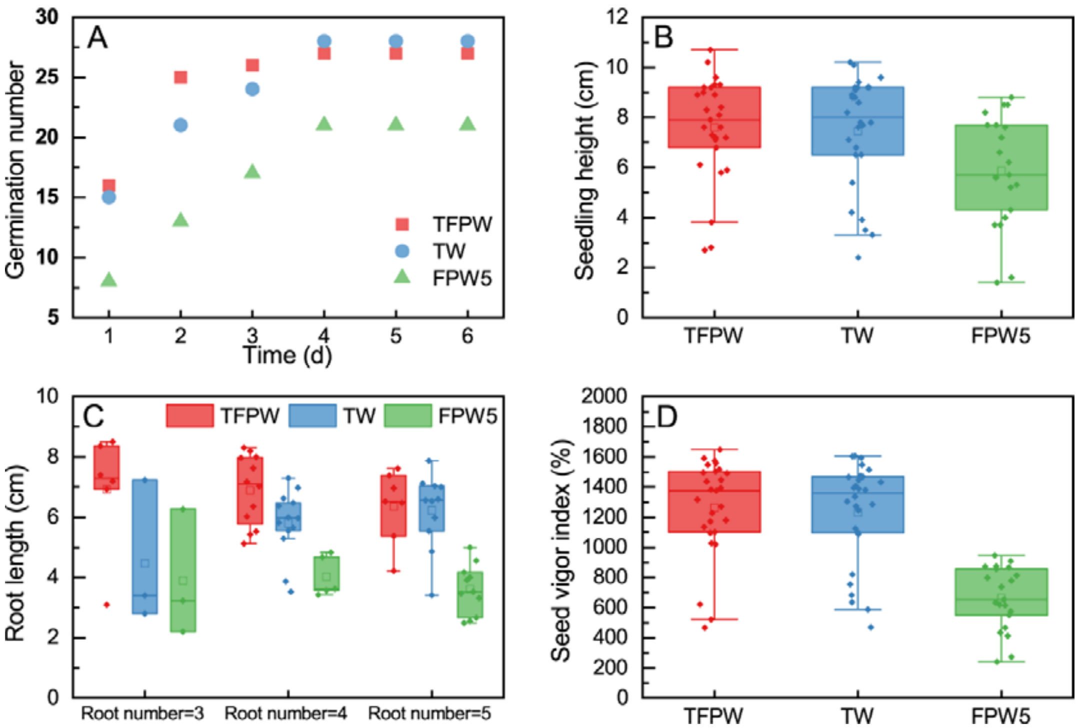


**Figure 1.** Germination performance of wheat seeds in different irrigation groups: treated flowback and produced water (TFPW, red), tap water control (TW, blue), diluted FPW (FPW5, green). (A) Germination number; (B) seedlings height on the sixth day; (C) root length for sampels in which the number 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 waters.

Irrigation with reused wastewater showed some stress effects. Compared to the control, TFPW irrigation significantly ($p < 0.05$) increased the root to shoot biomass ratio (R:S ratio). Additionally, irrigation with TFPW and FPW5 significantly ($p < 0.05$) decreased wheat total biomass by approximately 23 % and 10 %, respectively (**Figure 2C**). The results were in line with the reports suggesting that higher concentration of organic matter in the irrigation water leads to smaller biomass.[21] As presented in **Figure 2D**, the total yield per spike in TFPW and FPW5 groups decreased markedly ($p < 0.05$) by 34 % and 17 %; the grain weight reduced markedly by 12 % for TFPW irrigation ($p < 0.05$) and 20 % for FPW5 irrigation ($p < 0.05$). Among the three irrigation waters, the TOC concentration of TFPW was the highest and equal to 2.9 mg/L, which was however lower than the maximum value of 5 mg/L suggested in the literature.[21]

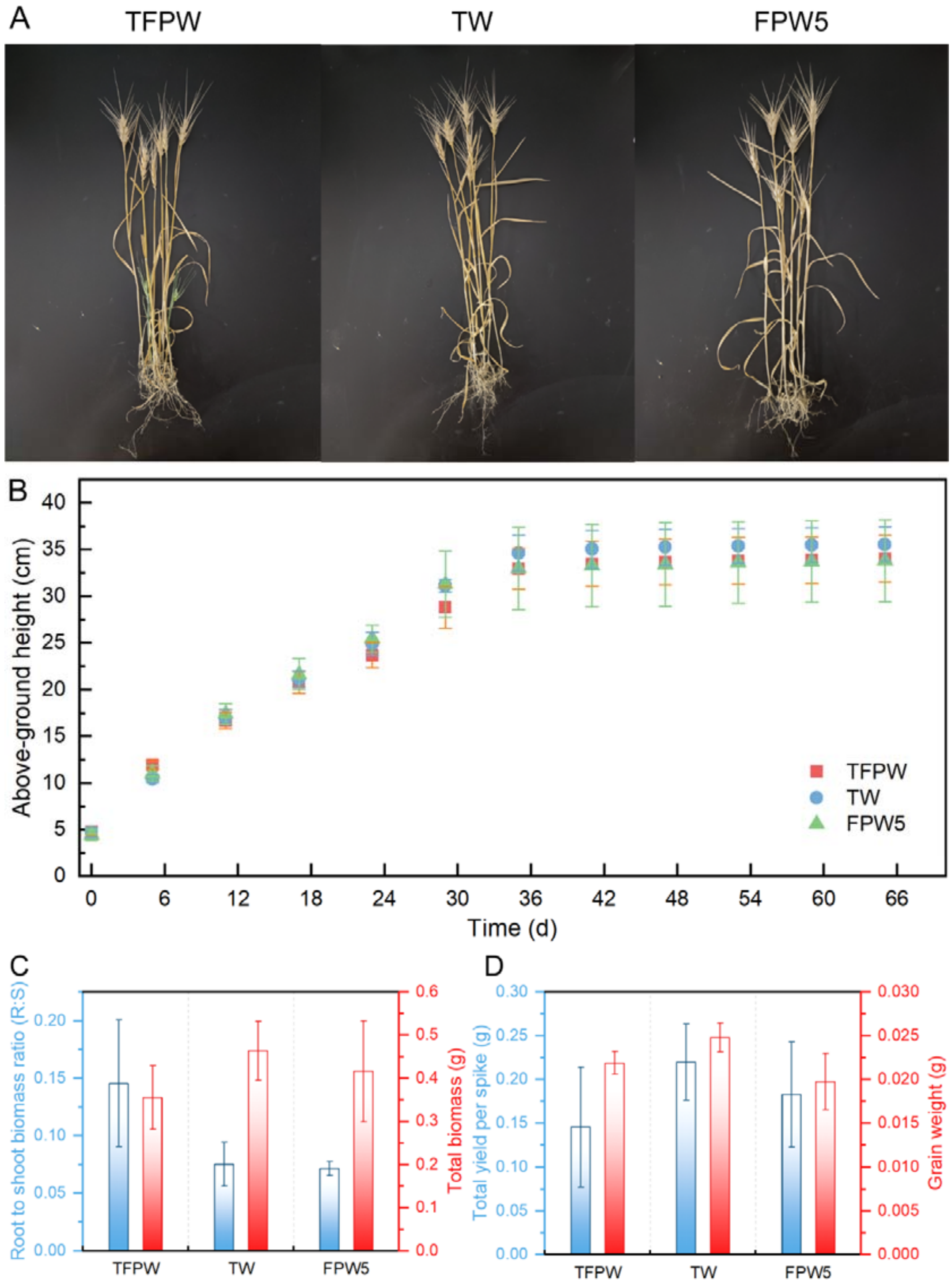


**Figure 2.** (A) Representative images of mature wheat irrigated with different waters. (B) Above-ground height of wheat irrigated with different waters in soil. (C) Wheat total biomass and root-shoot ratio. (D) Total wheat yield per spike and average grain weight.

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

**Effect on elements accumulation in wheat tissues.** Nutrient elements and toxic heavy metals in plant tissues were measured to provide insights into the translocation and accumulation effects.[40-42] The content of seven nutrient elements and eight toxic elements in 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.[20, 43] 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.[27, 44]

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.[45] 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 toxic when exceeding certain threshold levels.[20, 46] For instance, the presence of high concentrations of Zn and Cu in plants can reduce metabolic activity, generate reactive oxygen species, and induce oxidative damage.[46] Similarly, several toxic heavy metals and metalloids listed in **Figure 4** were classified as non-essential elements for plant growth and might cause serious acute and chronic health hazards to plants and humans. Pb and Cr are also toxic and/or carcinogenic to humans, and can cause damage to the nervous system and to the immune system, causing a variety of diseases similar to excessive As.[40, 47, 48]

The actual content of these toxic elements in the grains of wheat are a concern for food safety. As shown in **Figure 4B**, the accumulation of As in the roots of wheat was much higher than that in the shoots (stem, leaf, grain), which was consistent with what described in a previous study.[49] No significant differences were observed between the content of As in wheat grains irrigated by different waters. This study found that only Cr and Pb exceeded the maximum values of 1 mg/kg for Cr and 0.2 mg/kg for Pb recommended by China, while the other metals met the requirements.[50] Specifically, the Pb contents in wheat grains irrigated with TFPW and FPW5 were 0.217 mg/kg and 0.226 mg/kg, respectively, slightly above the limit and approximately half that of the TW group. The concentrations of Cr in the three groups of wheat grains were not significantly different (7-10 mg/kg), but were all considerably higher than the limit. Previous studies showed that plants cultivated under controlled indoor conditions were not only polluted by heavy metals from anthropogenic sources, but also more sensitive to heavy metal pollution than open field crops.[40, 51] In addition, the transport of many heavy metals in plants were regulated by the same transporter, which led to a competitive relationship in heavy metal accumulation. For example, the translocation and redistribution of Cd and Zn were regulated by plant cadmium resistance proteins.[52]

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.[53, 54] 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.

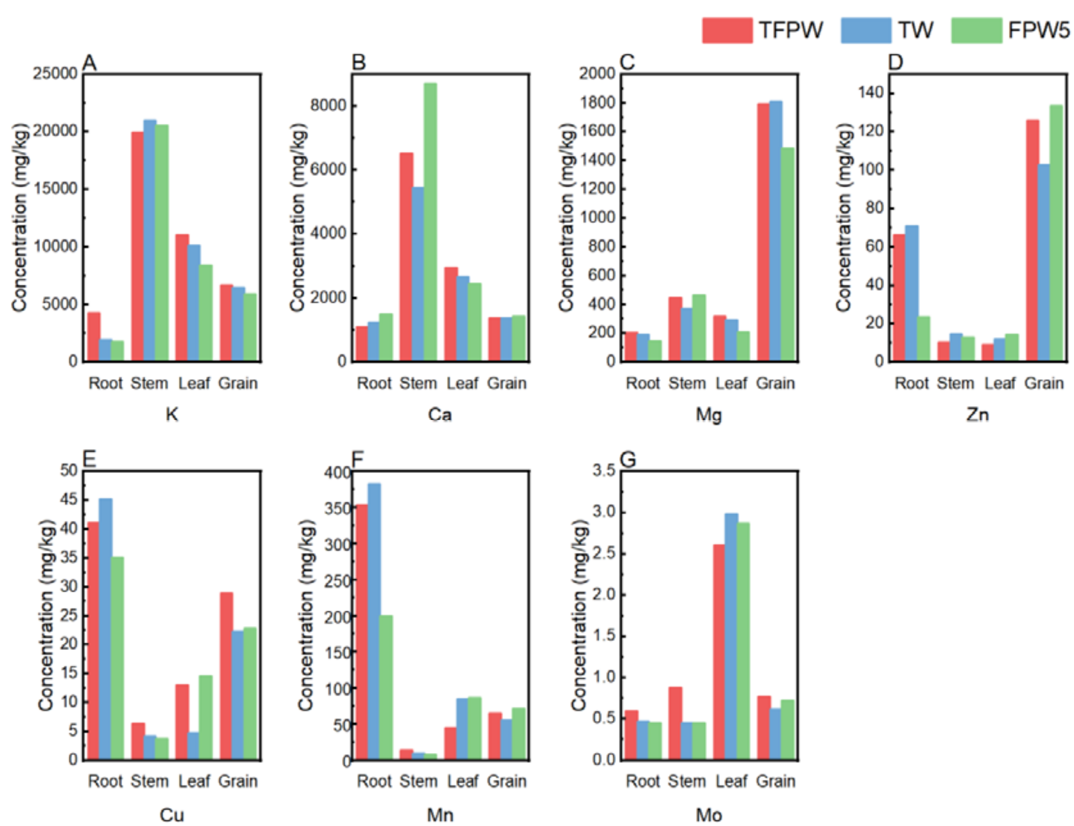


**Figure 3.** Concentrations of K (A), Ca (B), Mg (C), Zn (D), Cu (E), Mn (F), and Mo (G) in wheat tissues, including root, stem, leaf, and grain in wheat cultivated for 65 days applying

different irrigation waters.

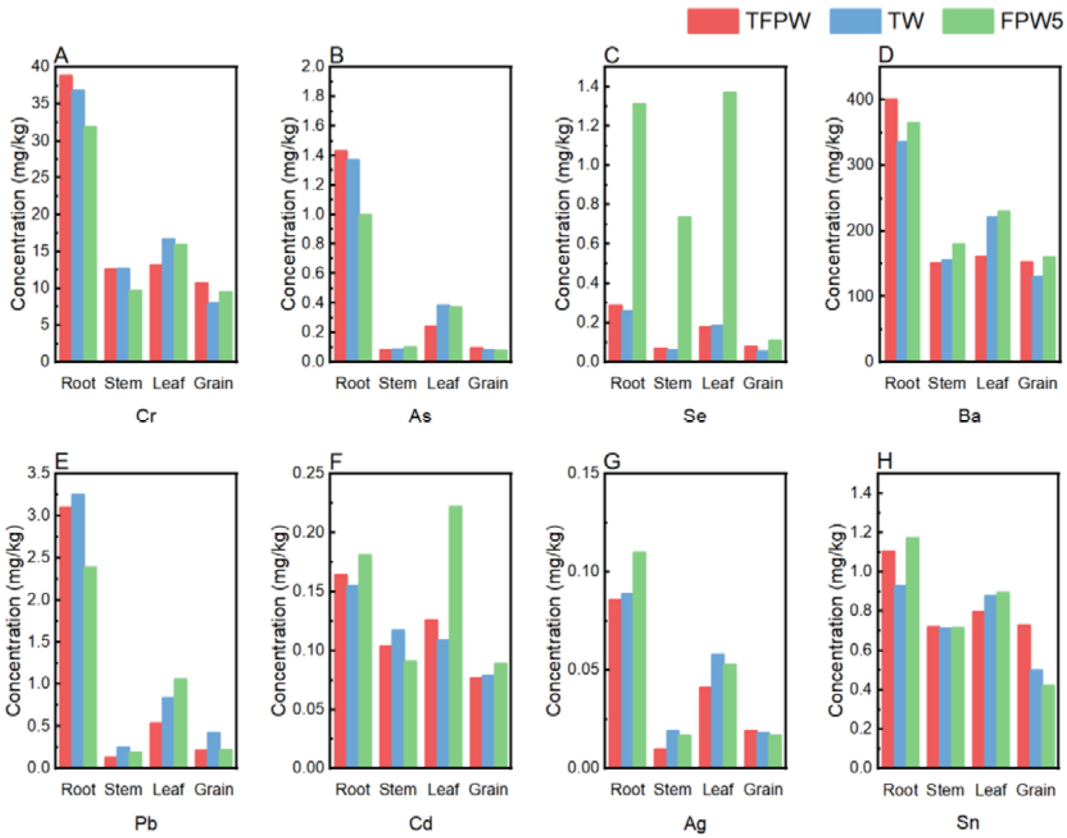


**Figure 4.** Concentrations of Cr (A), As (B), Se (C), Ba (D), Pb (E), Cd (F), Ag (G) and Sn (H) in wheat tissue including root, stem, leaf, and grain in wheat cultivated for 65 days applying different irrigation waters.

**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.[30, 55] 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.[56]

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

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

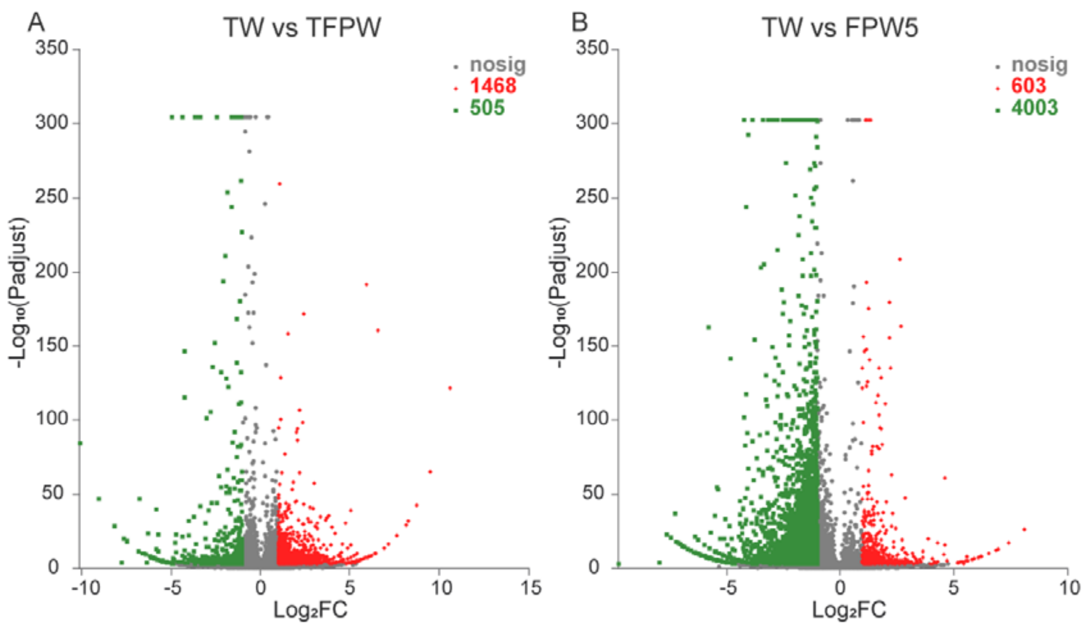


**Figure 5.** Volcano plots of differences in gene expression among (A) TW vs. TFPW and (B) TW vs. FPW5 analysis. Gray dots were not considered as significantly differentially expressed. Red (up-regulation) and green (down-regulation) dots indicate DEGs (|$\log_2$ (Fold Change) | > 1, Padjust (FDR) 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

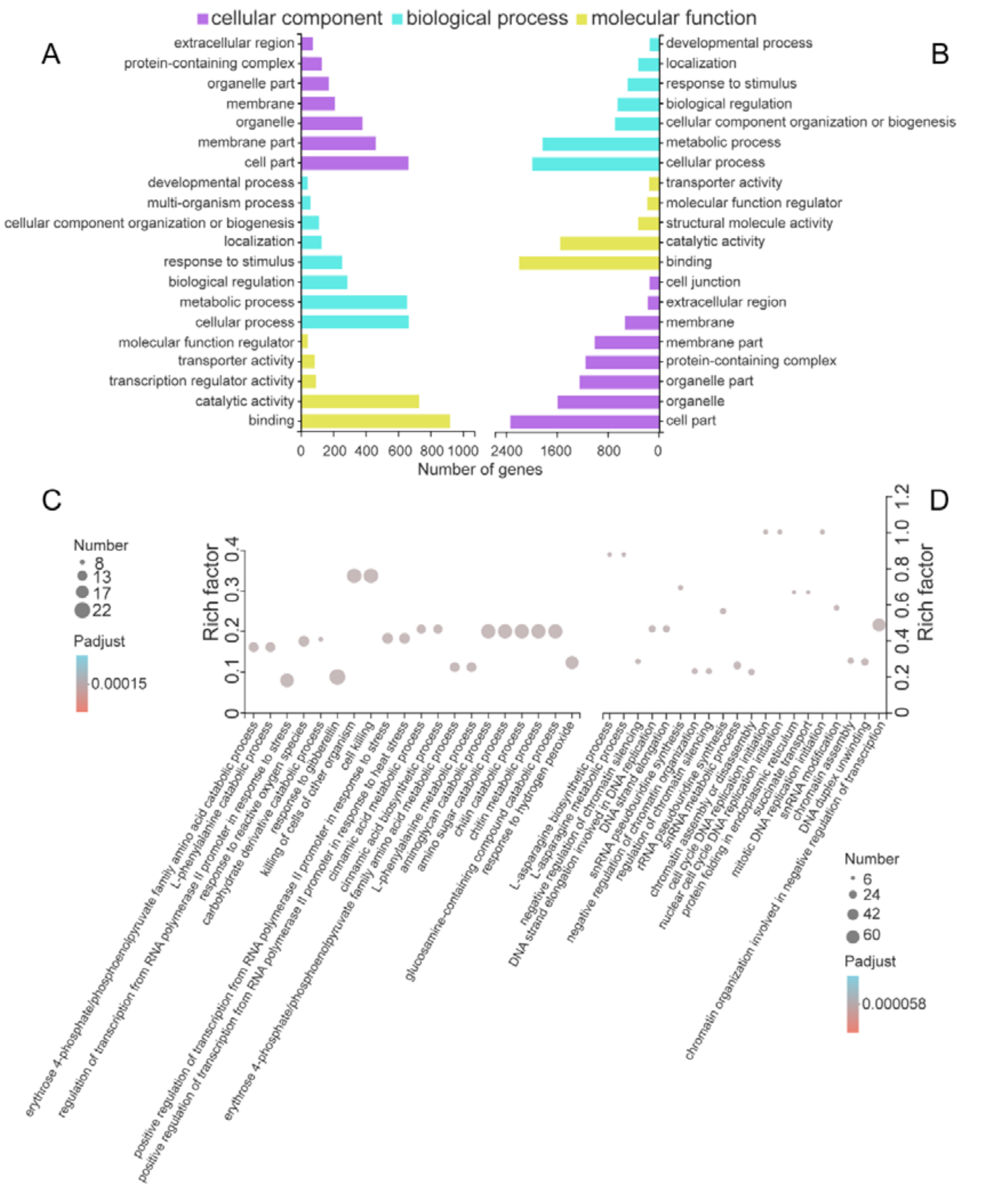


**Figure 6.** Function annotation and enrichment analysis of the intersection differentially expressed genes (DEGs) of wheat grain byased on gene ontology (GO) databases. (A, B): sub-categories of Gene Ontology (GO) terms of the DEGs in TW vs TFPW and TW vs FPW5 analysis, respectively. (C, D): bubble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

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

## ASSOCIATED CONTENT

### Supporting Information

The supporting information is available free of charge.

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### Notes

The authors declare no competing financial interests.

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