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Article

Impact of Rural Domestic Wastewater Irrigation on the Physicochemical and Microbiological Properties of Pakchoi and Soil

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Abstract: Great attention has been paid to the potential of wastewater irrigation as a sustainable water source, particularly due to water scarcity and water pollution issues. However, few studies have focused on its adverse effects and on the health risks it may pose. In this study, the physicochemical properties of soils and plants irrigated with rural domestic wastewater and associated microbiological risks were investigated. The results showed that sewage irrigation could increase the production of vegetables and improve soil fertility. While the nitrate content of plants increased significantly, pathogens on plants and in soils increased after irrigation with raw wastewater. In particular, there was a wide range of pathogenic bacteria in the phyllosphere, which may indicate risks if contaminated vegetables are consumed directly. Treated wastewater irrigation was not significantly different from controls, which were irrigated by tap water; consequently, it can be used as an

alternative water resource for agricultural irrigation. The presence of a wide spectrum pathogens in wastewater shows the necessity of long-term monitoring and further evaluation.

Keywords: rural domestic wastewater; pathogens; pakchoi; real-time quantitative PCR; phyllosphere

1. Introduction

The geographic distribution of water resources in China is very non-uniform, with 90% of surface water and 70% of groundwater distributed in various southern provinces. North China, which is situated in an arid and semi-arid climatic belt, receives less rainfall and experiences higher evaporation; it has consequently become one of the main water-deficient areas, particularly as the local demand for water has increased so rapidly with economic development and population growth, that it already surpasses local water supply capacity. Moreover, growing water pollution renders the situation even more serious. Water shortages and poor water quality are interacting with each other and seriously constraining economic and social development [1–3].

The scarcity of water resources has increasingly rendered recycled wastewater a valid substitute resource [4]. Treated wastewater is used directly for irrigation in many countries, especially in arid and semiarid areas. It contains essential plant nutrients, such as Ca, Mg, K, N, P and Fe, that are important for plant growth [5]. The reuse of treated wastewater avoids this being discharged into sensitive environments [6,7], helps conserve water resources, recycles nutrients (N and P) and minimizes pollution loads in receiving water bodies [8]. However, wastewater also contains various inorganic substances, including potential toxic elements and heavy metals (Cr, Cd, Pb, Ni and Hg) [9], which may be at phytotoxic levels or create health risks [10,11]. Safe and efficient management of this resource therefore cannot be achieved without appropriate monitoring [12], given that wastewater reuse also involves many potential risks to soil quality and to the growth and quality of crops [13–15].

Considerable research has been focused on the effect of wastewater irrigation on soil properties and on the quality of vegetables. Lado *et al.* [6] studied the effect of long-term secondary treated wastewater irrigation on soil chemical properties. Lv *et al.* [16] reported the effect of sewage irrigation on growth and development, yield and quality of potato. Similarly, Fayyad *et al.* [17] conducted a field experiment to investigate the effect of different treatments of potable and treated wastewater on the quality of tomato fruit. Singh *et al.* [18] described a year-long experiment to observe the effect of sewage wastewater irrigation on soil properties, crop yield and the environment. Maldonado *et al.* [19] conducted research on heavy metal content in soils under different wastewater irrigation patterns. However, little attention has been concentrated on the microbiological risk of wastewater irrigation.

The consumption of contaminated vegetables presents a potential risk to human health, with these containing large numbers of microbial pathogens in addition to heavy metals and other potential toxic elements. The presence of pathogenic bacteria may pose a health hazard for consumers of raw fruits and vegetables [20]. For example, *Arcobacter* spp. can cause persistent diarrhea, accompanied by abdominal pain, stomach cramps, nausea, vomiting, fever and other symptoms, and the infection rate is difficult to assess.

Given the significance of these risks, in this study, we tested the content of pathogenic microorganisms in rural domestic sewage and conducted research on pathogen residues on the surfaces of leaves and in soil of plant roots using real-time quantitative PCR. Additionally, the effects of wastewater irrigation on growth and nutrition plant indices were also investigated.

2. Experimental Section

2.1. Experimental Design

The experiment was performed in a greenhouse at Beijing Forestry University Forestry Science Co. Ltd. (40°00'96" N, 116°34'69" E, Beijing, China). The original experimental soil was collected from suburban farmland of Beijing. Table 1 lists the physicochemical characteristics of this soil.

Table 1. Physicochemical properties of the experimental soil sample.

Parameters	**Soil**	
Soil particle proportion (%)	>0.05 mm	31.25 sand
	0.05–0.02 mm	22.20
	0.02–0.005 mm	13.40 lime
	0.005–0.002 mm	6.30
	<0.002 mm	26.85 clay
pH	7.96	
EC (μs/cm)	571	
Organic matter (g/kg)	1.58	
NO_3-N (mg/kg)	8.56	
NH_3-N (mg/kg)	0.063	
Available P (mg/kg)	3.1	
Available K (mg/kg)	107.7	
Cu (mg/kg)	20.3	
Zn (mg/kg)	67.3	
Cd (mg/kg)	0.023	
Pb (mg/kg)	36.3	

Wastewaters were obtained from a rural domestic wastewater treatment plant, with a membrane bioreactor (MBR) processes (Figure 1), located in Huairou District of Beijing; the water samples' quality parameters are shown in Table 2.

Pakchoi seeds were purchased from the Chinese Academy of Agricultural Sciences (CAAS). The seeds were surface sterilized using 3% sodium hypochlorite solution disinfection for 15 min, followed by several cycles of rinsing with sterilized distilled water. The sterile seeds were then placed in a petri dish to sprout at room temperature and appropriate humidity.

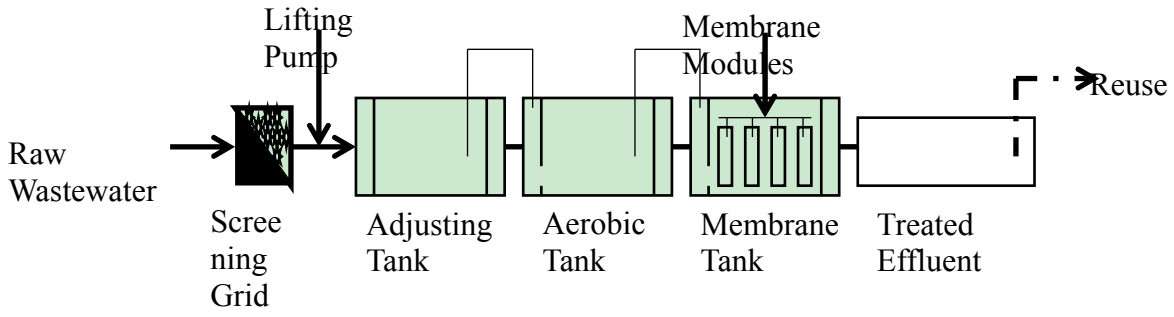

Figure 1. Process flow diagram of the wastewater treatment system with a membrane bioreactor.

Table 2. Physical and chemical properties of experimental water samples.

Parameters	Raw Wastewater	Treated Wastewater	Criteria of Tap Water [21]	Criteria of Irrigation Water [22]
pH	7.60	7.59	6.5–8.5	5.5–8.5
EC (μs/cm)	754	376	--	≤1,000
Salinity (mg/L)	415–566	207–282	≤1,000 (TDS)	-
Temperature (°C)	12.2	12.1	--	≤35
COD (mg/L)	346	35	--	≤100 [a] or 60 [b]
SS (mg/L)	26	21	--	≤60 [a] or 15 [b]
anionic surfactant (mg/L)	2.00	no detectable (<0.05)	≤0.3	≤5
TN (mg/L)	54.6	26.2	--	--
TP (mg/L)	4.97	7.66	--	--
Zn (mg/L)	0.153	no detectable (<0.006)	≤1.0	≤2
Cu (mg/L)	no detectable (<0.01)	no detectable (<0.01)	≤1.0	≤1
Pb (mg/L)	0.003	no detectable (<0.001)	≤0.01	≤0.2
Cd (mg/L)	0.0002	0.0002	≤0.005	≤0.01
Cl^- (mg/L)	44.2	56.1	≤250	≤350
NH_3 -N (mg/L)	45.7	8.59	≤0.5	--
NO_3- N (mg/L)	no detectable (<0.2)	16.0	≤20	--
Total bacteria count/mL	$(1.24 \pm 0.05) \times 10^6$	$(2.80 \pm 0.20) \times 10^5$	≤100	--
Total coliforms/mL	$6.05 \times 10^5 \pm 1.75 \times 10^5$	no detectable (<30)	no detectable	--
Fecal coliforms/mL	240,000	no detectable (<3)	no detectable	≤20 [a] or 10 [b]
Escherichia coli	8.9×10^4	no detectable (<30)	no detectable	--
Ascaris Lumbricoides (eggs/L)	34	no detectable	no detectable	≤2[a] or 1[b]

Notes: [a] Vegetables need processing, cooking or peeling; [b] rabbit food, melons and fruit; --, no data; TDS, total dissolved solids.

The soil was air-dried and sieved (20 mesh). Each pot (23 cm in top diameter, 16 cm bottom and 20 cm high) contained 2.5 kg dry soil and 20 seeds of pakchoi. After germination, the seedlings were thinned out, and 10 seedlings were left for growth. Irrigation was carried out from the seedling stage based on actual water requirement (irrigated approximately every 3 days, 100 mL at a time in one pot, 12 times and 1200 mL in total), with a specified amount of conventional fertilizer (0.15 g urea, 0.06 g calcium superphosphate, 0.15 g potassium sulfate per pot) added to the water. After the plants matured (about 6 weeks), the vegetables were harvested in summer 2013. Then, the second round of planting

was performed with the same method in autumn 2013. The third round of the experiments began on 19 March 2014 and extended until 5 May 2014 with the same method for the long-term investigation of the effect of wastewater irrigation.

Experimental treatments involved surface irrigation with three types of water: tap-water (DW), raw wastewater (WW) and treated wastewater (TW). At the same time, controls with nothing sowed and irrigated with tap water (CK-DW) and raw wastewater (CK-WW) were set up. Each treatment was applied in four replicates with a randomized block design.

2.2. Collection of Microbial Samples and DNA Extraction

After the third round of planting, vegetables were harvested at maturity (about 6 weeks) using sterile scissors, which separated the aboveground parts and roots. Harvested plants were placed in valve bags and transported immediately to the laboratory for analysis.

Leaves (10 g) were stuffed in an aseptic polypropylene tube containing 100 mL sodium phosphate buffer (0.2 M, pH 7.0), shaken for 30 min in a vapor-bathing constant temperature vibrator and then treated by ultrasonic waves (40 kHz) for 30 min using ultrasonic agitation (Ningbo Xingzhi Biological Technology Co. Ltd., Ningbo, China). The buffer solution was then filtered through a 0.22-μM cellulose nitrate microporous composite membrane. The microbes from the leaves were accumulated on the membrane, which was stored at −80 °C subsequently.

Surface soil and rhizosphere soil samples of each pot were air-dried in a sterile chamber at room temperature, then sieved through a 20 mesh sieve and stored at −80 °C.

Water samples were filtered through 0.22-μm membranes to collect microbes, then the membrane was put in a sterile centrifuge tube and stored at −80 °C.

Genomic DNA of the above microbes' samples was extracted using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions.

2.3. Physical and Chemical Properties Detection of Plants and Soil

The pH and electrical conductivity (EC) of soil samples were detected in aqueous extract (soil:deionized water = 1:5) using a multi-parameter water quality monitoring instrument [23]. Organic matter was measured using the potassium bichromate titrimetric method. The activity of urease and phosphatase was assayed based on the method of Tabatabai [24].

Fresh and dry weights of mature plants were measured by the gravimetric method, and the plants' height was measured by a ruler. The salicylic acid method was used to measure the vegetables' nitrate content [25], while soluble sugar was determined by the anthrone colorimetry method [26]. The Coomassie Brilliant Blue G250 staining method was used to determine the concentration of soluble protein in vegetable samples [27]. The vegetables' vitamin C content was determined by the 2,6-diohloroindophenol titration method [28].

2.4. Molecular Assays

This study focused on common pathogens, including pathogens present in the environment and several pathogens that have emerged as a focus of research interest in recent years. Specific pathogens considered were: *Aeromonas hydrophila*, *Arcobacter* spp., *Bacillus cereus*, *Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli*, *Legionella* spp. and *Mycobacterium* spp.

Traditional PCR was conducted to confirm the presence of pathogens. Based on the PCR results, positive pathogens were investigated further, with the corresponding primer pairs for each target and expected product size shown in Table 3.

Table 3. Primers of pathogenic bacteria for real-time PCR assays.

Pathogenic Bacteria	Targeted Gene	Primer Sequences	Amplicon Size (bp)	Ref.
Aeromonas hydrophila	Cytolytic enterotoxin	AHCF1: GAGAAGGTGACCACCAAGAACA AHCR1: AACTGACATCGGCCTTGAACTC	232	[29]
Arcobacter spp.	23S rRNA	ARCO1: GTCGTGCCAAGAAAAGCCA ARCO2: TTCGCTTGCGCTGACAT	331	[30]
Bacillus cereus	16S rRNA	F: TCGAAATTGAAAGGCGGC R: GGTGCCAGCTTATTCAAC	288	[31]
Clostridium difficile	16S rRNA	Clo-16F: TTGAGCGATTTACTTCGGTAAAGA Clo-16R: CCATCCTGTACTGGCTCACCT	157	[32]
Clostridium perfringens	16S rRNA	Clp-F: ATGCAAGTCGAGCGA(G/T)G Clp-R: TATGCGGTATTAATCT(C/T)CCTTT	120	[32]
Escherichia coli	*uidA*	Eco-F: CTGCTGCTGTCGGCTTTA Eco-R: CCTTGCGGACGGGTAT	205	[33]
Legionella spp.	16S rRNA	LEG448:GAGGGTTGATAGGTTAAGAGC LEG858:GTCAACTTATCGCGTTTGCT	430	[34]
Mycobacterium spp.	16S rRNA	Myco F: ATGCACCACCTGCACACAGG Myco R: GGTGGTTTGTCGCGTTGTTC	470	[35]

Positive PCR products were purified with the E.Z.N.A.™ Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA) and ligated to a pGEM-T Easy vector system I (Promega, Madison, WI, USA). Recombinant plasmids were transferred into *E. coli* DH5α-competent cells (Biomed, Beijing, China) and coated on LB agar plates containing ampicillin, X-gal and IPTG, as recommended by the manufacturer. PCR was performed to confirm the sizes of insert fragments and sequenced by Ruibio BioTech Co., Ltd. (Beijing, China). The gene sequences were aligned by BLAST [36] on NCBI. Plasmid DNA was extracted using the E.Z.N.A.™ Plasmid Mini Kit I (Omega Bio-tek, Norcross, GA, USA). The gene copy number was calculated according to concentrations of plasmid DNA determined by a Nanodrop2000 UV-VIS Spectrophotometer (Thermo Scientific, Waltham, MA, USA) [37]. The plasmid was diluted using the ten-fold dilution method and used as the standard curve for the quantitative PCR.

Real-time PCR amplification was performed in a 20-μL reaction volume using GoTaq qPCR Master Mix (Promega, Madison, WI, USA) on a Stratagene Mx3005P instrument (Agilent Technology, Santa Clara, CA, USA). The PCR mixture contained 10 μL of Master Mix, 0.5 μL of each primer

(10 μM·L^{-1}), 2 μL of template DNA and 7 μL nuclease-free water. The reaction procedure was as follows: 95 °C for 2 min, 45 cycles at 95 °C for 15 s, 58–62 °C for 45 s, 72 °C for 45 s. All experiments were performed in triplicate. For each run, template DNA was replaced with double-distilled water as a negative control.

2.5. Statistical Analysis

Statistical analyses were performed with Microsoft Office Excel 2013 and SPSS Statistics 19.0. In all cases, differences were considered to be significant if the *p*-value for the χ^2 test was less than 0.05.

3. Results and Discussion

3.1. Effects of Sewage Irrigation on Soil Properties

In order to investigate the influence of rural domestic wastewater on the soil and plant properties, we compared the basic soil chemical properties after irrigating with tap and wastewater for three seasons. The results showed that the soil pH decreased after planting and irrigation, mainly because of the neutralization. There was no significant difference in soil pH between non-planted soils irrigated with tap water and wastewater, while soil pH was significantly higher in non-planted than in planted soil (Figure 2), because the pH of plant root exudates is lower than soil. On the other hand, it can be seen from Figure 1 that soil pH declined significantly after planting and irrigation, regardless of whether this took place with wastewater or tap water. The result indicated that wastewater irrigation may not significantly affect soil pH, but that vegetables may indicate the significant influence of this parameter. The pH of all of the soil samples meets the standards for farmland soil [38].

Electrical conductivity(EC) and salinity (positively correlated with each other) of soil irrigated with wastewater were significantly lower than in soil irrigated with tap water and treated wastewater, due to the wastewater irrigated plants growth being better, so that more mineral salts were absorbed by the plant. EC and salinity of soil increased regardless of the source of irrigation water, due to the mineral salts from irrigation water being concentrated in soil with water evaporation. Soil salinities of all soil samples are lower than the standard limit (1.0 g/kg) of the environmental quality evaluation standards for farmland for edible agricultural products [38].

The difference in organic matter (OM) among different treatments is not significant ($p > 0.05$). However, we can see from Figure 1 that OM increased after the irrigation. This might be because the irrigation promoted lichen and autotrophic bacteria growth. Soil organic matter contains a large number of major elements and trace elements that are essential for plant growth, and it is therefore one of the important indicators of soil fertility [39]. On the other hand, growth-promoting substances, such as vitamins, amino acids, plant hormone and gibberellin, are released when soil organic matter degrades, stimulating the growth of higher plants and microorganisms [40].

The urease activities of soil irrigated with wastewater have no significant difference with the control and those of soil irrigated with treated water. This activity increased in all treatments after the experiment, possibly because N and P fertilizers were added during the course of the experiment. The phosphatase activity of soil irrigated by wastewater was not significantly different than that of soil irrigated with tap water or treated wastewater. However, values were lower in planted treatments than

in unplanted ones. The results indicate that changes in N and P fertilizers led to an increase of phosphatase activity, and P was greatly in demand by the plant. The conversion speed of organic and inorganic nutrients in soil mainly depends on the enzymatic reaction of redox enzymes. Soil enzyme activity could reflect the strength and direction of biochemical processes and is an important index for evaluating soil fertility and self-purification ability. It is therefore important to improve soil enzyme activity in order to improve the soil ecological environment and soil fertility.

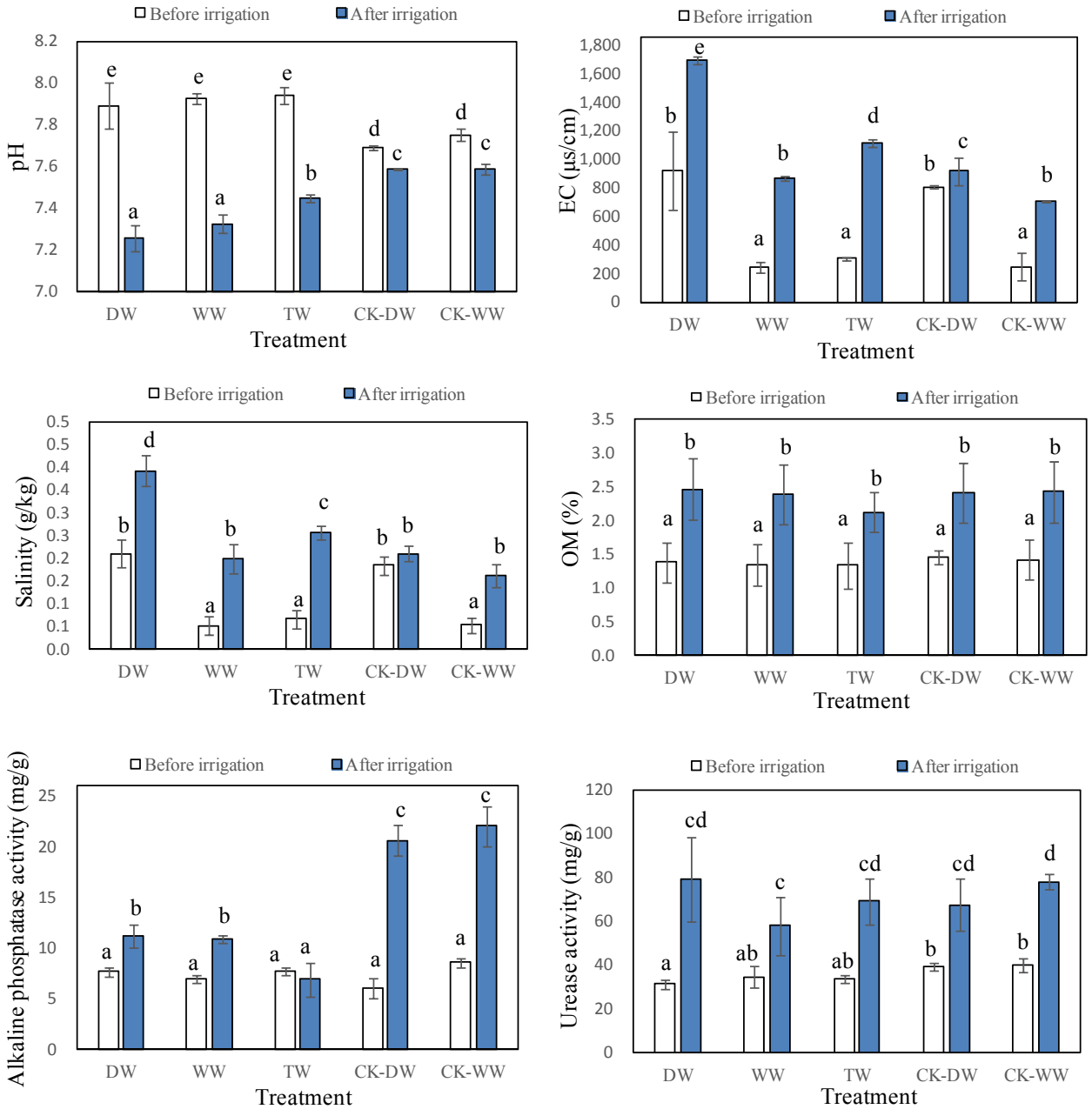

Figure 2. Properties of soil before and after irrigation of the third season planting. Abbreviations: DW, treatment irrigated with tap water and plant; WW, treatment irrigated with wastewater and plant; TW, treatment irrigated with treated wastewater and plant; CK-DW, treatment with no plant and irrigated with tap water; CK-WW, treatment with no plant and irrigated with wastewater. The standard deviation of the mean (n = 3) is shown; different letters demonstrate a significant difference at $p < 0.05$ based on ANOVA test analysis.

3.2. Effects of Sewage Irrigation on Plant Properties

Table 4 shows the quality parameters of pakchoi after the experiment. The height and fresh weight of vegetables irrigated with wastewater were significantly higher than those of vegetables irrigated with tap water and slightly higher than those of vegetables irrigated with treated wastewater. The height and fresh weight of vegetables irrigated with treated wastewater were higher than those of vegetables irrigated with tap water, but the difference was not significant ($p > 0.05$). The result indicates that wastewater irrigation could greatly increase the output of pakchoi and that irrigation with treated wastewater led to a slight increase in output.

Table 4. Quality parameters of pakchoi following irrigation with DW (control), WW and TW.

Samples	Height (cm)	Fresh Weight (g)	Soluble Sugar (%)	Soluble Protein (mg/g)	Nitrate (g/kg)	Vc (mg/g)
DW	15.25 ± 0.76 [a]	5.06 ± 0.57 [a]	0.18 ± 0.04 [a]	9.74 ± 0.33 [a]	1.19 ± 0.10 [a]	1.02 ± 0.11 [a]
WW	19.18 ± 0.76 [b]	10.83 ± 1.22 [c]	0.16 ± 0.01 [a]	10.16 ± 0.42 [a]	1.53 ± 0.09 [a,b]	1.06 ± 0.19 [a]
TW	17.75 ± 0.89 [a,b]	7.31 ± 1.05 [a,b]	0.15 ± 0.01 [a]	9.26 ± 0.11 [a]	1.58 ± 0.07 [b]	0.89 ± 0.08 [a]

Notes: The standard deviation of the mean ($n = 3$) is shown; [a,b,c] different letters demonstrate a significant difference at $p < 0.05$ based on ANOVA test analysis.

Wastewater irrigation did not make significant changes in the content of soluble sugar, soluble protein and vitamin C (Vc), which means that wastewater irrigation had no clear influence on the nutritional quality of pakchoi. However, nitrate content significantly increased after irrigation with wastewater. Human consumption of vegetables in which nitrates exceed relevant standards may be seriously harmful to health; for this reason, even if the nitrate content of vegetables irrigated with wastewater conformed to safety standards (agricultural product-safety requirements for non-environmental pollution vegetables: GB18406.1-2001), further studies should be carried out on this aspect.

3.3. Quantitative PCR Detection of Pathogenic Bacteria

Real-time PCR detection of pathogenic microorganisms was conducted to monitor the amount of pathogens in wastewater, plant phyllosphere, rhizosphere and non-rhizosphere soil. The number of pathogenic bacteria in each sample is shown in Table 5 and Figure 3.

Table 5. Number of pathogens in wastewater and treated wastewater.

Pathogens	WW (Copies/L) [a]	TW (Copies/L) [b]
Aeromonas hydrophila	$(5.48 \pm 0.22) \times 10^{10}$	$(1.96 \pm 0.02) \times 10^{7}$
Arcobacter spp.	$(1.01 \pm 0.04) \times 10^{11}$	$(1.45 \pm 0.01) \times 10^{8}$
Bacillus cereus	$(2.01 \pm 0.10) \times 10^{8}$	$(2.02 \pm 0.06) \times 10^{7}$
Clostridium difficile	$(4.20 \pm 0.10) \times 10^{6}$	$(6.35 \pm 0.11) \times 10^{4}$
Clostridium perfringens	$(2.85 \pm 0.09) \times 10^{9}$	$(5.00 \pm 0.25) \times 10^{4}$
E. coli	$(1.02 \pm 0.06) \times 10^{9}$	$(2.40 \pm 0.36) \times 10^{5}$
Legionella spp.	$(1.27 \pm 0.09) \times 10^{6}$	$(6.10 \pm 0.77) \times 10^{5}$
Mycobacterium spp.	$(5.71 \pm 0.40) \times 10^{7}$	$(3.17 \pm 0.35) \times 10^{7}$
Total bacteria	$(3.13 \pm 0.38) \times 10^{11}$	$(1.56 \pm 0.08) \times 10^{10}$

Notes: The standard deviation of the mean ($n = 3$) is shown; different letters ([a, b]) demonstrate a significant difference at $p < 0.05$ based on ANOVA test analysis.

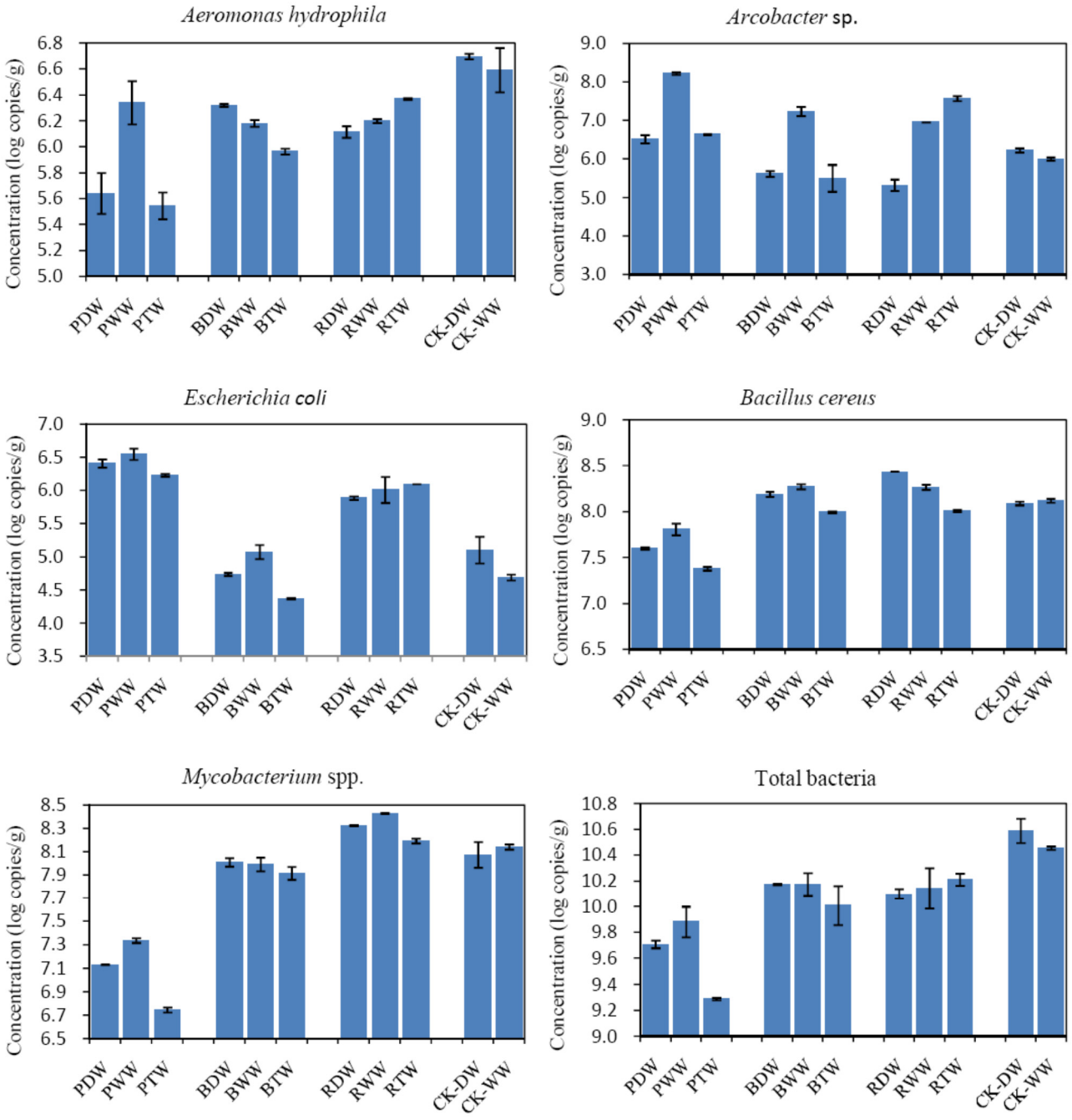

Figure 3. Pathogenic bacteria in each sample. Abbreviations: PDW, phyllosphere irrigated with tap water; PWW, phyllosphere irrigated with wastewater; PTW, phyllosphere irrigated with treated wastewater; BDW, non-rhizosphere irrigated with tap water; BWW, non-rhizosphere irrigated with wastewater; BTW, non-rhizosphere irrigated with treated wastewater; RDW, rhizosphere irrigated with tap water; RWW, rhizosphere irrigated with wastewater; RTW, rhizosphere irrigated with treated wastewater.

In raw wastewater and treated wastewater, Aeromonas hydrophila, Arcobacter spp., Bacillus cereus, Clostridium difficile, Clostridium perfringens, *E. coli*, *Legionella* spp. and Mycobacterium spp. were detected. Of these, Clostridium difficile, Clostridium perfringens and *Legionella* spp. were not found in other samples. Most of the pathogens decreased after treatment. The result shows that although the

sewage treatment could remove pathogenic microorganisms significantly, treated wastewater would still contain numerous pathogens, the risks to human health of which were not clear till now. Further study should be conducted for the standard limit of these pathogens in treated wastewater.

Aeromonas hydrophila was very common in raw wastewater, and its numbers were still very high after treatment. There were no significant differences between the frequency of *A. hydrophila* in the phyllosphere, rhizosphere soil and non-rhizosphere soil. There were also no significant differences between treatments irrigated with raw wastewater and treated wastewater, as compared to the control. The quantity of *A. hydrophila* was still up to seven orders of magnitude. *A. hydrophila* is widely distributed in different aquatic environments in nature; it is an opportunistic pathogen, which could lead to food poisoning, waterborne diseases, infectious diarrhea, secondary infections and sepsis. It is therefore very dangerous to come into contact with *A. hydrophila* directly or by eating vegetables infected with the bacteria.

The number of *Arcobacter* spp. in wastewater was very high and could reach the same order of magnitude as the total bacteria. Quantities of *Arcobacter* spp. in the phyllosphere were higher than in soil; similarly, the bacterium was more frequent in plants irrigated with wastewater than in the control, while plants irrigated with treated wastewater were not significantly different from the control. *Arcobacter* spp. is a foodborne and waterborne pathogen. Of all of the species, *A. cryaerophilus*, *A. butzleri* and *A. skirrowii* are pathogens associated with human gastroenteritis and bacteremia [41,42]. So far, the pathogenesis of *Arcobacter* spp. is not clear. The possible infection pathways of *Arcobacter* spp. may include direct contact or eating contaminated food or drinking water.

Human pathogenic bacteria can be colonized both in phyllosphere and rhizosphere, and accordingly, some outbreaks of foodborne illnesses have been reported, which were associated with human consumption [43]. Contamination with *E. coli* in all treatments followed the order of phyllosphere > rhizosphere > non-rhizosphere. This was quite different from other bacteria mentioned above; *E. coli* more easily to survived in the phyllosphere, which might be more easily colonized than other pathogens. Some studies have proven the ability of intestinal pathogens, such as *E. coli*, *Salmonella* spp. and *Bacteroides*, to internalize and colonize into plant leaves through wounds, stomata or root uptake and migration [44,45], which could be contaminated via rainwater and irrigation water. *E. coli* is a foodborne pathogen used as a health standard for drinking water and food. Pathogenic *E. coli* could cause a disease outbreak through contaminated drinking water, food and aquatic environment, which could potentially be life-threatening.

In summary, there were no significant differences between the control and plants irrigated with treated wastewater, suggesting that treated wastewater can be used as a valid alternative for agricultural reuse. This result is coincident with the report by Jang *et al.* [46], which concluded that reclaimed wastewater irrigation in rice paddies presents no increased human health or eco-environmental risks. However, the pathogens in both phyllosphere and soils irrigated by raw wastewater showed higher contents than the control. Pathogens in sewage are directed into the soil through irrigation, and soil can provide more suitable conditions (such as temperature, moisture, nutrients, and so on) for the survival of microorganisms. The potential risks of pathogens in the wastewater irrigation process need further assessment.

4. Conclusions

Although rural domestic wastewater irrigation could improve soil fertility and plant yields, pathogens might be transported into soil through irrigation with raw wastewater and then transmitted to the plant in greenhouse conditions. Since vegetables contaminated with pathogenic bacteria are an important pathway of intestinal infectious diseases, more attention should be paid to the potential risks of wastewater irrigation in greenhouses for vegetable planting. The pathogens in the wastewater irrigation process should be further investigated in field conditions.

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Author Contributions

Bo Yang, Xiao Kong, Bingjian Cui, Decai Jin and Zhihui Bai designed and carried out the experiments and analyzed the data. Xiao Kong wrote the main manuscript text. All authors reviewed and improved the manuscript. Zhihui Bai supervised the project.

Conflicts of Interest

The authors declare no conflict of interest.

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