

Article

Monitoring Opportunistic Pathogens in Domestic Wastewater from a Pilot-Scale Anaerobic Biofilm Reactor to Reuse in Agricultural Irrigation

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Abstract: Wastewater reuse for agricultural irrigation in many developing countries is an increasingly common practice. Regular monitoring of indicators can help to identify potential health risks; therefore, there is an urgent need to understand the presence and abundance of opportunistic pathogens in wastewater, as well as plant phyllosphere and rhizosphere. In this study, an anaerobic biofilm reactor (ABR) was developed to treat rural domestic wastewater; the performance of pollutants removal and pathogenic bacteria elimination were investigated. Additionally, we also assessed the physicochemical and microbiological profiles of soil and lettuces after wastewater irrigation. *Aeromonas hydrophila*, *Arcobacter* sp., *Bacillus cereus*, *Bacteroides* sp., *Escherichia coli*, *Legionella* sp., and *Mycobacterium* sp. were monitored in the irrigation water, as well as in the phyllosphere and rhizosphere of lettuces. Pathogens like *B. cereus*, *Legionella* sp. and *Mycobacterium* sp. were present in treated effluent with relatively high concentrations, and the levels of *A. hydrophila*, *Arcobacter* sp., and *E. coli* were higher in the phyllosphere. The physicochemical properties of soil and lettuce did not vary significantly. These data indicated that treated wastewater irrigation across a short time period may not alter the soil and crop properties, while the pathogens present in the wastewater may transfer to soil and plant, posing risks to human health.

Keywords: domestic wastewater; bacterial pathogens; qPCR; agricultural reuse

1. Introduction

Currently, water scarcity has become a global problem; many countermeasures have been taken to meet the challenge in China and other countries. Reclaimed wastewater provides an effective leverage to complement other conventional water resources supply, as well as alleviate water quality problems associated with pollutants discharge. In particular, wastewater reuse for agricultural irrigation is regarded as a viable way to address the imbalance between water demand and supply, and may contribute to a sustainable agriculture [1,2]. However, wastewater reuse for agricultural irrigation may have two implications: the first may affect soil fertility and crop productivity, and the second may pose human health risks and environmental hazards due to the accumulation of chemical and microbiological contaminants [3]. Wastewater and agriculture are two sectors where the economic and environmental benefits of joint water management have been demonstrated through several case studies around the world [4,5]. Conventional end-of-pipe solutions for wastewater treatment have been criticized from a sustainable view-point, in particular regarding recycling of nutrients in effluent, instead of being discharged directly [6]. A novel planning model, consisting of a reuse-centric performance assessment and optimization model to help design wastewater treatment plants for reuse

in agriculture. The coupled application indicated that wastewater supplementation could increase profits by \$20 million annually, and conserve 35 mm³ of water in local rivers each year in China [7].

Wastewater is usually considered as a reservoir and vehicle for various human pathogens, and discharges into the natural environment may pose direct or indirect exposure risks. Comparing estimates from quantitative microbial risk analyses and epidemiological studies have raised public concerns regarding the health risks in wastewater irrigation [8,9]. Apart from conventional indexes related to water quality criteria, there is a need to be concerned about the microbiological quality of irrigation water in terms of its effects on the soils and crops. In particular, the principal concern was focused on the microbial contamination of eaten raw or simple processed vegetables and fruits, such as lettuces and tomatoes [10,11]. Opportunistic pathogens are defined as pathogens that usually do not cause diseases in healthy individuals, however, may cause disease in the immunocompromised population [12]. During the past decade, the numbers of human infections caused by opportunistic pathogens has increased dramatically. Wastewater is commonly viewed as a vector that transfers opportunistic pathogens to soil and fresh produce during irrigation. A study has identified the microbial risk factors in the preharvest fruits and vegetables, including *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7, and demonstrated that contaminated water was a crucial issue for microbiological safety of fresh produce [13]. Contaminated water may be a significant source of foodborne pathogens associated with ready-to-eat fresh vegetables. To better understand the transfer of *Salmonella enterica* from contaminated water to the crops, the persistence of *Salmonella enterica* in the phyllosphere and rhizosphere of parsley was investigated following spray irrigation with contaminated water [14]. Utilization of reclaimed water for irrigation involved in high microbial loads that depended on the degree of sewage treatment, raw or partially treated wastewater for agricultural irrigation should be considered for assessment of microbial water quality.

Many reports were mainly focused on fecal indicators such as Fecal Coliforms and *Escherichia coli* to evaluate potential health risks, neglecting pathogenic bacteria, viruses, and protozoa groups [15,16]. Consequently, current standards and guidelines for microbiological quality of wastewater may result in underestimation or overestimation of the risks for public health. The aim of this study was to evaluate the removal of contaminants using an anaerobic biofilm reactor to allowable irrigation limits, and characterize the presence of opportunistic pathogens from the processes to the irrigated soils and vegetables.

2. Materials and Methods

2.1. Reactor Operation and Experimental Design

The experimental site is located in the suburb of Beijing. The reactor has a self-made set of small-scale anaerobic reactors with nylon materials placed into the reactor in a radial pattern. The reactor was composed of three stages and a cylinder shaped with a conical bottom, which has shown to be capable of conducting loads of 360 L at the hydraulic retention time (HRT) of 72 h (Figure 1). No disinfection facility was installed with the reactor.

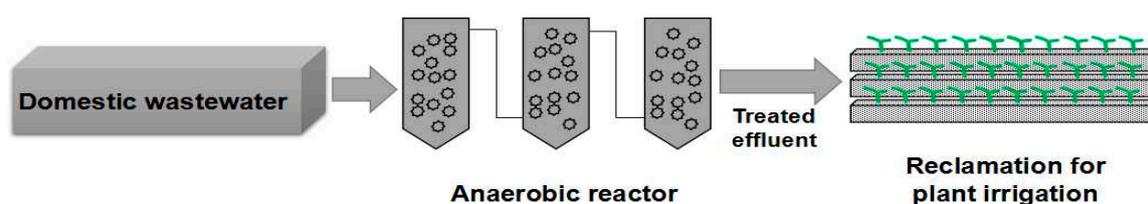


Figure 1. Schematic diagram of domestic wastewater from processing to recycling.

Lettuce (*Lactuca sativa* L.) was used as the testing object to evaluate microbial contamination irrigated with different water sources. The lettuce seeds were planted under an open field in 2×2 m trial plots. A total of 12 plots were designed as three irrigation treatments using potable water (PW), raw wastewater (RW), and treated effluent (TE). Surface irrigation was performed from the seedling stage; each plot was irrigated according to water demand (5 L per square meter every two days in one plot). The experiment plots lasted for approximately two months from planting to harvesting. Each treatment was applied in four replicates with a randomized block design.

2.2. Sample Collection, Processing, and DNA Extraction

For physicochemical parameters and pathogenic bacteria testing, raw wastewater and treated effluent samples were collected between June and October of 2017 at a monthly interval. Approximately 1 L water samples were pooled in sterilized polyethylene bottles in triplicates. The water samples were transported to the laboratory immediately for further analysis. The pH and electrical conductivity (EC) were tested using a portable multi-parameters meter (HACH HQ40d, HACH Company, Loveland, CO, USA) on site, and chemical oxygen demand (COD), total nitrogen (TN), ammonia nitrogen ($\text{NH}_3\text{-N}$), and total phosphorus (TP) were measured based on the standard methods of China's discharge standard of water pollutants for municipal wastewater treatment plants [17]. The levels of total heterotrophic bacteria, Total Coliforms, and *Salmonella* were determined by spread plate method using selective chromogenic media (Hopebio, Qingdao, China). Fecal Coliforms were enumerated using the multi-tube fermentation method with EC broth (Hopebio, Qingdao, China). Helminths were detected by microscopic examination. For pathogens detection, 100 mL of each water sample was filtered through $0.22 \mu\text{m}$ mixed cellulose membranes (47 mm diameter, Millipore Filter Corporation, Bedford, MA, USA) with glass filtrator (Jinteng T-50, Jinteng Experimental Equipment Corporation, Tianjin, China). The filter membrane was folded in half four times and placed into a 1.5 mL microcentrifuge tube, and then frozen at -80°C until required analyses.

Samples of lettuces were collected using random sampling two months after sowing. The leaves were sampled aseptically using sterilized scissors and placed into sterile homogeneous bags (Hopebio, Qingdao, China). The method for collection of epiphytic microbial pellets was performed as described by Zhang et al. [18]. Briefly, leaves were aseptically transferred into polypropylene tubes containing 100 mL potassium phosphate buffer (0.1 M, pH 7.0), and then ultrasonication was performed at a frequency of 40 KHz for 7 min in an ultrasonic cleaning bath to dislodge the microbial pellets from the leaf surface. The rhizosphere samples containing soil were sampled from each plot by cutting above the ground with a sterile blade, and the soil adhering to the lettuce was removed by shaking loosely. The soil samples homogenized and sieved after freeze drying to wipe off crude particles for further analyses.

Genomic DNA from each sample was extracted in Lysing Matrix tubes using FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol. Raw DNA was purified, and the concentrations were determined by Nanodrop-2000 spectrophotometer (Nanodrop Inc., Wilmington, DE, USA) and frozen at -80°C until used.

2.3. Preparation of qPCR Standard Curves

Conventional PCR was conducted to identify the targeted microbes, and the PCR product was loaded onto 1% (W/V) agarose gel electrophoresis to confirm expected band size.

The obtained PCR products were gel-purified and ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA), then transformed into competent *E. coli* DH5 α (Biomed, Beijing, China). The positive clones were screened on X-Gal-IPTG-Ampicillin-indicator plates by color-based recombinant selection, and further confirmed insert fragments by PCR amplification with T7 and SP6 primers and sequenced by Ruibo BioTech Co, Ltd. (Beijing, China). The nucleotide sequences were submitted and aligned by BLASTn in NCBI. After determination, the positive clones were selected to extract plasmid DNA using E.Z.N.A.[®] Plasmid Mini Kit Spin Kit (Omega Bio-tek, Doraville, GA, USA)

according to manufacturer's instructions. The concentration of the plasmid DNA was determined by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), which was used as pathogenic gene standards. The gene copy number was calculated directly from the concentration of the extracted plasmid DNA [19]. Concentrations of these pathogens in wastewater, phyllosphere, and rhizosphere samples were quantified by assaying 10-fold serial dilutions of standard plasmid DNA.

2.4. Quantitative PCR Assay

Quantitative analysis of selected pathogens in prepared samples was performed by real-time quantitative PCR with SYBR green. The sequences of the primers targeting genes are listed in Table S1. Amplification was performed with the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). 2 µL of template DNA was added to 23 µL of reaction mixture in a total volume of 25 µL containing 2 × SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Biotechnology, Dalian, China), 0.2 µM of each primer. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 30 s, 45 cycles of 95 °C for 5 s, 53–60 °C for 30 s, 72 °C for 30 s, followed by a melt curve stage: from 65 °C, gradually increasing 0.5 °C·s⁻¹ to 95 °C. All qPCR samples were run in technical triplicate, and corresponding negative (DNase/RNase-free distilled water) control was included. PCR efficiency and correlation coefficients of the standard curves were in the range of 89.40%–110.00% and R^2 from 0.989 to 0.999, indicating a good linear relationship to quantitative requirements (Table S2). The detection limit for qPCR was 10–100 copies of plasmid from three independent runs.

2.5. Data Analysis

To test the linearity and the dynamic range of the real-time quantitative PCR reaction, the external standard curves were generated by 10-fold serial dilutions of known copy number of plasmid DNA. The qPCR results were analyzed with CFX Manager Software (v 2.0, Bio-Rad), and the amplification efficiency (E) was estimated by the formula $E = (10^{-1/\text{slope}}) - 1$. All graphs were generated by OriginPro 8.0 (OriginLab, Northampton, MA, USA). Data were analyzed using One-way ANOVA and Tukey's HSD test ($p < 0.05$) was used for comparison between treatments. All statistical analyses were performed using SPSS 20.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Operational Performance of the Reactor

All the quality parameters of wastewater were measured according to the discharge standard of pollutants for municipal wastewater treatment plant [17]. Details on reactor parameters are described in Table 1. During the five months of this study, there was a steady increase in chemical oxygen demand (COD) removal efficiencies of treated wastewater from 75% to 92%. The concentrations of TN, NH₃-N, and TP were steadily increased in effluent, and its direct discharge would degrade surface water quality. The treatment process achieved 1–2 logs removal of heterotrophic bacteria and Total Coliforms. Total bacteria count in the raw wastewater was 10⁸ per 100 mL and 10⁶–10⁷ for the treated effluent, 10² for the potable water. No Faecal Coliforms were found in the treated effluent and potable water.

Table 1. Operation parameters of raw domestic wastewater and treated effluent.

Water Parameters	Raw Wastewater	Treated Effluent	Potable Water	Criteria of Irrigation Water [20]	Discharge Standard [17]
pH	6.75–7.41	6.36–7.86	7.53	5.5–8.5	6–9
EC ($\mu\text{S}\cdot\text{cm}^{-1}$)	556–814	382–866	293	≤ 1000	-
COD ($\text{mg}\cdot\text{L}^{-1}$)	135–218	11–54	10	$\leq 100^a$ or 60^b	50–60
TN ($\text{mg}\cdot\text{L}^{-1}$)	11.8–22.5	13.2–16.4	<0.5	c^-	15–20
$\text{NH}_3\text{-N}$ ($\text{mg}\cdot\text{L}^{-1}$)	8.4–17.7	6.7–17.6	0.16	-	5–8
TP ($\text{mg}\cdot\text{L}^{-1}$)	2.5–5.2	2.0–7.9	0.21	-	0.5–1
Total bacteria (CFU $\cdot\text{mL}^{-1}$)	3.6×10^6 – 4.3×10^6	1.67×10^4 – 3.4×10^5	<100	-	-
Total Coliforms (CFU $\cdot\text{mL}^{-1}$)	1.0×10^4 – 6.0×10^5	1.0×10^3 – 7.17×10^4	not detectable	-	-
Faecal Coliforms (CFU $\cdot 100\text{ mL}^{-1}$)	2.4×10^4	not detectable (<3)	not detectable	$\leq 20^a$ or 10^b	1–10

^a Vegetables need processing, cooking or peeling; ^b rabbit food, melons and fruit; ^c -: no data.

3.2. Physicochemical Properties of Soils and Lettuces

The physicochemical and microbiological properties of the soils irrigated by potable water and wastewater are summarized in Table 2. The observed data showed no difference in pH among the soils irrigated with potable water and wastewater, while EC was significantly higher in soils irrigated with wastewater than potable water. The organic matter (OM) content was similar in all soils irrigated with different water sources. Compared with PW, the concentrations of total nitrogen and available potassium in the soils irrigated with RW and TE were higher; these nutrients might be absorbed and converted from soils to crops [21]. As regards heavy metals, the average concentrations of Pb, Cu, Zn, Hg, As, Cr, and Cd were comparable in all treatments, except for the lower concentration of Zn and Cr in the soils irrigated with PW. The values of these metals were below the recommended maximum levels proposed by the Environmental Quality Standard for Soils [22]. Compared with PW and RW, total bacteria in soils irrigated with TE showed a significant increase ($p < 0.05$). Total Coliforms in soils irrigated with RW and those irrigated with TE resulted in significant increases ($p < 0.05$).

Table 2. Characteristics of the irrigated soils as compared with the guidelines for soil quality.

Parameters	LPW	LRW	LTE	[22]	[23]
pH	7.36 a	7.18 a	7.50 a	6.5–7.5	6.5–7.5
EC ($\mu\text{S}\cdot\text{cm}^{-1}$)	563 a	1210 b	1587 b	-	-
Organic matter (%)	1.57 a	1.67 b	1.73 b	-	-
Total nitrogen (%)	0.113 a	0.123 a	0.130 a	-	-
Available P ($\text{mg}\cdot\text{kg}^{-1}$)	53.8 a	51.7 a	55.1 a	-	-
Available K ($\text{mg}\cdot\text{kg}^{-1}$)	154 a	172 a	169 a	-	-
Pb ($\text{mg}\cdot\text{kg}^{-1}$)	18.4 a	23.6 b	18.9 a	35–500	50–80
Cu ($\text{mg}\cdot\text{kg}^{-1}$)	24.4 a	25.5 a	25.4 a	35–400	50–200
Zn ($\text{mg}\cdot\text{kg}^{-1}$)	81.1 a	98.1 a	136.5 b	100–500	200–300
Cd ($\text{mg}\cdot\text{kg}^{-1}$)	0.23 a	0.22 a	0.24 a	0.2–1.0	0.3–0.6
Cr ($\text{mg}\cdot\text{kg}^{-1}$)	34.45 a	40.40 a	43.65 a	90–300	250–300
Hg ($\text{mg}\cdot\text{kg}^{-1}$)	0.111 a	0.119 a	0.139 a	0.15–1.5	0.25–0.35
As ($\text{mg}\cdot\text{kg}^{-1}$)	9.74 a	9.76 a	10.55 a	15–40	20–30
Total bacteria (CFU $\cdot\text{g}^{-1}$)	2.1×10^7 a	2.0×10^7 a	7.5×10^7 b	-	-
Total Coliforms (CFU $\cdot\text{g}^{-1}$)	1.3×10^6 a	6.1×10^6 b	3.1×10^6 c	-	-

Abbreviations: LPW, treatment irrigated with potable water; LRW, treatment irrigated with raw wastewater; LTE, treatment irrigated with treated effluent. Mean values ($n = 3$) in each row followed by the same lowercase letter are not significantly different using Tukey's HSD test at $p < 0.05$.

Average values of the main growth and quality parameters of lettuces measured at harvesting time are listed in Table 3. Plant height, fresh weight, soluble sugars, and soluble proteins did not show significant differences between the compared treatments. Nitrate content in the leaves of each treatment was lower than that in the stems. Data showed significant accumulation in the level of Total

Coliforms in edible vegetables irrigated with RW ($p < 0.05$), although possessing the same orders of magnitude as the control.

Table 3. Quality and microbiological parameters of lettuces irrigated with potable water (PW), raw wastewater (RW), and treated effluent (TE).

Parameters	LPW	LRW	LTE
Height (cm)	25.75 a	24.55 a	25.69 a
Fresh weight (g)	50 a	47 a	49 a
Soluble sugars (%)	0.79 a	0.67 a	0.73 a
Soluble proteins (mg·g ⁻¹)	10.99 a	10.64 a	10.67 a
Nitrate-Stem (mg·kg ⁻¹)	515 a	521 a	494 a
Nitrate-Leaf (mg·kg ⁻¹)	261 a	145 b	170 b
Total bacteria (CFU·g ⁻¹)	3.5 × 10 ⁶ a	5.3 × 10 ⁶ a	4.7 × 10 ⁶ a
Total Coliforms (CFU·g ⁻¹)	1.2 × 10 ⁴ a	5.3 × 10 ⁴ b	2.0 × 10 ⁴ a

Note: Mean values ($n = 3$) in each row followed by the same lowercase letter are not significantly different using Tukey's HSD test at $p < 0.05$.

3.3. Abundances of Selected Opportunistic Pathogens in Wastewater

The detection and quantification of the wastewater samples using real-time qPCR equipped with nine primer sets, including *A. hydrophila*, *Arcobacter* sp., *B. cereus*, *Bacteroides* sp., *E. coli*, *Legionella* sp., *Mycobacterium* sp., total bacteria, and total fungi are displayed in Figure 2. The overall abundances of pathogens per litre of wastewater ranged from 10⁴ to 10¹¹ gene copies when targeted genes were quantified over the time. The levels of *A. hydrophila*, *Arcobacter* sp., *Bacteroides* sp., and *E. coli* in TE were declined by 1–3 orders of magnitude. *A. hydrophila* in this study was not significantly different in RW, except it was increased by 3 logs in September. The other dominant potential pathogen in wastewater was *Arcobacter* sp., one typical of emerging pathogens. It was found that the mean level of *Arcobacter* sp. in June, July, and October was markedly higher in RW than other periods of time. In particular, the removal efficiency reached a maximum in September and October. However, concentrations of *B. cereus*, *Legionella* sp., and *Mycobacterium* sp. in TE experienced an increase in gene copies of 0.9 to 2.84, 0 to 1.47, and 0.68 to 1.77, respectively. The abundances of 16S and 18S rRNA genes in both RW and TE had the same changing trends, and ranged from 9.86 log₁₀ to 12.13 log₁₀ copies·L⁻¹ and 6.75 log₁₀ to 9.13 log₁₀ copies·L⁻¹, respectively. The results showed that TE harboured large amounts of potential pathogens even after treatment; the risk assessment of these pathogens in environments by wastewater reuse should be further researched. *B. cereus*, *Legionella* sp., and *Mycobacterium* sp. were detected in TE by which the concentrations increased; this indicated that opportunistic pathogens commonly found in the wastewater and an ineffective elimination in the effluent with or without disinfection. The amounts of *Legionella* sp. and *Mycobacterium* sp. in TE reached 7–8 logs, but their infective doses are unknown [24]. Many species and total abundances of opportunistic pathogens were found in wastewater, which may threaten public health.

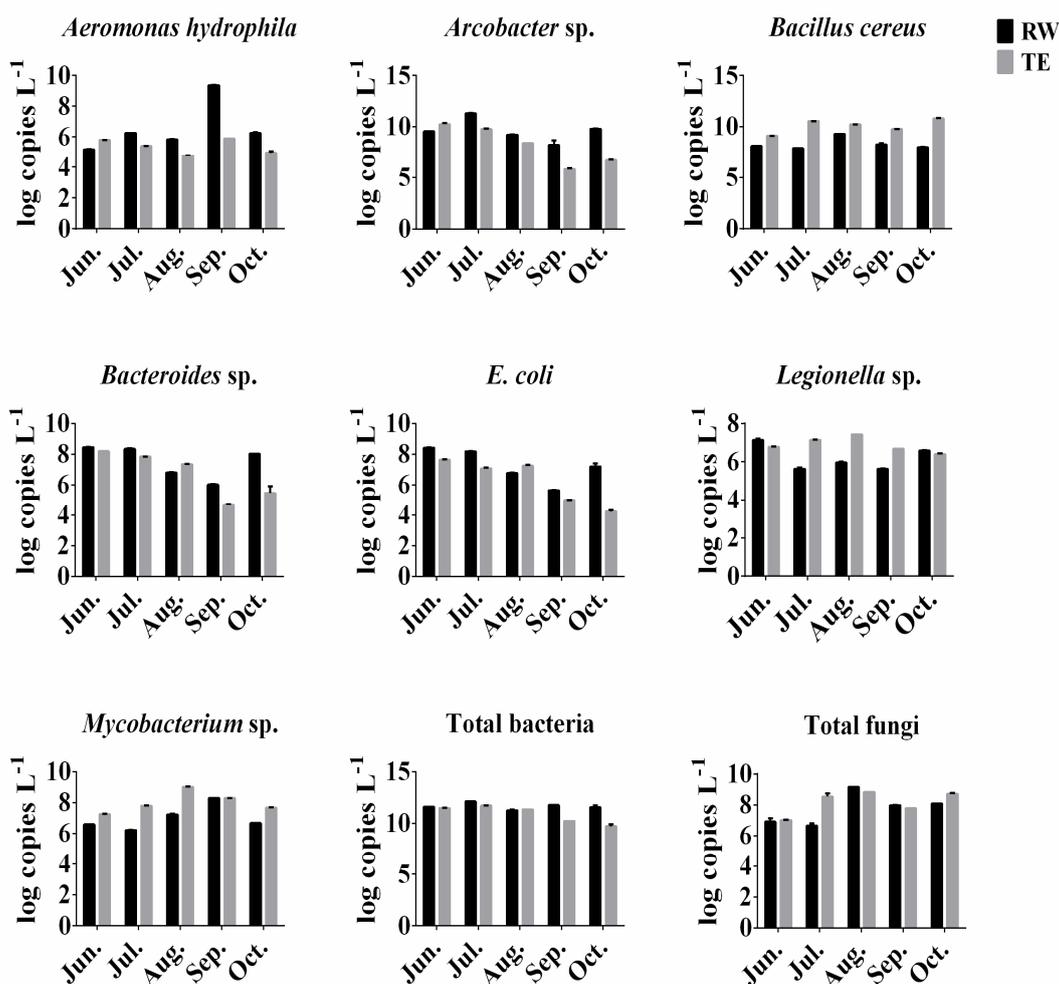


Figure 2. Pathogenic bacteria gene copy numbers in wastewater at different sampling stages (RW = raw wastewater, TE = treated effluent. Error bars indicate standard deviation from average, $n = 3$).

3.4. Abundances of Potential Pathogens in Phyllosphere and Rhizosphere

One of the main concerns for wastewater reuse is the microbiological quality due to the possibility of disease transmission. It has been found that pathogenic microorganisms are not completely eliminated by conventional wastewater treatment processes and also detected in final effluents for reclamation purposes [25]. Wastewater reclaimed through the anaerobic biofilm reactor, as well as raw wastewater and potable water was employed to cultivate lettuces in a greenhouse, by means of surface irrigation. Our study highlighted that potential risks associated with the reuse of treated wastewater occurred not only from conventional fecal indicators, but also from known and emerging pathogens. In the present study, the presence of pathogenic bacteria was investigated in the phyllosphere and rhizosphere of lettuce following irrigation with different water sources. The concentrations of opportunistic pathogens were determined on leaf surfaces ranging between 2.5 log₁₀ copies·g⁻¹ and 9.0 log₁₀ copies·g⁻¹, leaf surfaces of lettuce were contaminated with wastewater reuse (Figure 3A). Compared to the potable water, the abundances of *A. hydrophila*, *Arcobacter* sp., and *E. coli* in phyllosphere were higher in lettuces irrigated with raw wastewater and treated wastewater. Lettuce plants irrigated with wastewater carry the human pathogens, and thereby result in the contamination of sprouts, leaves, and roots. Data indicated that the abundances of *B. cereus*, *Bacteroides* sp., *E. coli*, *Legionella* sp., and *Mycobacterium* sp. in rhizosphere were higher than other pathogens (Figure 3B). The quantities of *B. cereus* and *Mycobacterium* sp. were introduced into the soils up to six orders of magnitude, although there were

no differences among different treatments ($p > 0.05$). Irrigation water used in this study was found to be a potential risk factor for the introduction of pathogens, but high concentrations of some pathogens were detected on lettuce rhizosphere regardless of the irrigation treatment.

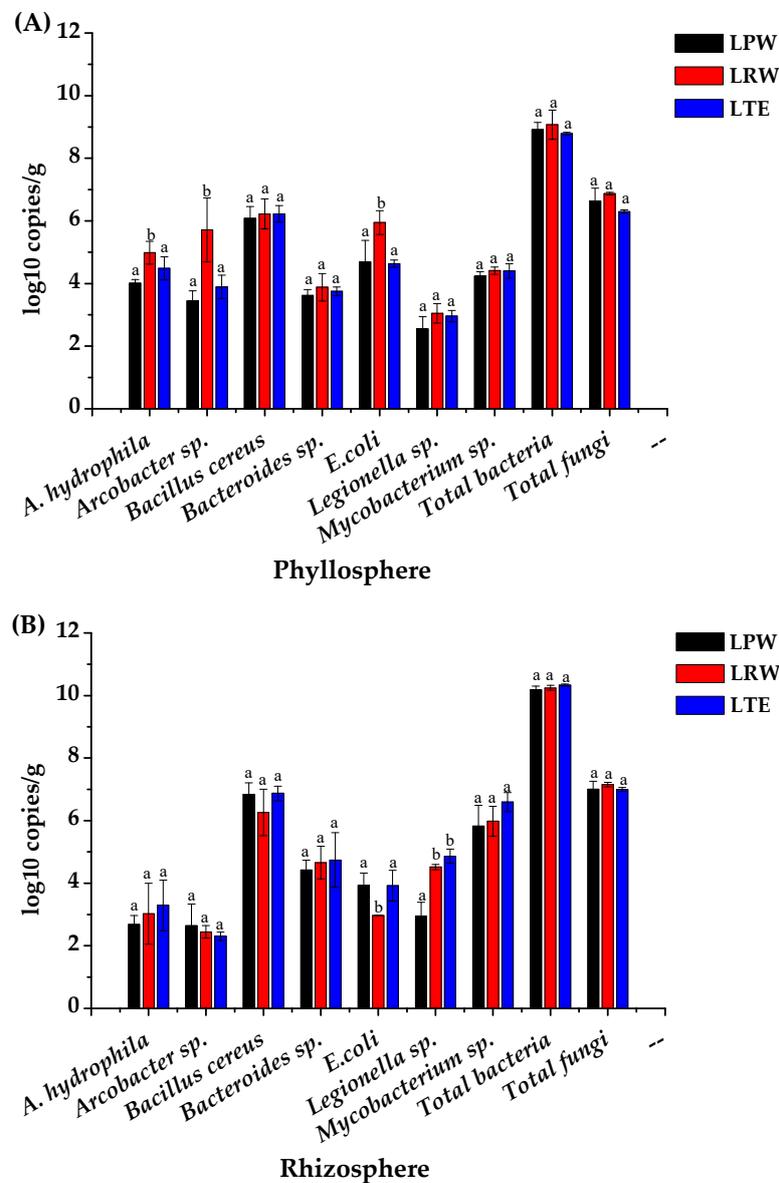


Figure 3. Pathogenic bacteria in the phyllosphere (A) and rhizosphere (B) of lettuce irrigated with potable water and wastewater for two months irrigation (error bars indicate standard deviation from average, $n = 3$). Mean values ($n = 3$) in each group followed by the same lowercase letter are not significantly different using Tukey's HSD test at $p < 0.05$.

4. Discussion

Wastewater treatment is intended to reduce organic and inorganic pollutants, as well as microbial contaminants. In this study, we found that the wastewater treated by the anaerobic reactor was less efficient in removing nitrogen and phosphorus; Fecal Coliforms, *Salmonella*, and helminths were undetectable in TE. Although the treated wastewater did not meet the discharge standards (TN less than $15 \text{ mg}\cdot\text{L}^{-1}$, $\text{NH}_3\text{-N}$ less than $5 \text{ mg}\cdot\text{L}^{-1}$, TP less than $0.5 \text{ mg}\cdot\text{L}^{-1}$) [17], the water quality could conform to the guideline standards for reuse in irrigation. The concentrations of heavy metals in all water sources were far below the guidelines values of farmland irrigation, and then the cumulative risk

of heavy metals in soils and crops was low. Irrigation using RW and TE resulted in significantly higher soil EC levels, and it might increase the risk of soil salinization. Microbial contamination was assessed by measuring total bacteria and Total Coliforms on samples from soils and crops at harvesting time, results showed total bacteria in soils and on crops irrigated with water from PW and RW were not significantly different ($p > 0.05$), while the quantities of Total Coliforms were higher in RW than PW. The low quantities of Fecal Coliforms (3 CFU 10 g^{-1} to 56 CFU 10 g^{-1}) and *E. coli* (1 CFU 10 g^{-1} to 4 CFU 10 g^{-1}) observed on vegetable crops and soil showed that it was possible to irrigate with treated wastewater [26]. Another study indicated that the treated wastewater source did not significantly affect fecal indicators of tomato fruit and soil, but the community composition and dynamics of bacterial population in soil is influenced by the different water sources used for irrigation [27]. As compared to freshwater, treated effluent did not present any adverse effect of fecal pollution on crop quality and soil, and therefore did not cause risks for human health.

Considerable efforts have been devoted to the development of anaerobic treatment processes, suitable for treating low strength wastewater, such as decentralized treatment of domestic wastewater from the rural area. Over the past decades, the growth of interest in the use of wastewater for agriculture irrigation in arid and semi-arid regions was robust on account of the scarcity of conventional water supplies. The microbial contamination of raw and treated wastewater is limited to focus on the presence and enumeration of fecal indicators and helminths in many studies, for evaluating the pathogens removal performance [28]. Very strict microbiological standards in terms of fecal indicators for treated wastewater have been adopted in many countries. Investigating the fate of bacterial indicators is relevant to assess their persistence in the environment and possible transfer to the receiving surface water or to the food chain. This study showed that fecal indicators and other physicochemical properties in treated effluent conformed to irrigation standard in China, despite not meeting discharge standards. However, the quantities of these indicators may not reflect the actual abundances and species of pathogenic microorganisms in the environment.

Wastewater has been reported many times as a potential reservoir of pathogenic microorganisms, indicating that wastewater contains abundant dissolved nutrients that can be used for bacterial growth and multiplication. This study presents different concentrations of opportunistic pathogens and fecal indicators in domestic wastewater over the duration of five months. *Aeromonas* species are generally considered to be aquatic pathogens that are resident in water environments, which could pose a risk to public health. In a recent study, a two-season microbiological investigation of treated effluent with emphasis on *Aeromonas* sp. was conducted. They found that a rise of *A. hydrophila* was observed in summer in raw sewage, treated wastewater, and effluent-carrying canal [29]. The removal efficiency of microbial contaminants was evaluated from a local wastewater treatment plant and highlighted the potential risk associated with wastewater reuse for agricultural irrigation. *A. hydrophila* could still be recovered from the chlorinated effluent; despite the treatment process achieving 3.5 logs removal of heterotrophic bacteria and Fecal Coliforms [30]. A relatively high concentration of *A. hydrophila* was detected in treated effluent, and the quantity was maintained at high levels (4–5 logs). *Arcobacter* sp., *Bacteroides* sp., and *E. coli* from wastewater possessed high abundances that belonged to opportunistic pathogens in the intestine and indicators of fecal pollution, these entering agricultural environments might pose a potential risk to human health. Among the *Arcobacter* species only *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, and *A. cibarius* were identified as being associated with animal and human infections [31]. *Arcobacter* sp. as an emerging human pathogen of animal origin was one of the most dominant bacterium in domestic wastewater, and the concentration was high as a previous study revealed [32]. Few studies have assessed the occurrence of *Arcobacter* sp. in wastewater, their quantities were found to be reduced during the treatment processes but were not entirely eliminated in effluent [33,34]. In reality, people would expect a functioning treatment system to produce treated effluent with lower pathogen loads than raw wastewater, but often this is not the case. The concentrations of *B. cereus*, *Legionella* sp., and *Mycobacterium* sp. were higher in TE than RW. *B. cereus* is a common food-borne pathogen, and the risk of disease transmission is influenced by the level and persistence of contamination in

water, soil, and crops. Irrigation with poor-quality water is one way that fruit and vegetables can be contaminated with food-borne pathogens [35]. *Legionella* sp. and *Mycobacterium* sp., which caused non-enteric illnesses, had been detected in wastewater and potable water systems, and they could proliferate within free-living amoebae where they are protected from the adverse environment [36,37]. *Legionella* sp. and *Mycobacterium* sp. have been observed to regrow in reclaimed water and distribution systems due to biofilm development and disinfectant dissipation [38,39]. Therefore, there is a need to develop management strategies for prevention relevant to bacterial regrowth before treated wastewater is used for agriculture irrigation. Spray irrigation, toilet flushing, and cooling towers that generated aerosols were evaluated for *Legionella* health risks in reclaimed water using quantitative microbial risk assessment, data showed that *Legionella* median annual infection risks and annual clinical severity infection risks for toilet flushing can exceed a 10^{-4} annual risk of infection benchmark [9]. The levels of opportunistic pathogens detected in treated effluent samples may not pose any risks to healthy humans, however, young children, the elderly, and the immunocompromised population could be at risk.

Contaminated water could enhance the persistence and survival of pathogens in soils and vegetables, thus increasing human health risks. Agricultural irrigation is the most commonly utilized way of reusing wastewater, however, the presence of pathogenic microorganisms in wastewater is one major impediment for wastewater reuse. The accumulation and persistence of fecal-sourced microbes from wastewater in soil and crop is one of the major concerns associated with wastewater irrigation. Irrigation with wastewater may introduce the high bacteria counts in soil, showing that pathogens could invade the roots and colonize crops [40]. The phyllosphere and rhizosphere are known to be source-reservoir combinations for opportunistic human pathogenic bacteria. A range of bacterial pathogens were monitored in the phyllosphere and rhizosphere of lettuce irrigated with different water sources, it was found that there were no significant differences in some pathogens (*B. cereus*, *Bacteroides* sp., *Mycobacterium* sp.) among the irrigation treatments. A case study related to drip irrigation with treated wastewater showed that fecal indicator contamination (Total and Fecal Coliforms) was not associated with the different water sources, and thus did not result in the transfer of fecal indicator bacteria or microbial pathogens to the irrigated soil or crop [41]. Other studies have investigated the effectiveness of onsite wastewater reuse systems in reducing human-originated fecal contaminants, the risks of diverse pathogens in wastewater that expose pathogens in the wastewater discharge was well studied within the Environment Canada guidelines [42,43]. The abundances of pathogens in soils irrigated with treated graywater and fresh water were of the same orders of magnitude, suggesting treated graywater irrigation has no effect on soil pathogens diversity or abundances [44]. *Aeromonas* has been isolated from a wide range of fresh produce [45]. The potential for contamination via irrigation water showed an increased incidence of enteropathogens. Traditional eating habits of consuming raw eaten or lightly cooked vegetables may represent an important source of risk for human health due to the fact that they are carriers of food-borne pathogens associated with contaminated water irrigation. Wachtel et al. described *E. coli* contamination of the roots of cabbage irrigated with sewage-contaminated water, although the edible part of the plant was unaffected [46]. Occurrence of emerging food-borne pathogenic *Arcobacter* sp. was assessed from pre-cut ready-to-eat vegetables, the results revealed a widespread distribution of virulence-associated genes among the *Arcobacter* on raw vegetables [47]. The quantities of *A. hydrophila*, *Arcobacter* sp. and *E. coli* in the phyllosphere and rhizosphere were significantly increased in raw wastewater irrigation. This could be attributed to the plant direct contact with contaminated irrigation water. *B. cereus* is commonly found in soil and spoilage food, a notable result from the study showed the difficulties in eliminating *B. cereus* and *E. coli* on raw fruits and vegetables [40]. In this study, no significant differences were observed in *B. cereus* and *Mycobacterium* sp. between the phyllosphere and rhizosphere irrigated with different water sources, while the concentrations were maintained at a high level. The contamination of vegetables may occur during the production steps, where either contaminated organic fertilizers or irrigation

water obtained from different sources. It is suggested that agricultural practices need to be highlighted to help protect human health.

In summary, understanding the ecology of pathogens and naturally occurring microorganisms is essential before interventions for elimination or control of growth can be devised. It should be pointed out that the qPCR used in this study might overestimate the abundances of opportunistic pathogens due to insufficient discrimination between live and dead bacterial cells. Therefore, the abundances and fates of active pathogens in different environmental media should be determined in future studies. Considering the dominant sources of microbial contamination in the environment, more specifically in raw or treated wastewater, efforts should be focused on maximizing the benefits and minimizing any detrimental effects on public health or environments.

5. Conclusions

The reuse of domestic wastewater for agricultural irrigation is regarded as an option to address water scarcity, which could partially substitute for chemical fertilizer as nutrient sources. In the present study, we revealed that the phyllosphere and rhizosphere contained different levels of opportunistic pathogens, which deserves greater consideration. Apart from fecal indicators, some pathogens can still persist in the final effluent, which pose risk to environmental and human health. Wastewater for reuse in irrigation could act as a potential source of pathogens. Although irrigation of treated wastewater appeared to pose potential risks, treated wastewater for reuse presented a promising practice to alleviate water scarcity through appropriate management strategies. The detection of opportunistic pathogens in wastewater would facilitate decision-making in effective technology and management solutions to reduce microbial risks in receiving soils and crops. Meanwhile, although fecal contamination pertaining to wastewater reuse in agriculture is a crucial indicator in current standards, comprehensive microbial risk assessments are recommended for wastewater irrigation of crops. It is concluded that wastewater irrigation should track microorganisms including viral, bacterial, and protozoan pathogens, rather than only focusing on fecal indicators.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4441/11/6/1283/s1>, Table S1: qPCR primer sequences and reaction conditions. Table S2: The standard curve showed a linear relationship.

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