Pathogenic determination from rural wastewater treated by MBR process and effect of wastewater on lettuce pot planting

Bingjian Cui [1,2], Xiao Kong [2,3], Xi Chen[1,2], Decai Jin [1,2], Hongxun Zhang[2*]

[1]Key Laboratory of Environmental Biotechnology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

[2]University of Chinese Academy of Sciences, Beijing 100049, China

[3]College of Environment and Safety Engineering, Qingdao University of Science and Technology, Qingdao 266042, China

[*]**Corresponding** author. E-mail: hxzhang@ucas.ac.cn, Tel: +86 10 62849155

Abstract

Most regions of developing countries use wastewater to irrigate farmlands that alleviate the crisis of water shortage, but to arise numerous potential health risks associate with wastewater irrigation for human and crops. In this study, the microbiological quality of Membrane Bio-Reactors (MBRs) processes of the domestic wastewater treatment plant was assessed by monitoring the concentrations of *Aeromonas hydrophila*, *Arcobacter* sp., *Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli*, *Hartmannella vermiformis*, *Legionella* sp.. A pot planting experiment is conducted to investigate the persistence of pathogens and impact to vegetables and soils by using different water. The results showed that wastewater slightly impacted physiochemically to soil and vegetable, and the pathogens on vegetables and in soils varied exponentially among different treatments. Compared with controls, pathogens presented in the phyllosphere slightly higher which irrigated with wastewater, whereas no significant difference in the rhizosphere between treated effluent and controls. Accordingly, it is recommended that the treated effluent can be used as an alternative water resource for agricultural irrigation, the variety of pathogens in wastewater should be monitored over an extended period of time. But for the irrigation with untreated wastewater it has health risks.

Keywords: domestic wastewater; irrigation; pathogens; real-time PCR

{**Citation:** Bingjian Cui, Xiao Kong, Xi Chen, Decai Jin, Hongxun Zhang. Pathogenic determination from rural wastewater treated by MBR process and effect of wastewater on lettuce pot planting. American Journal of Research Communication, 2015, 3(6): 1-27} www.usa-journals.com, ISSN: 2325-4076.

Introduction

During the past decades, with water increasingly scarce and a growing demand, agricultural irrigation with wastewater becomes a common practice in China and other countries located in semi-arid and arid regions of the world, particularly of vegetable crops (Hamilton et al., 2007). Wastewater reuse presents a vast potential to a widely range of applications. It is reported that main uses include irrigation for agriculture, aquaculture, groundwater recharge, industrial reuse, recreational impoundments, formation of wetlands, make-up water for watershed, potable water. The distribution for reclaimed water reuses is reflecting regional characteristics, these applications are provided internationally (Baydal et al., 2009) (FDEP, 2011).

Agriculture irrigation is the biggest uses of recycled wastewaters so far. For example, 41% of reclaimed water in Japan and 60% of Californian reclaimed wastewaters are used for the irrigation of crops (Asano and Levine, 1996). Due to massive human population increased, greater strains will be pressed on available resources and pose a serious threat to environment sources (Day, 1996). Reclamation of domestic wastewater for irrigation in agriculture is viewed as a reasonable and sustainable option to improve water usage and address water scarcity. In addition to the increasing need to meet potable water supply demands and other essential demands (e.g. landscape irrigation, commercial, and industrial needs). With increasingly agriculture demands for water, alternative sources are quite imperative, such as domestic wastewater, industrial wastewater and rainwater runoff etc (Pimentel et al., 2003). Among these alternatives, domestic wastewater is a valuable but under-utilized resource due to possible environmental contamination and fears of

health risks (Frank et al., 2007). While nutrients (as organic carbon, nitrogen, phosphorus and potassium) contained in it is considered as beneficial to crop growth in agriculture and greatly reduced the costs of crop production by reducing the need for application of artificial fertilizers (WQPN, 2008) (do Monte et al., 1992).

Untreated wastewater is reused for agricultural irrigation as well as treated wastewater. However, the reuse of untreated wastewater for irrigation may rise potential risks to public health, soil characteristics, and crop productivity and quality (Akponikpe et al., 2011). It was reported that soil, crop leaves, and fruits were contaminated by fecal coliforms when untreated wastewater was reclaimed, reported in countries of the Mediterranean region, such as Turkey, Morocco and Mexico. The majority of countries produced large amounts of wastewater but scant attention was paid to adequate treatment due to socioeconomic conditions, farmers irrigate their land with diluted, untreated, or partially treated wastewater (Pedrero et al., 2010; Battilani et al., 2010). Consequently, it was causing widespread public health problems, limiting for economical and agricultural development, further hazard natural ecosystems.

Hazardous agents in wastewater usually contain microbial pathogens, toxic chemicals (inorganics and organics) and heavy metals (Sanjay et al., 2013). Public health considerations are centered on pathogenic organisms that could be present in sewage in great numbers and variety, which may cause disease to farm workers, consumers, and those who inhale aerosols generated when the wastewater is applied (Shainberg et al., 1978). Many opportunistic pathogens can be major members of natural microbial populations, which present in wastewater have the ability to rapidly growth and increase the risk of infections for immune-compromised (Ashbolt et al., 1995). Gastrointestinal infections are the most common diseases caused by pathogenic bacteria in wastewater. Such as *Aeromonas* sp. and *Arcobacter* sp. are attributed with being the major causes of human acute enteritis and gastroenteritis (Wesley et al., 1996).

On the whole, it is well known that microorganisms are widely present in the wastewater treatment processes, especially pathogenic bacteria. The key concern of

this study that focus on wastewater reuse impact on soil and the health risks derived from the presence of manifold microbial pathogens in domestic wastewater and irrigated soil and leafy greens. The objectives of this study are to evaluate the occurrence and prevalence of microbial pathogens involved in wastewater treatment processes, and then to evaluate presence of pathogenic bacteria in the phyllosphere with that in the rhizosphere of lettuce after irrigated with different treatments. The effects of wastewater reuse on physicochemical characteristics of soils and vegetables investigated as well to further discuss the feasibility of wastewater irrigation.

Materials and methods

Wastewater treatment plant and water sampling

The wastewater treatment plant used in this study was located in Huairou district, Beijing, China, which was used to treat the rural domestic wastewater for 600 m^3/d produced by almost 1800 local residents. The untreated wastewater entered the wastewater treatment plant and went through various treatment procedures including screening grid, adjusting tank, aerobic tank and Membrane Bio-Reactor (MBR) tank (Fig. 1). Then the treated effluent was used to farmland irrigation, as well as landscape and recreational impoundments. Five water samples were collected from the wastewater treatment plant, which were from the untreated wastewater, adjusting tank, aerobic tank, membrane tank and treated effluent, using sterile 10-L plastic containers.

Water physiochemical quality detection

The physiochemical qualities were monitored for the water samples from the untreated wastewater, aerobic tank and treated effluent. When sampling, some water quality parameters including pH and electrical conductivity (EC) were detected using multi-parameters water quality monitoring instrument (DZB-718 portable multi-parameter meter, REX Instrument Factory, Shanghai) *in situ*. Chemical oxygen

demand (COD) was determined by HACH DRB200 (HACH, USA) and total suspended solids (TSS) was measured using the gravimetric method (GB/T11901-1989). Some other parameters including total nitrogen (TN), total phosphorus (TP) and the cations of Cu, Pb, Zn, Cd were sent to the Pony Testing International Group (Beijing, China) and strictly tested with the standard methods (DB 11/ 307–2013) in China.

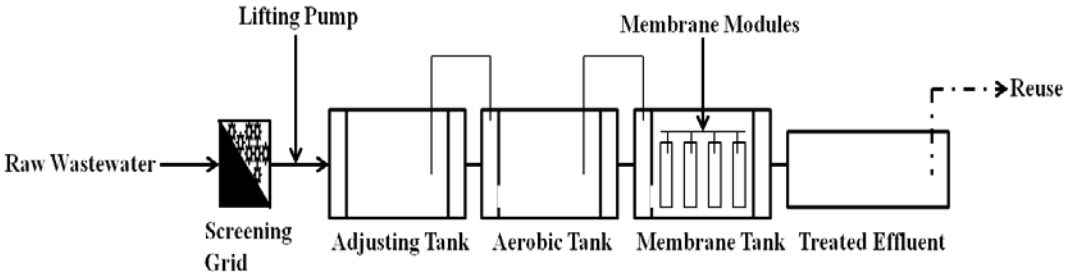

Fig. 1: Schematic diagram of the wastewater treatment plant used in this study.

Culture-based analysis

The numbers of the total heterotrophic bacteria, *Escherichia coli*, total coliforms and fecal coliforms in the water samples from the untreated wastewater, aerobic tank and treated effluent was monitored using conventional culture-based methods as described in the standards (GB 4789.3-2010). And the helminths were enumerated with the sedimentation method (GB/T 5084-2005, China). All the mediums used in this study were purchased from Hope Bio-Technology Co., Ltd. (Qingdao, China). For the bacterial enumeration experiments, all the water samples were tested in triplicate.

Real-time quantitative PCR analysis

The Real-time quantitative PCR was used to analyze the pathogens in all the 5 water samples. The microbial biomass was harvested from 100 mL of the untreated

wastewater and 2 L of the treated effluent samples respectively filtered with 0.22 μm polycarbonate membranes (47 mm diameter, Millipore, USA) (Poitelon et al., 2009a). And a volume of 5 mL for the other water samples was centrifuged (8000 rpm, 4℃, 15 min) to get the microorganisms. The total genomic DNA was extracted as described in Section 2.1.3.2 as above.

PCR products amplified from the extracted DNA with the primer sets for real-time quantitative PCR to detect 8 pathogenic bacteria (Table 1) were gel-purified and ligated into the pGEM-T Easy Vector (Promega, Madison) based on Section 1.1.3.2 described above. After determination, the positive clones were selected to extract plasmid DNA using a E.Z.N.A.® Plasmid Mini Kit I Spin Kit (Omega Bio-tek, Inc.). The concentration of the extracted plasmid DNA was detected with a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA), which was used as pathogenic gene standards. The gene copy number was calculated directly from the concentration of the extracted plasmid DNA (Joseph et al., 2003).

Both genomic and plasmid DNA were used as references for calibrating the bacterial quantities. The real-time quantitative PCR was performed using GoTaq® qPCR Master Mix with BRYT Green® dye (Promega, Madison) and ran on a Stratagene Mx3005P instrument (Agilent Technology, Santa Clara, CA). The following reaction mix was used: 2 μL template genomic DNA, 0.4 μL of 10 μM of each set of primers, 10 μL GoTaq qPCR Master Mix (Promega, Madison), and 7.2 μL Nuclease-Free water, to a final volume of 20 μL. The following procedure was used: denaturation of 95℃for 2 min, 45 cycles of 95℃ for 15 s, 58℃/62℃ for 45 s, 72℃ for 45 s. The third segment of dissociation stage: 95℃ for 1 min, 55℃ for 30 s, 95℃ for 30 s. For each PCR experiment, corresponding negative (sterile water) control was included. Samples were tested in triplicate to obtain positive/negative results.

To test the linearity and the dynamic range of the real-time quantitative PCR reaction, tenfold serial dilutions of known copy number of pathogenic plasmid DNA were subjected to real-time quantitative PCR in triplicate to generate an external standard curve. PCR efficiency and correlation coefficients for standard curves were ranged from 89.50% to 99.20% and r^2 values from 0.952 to 0.993. The primer pairs

used for real-time quantitative PCR are listed Table 1 as above.

Table 1: The primer sets for real-time quantitative PCR to detect 8 pathogen bacteria

Group	Target gene	Primer sequences (5'-3')[a]	Amplicon Size (bp)
Aeromonas hydrophila	Cytolytic enterotoxin	F: GAGAAGGTGACCACCAAGAACA R: AACTGACATCGGCCTTGAACTC	232
Arcobacter sp.	23S rRNA gene	F: GTCGTGCCAAGAAAAGCCA R: TTCGCTTGCGCTGACAT	331
Bacillus cereus	16S rRNA gene	F: TCGAAATTGAAAGGCGGC R: GGTGCCAGCTTATTCAAC	288
Clostridium difficile	16S rRNA gene	F:TTGAGCGATTTACTTCGGTAA AGA R: CCATCCTGTACTGGCTCACCT	157
Clostridium perfringens	16S rRNA gene	F: ATGCAAGTCGAGCGA(G/T)G R:TATGCG GTATTAATCT(C/T)CCTTT	120
Escherichia coli	*uidA*	F: CTGCTGCTGTCGGCTTTA R: CCTTGCGGACGGGTAT	205
Legionella sp.	16S rRNA gene	F: GAGGGTTGATAGGTTAAGAGC R: GTCAACTTATCGCGTTTGCT	430
Mycobacterium sp.	16S rRNA gene	F: ATGCACCACCTGCACACAGG R: GGTGGTTTGTCGCGTTGTTC	470
Hartmannella vermiformis	18S rRNA gene	F: TTACGAGGTCAGGACACTGT R: GACCATCCGGAGTTCTCG	502

[a]**F**, forward primer; **R**, reverse primer.

To verify the identity of the PCR products obtained from a set of samples. Each targeted gene products were purified using a Gel Extraction Kit (Promega, Madison) as recommended by the manufacturer's instructions, then sequenced and aligned using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pot planting experiment

The planting experimental site was located in a solar greenhouse affiliated to Beijing Forestry University Forest Science Co. Ltd, Beijing, China. Vegetable of lettuce (*Lactuca Sativa*) was grown in 2.5-L plastic pots filled with soil randomly collected from a suburb forestry farm in Beijing. The seeds of lettuce (*Lacuca sativa*) were purchased from Chinese Academy of Agricultural Sciences (CAAS.), and then pretreated as Quilliam RS et al. described. Each pot was planted with 20 seeds. The pots were placed in the greenhouse where the temperature was kept between 28℃ and 32℃, the relative humidity 40%-60% and the photoperiod of 16-h light and 8-h darkness each day.

In order to investigate the effect of the wastewater on the soil and the plants, we compared the original soil physicochemical properties after irrigating with potable water and wastewater for 3 quarters. 4 irrigation treatments were utilized: complete fertilization (Urea 70 kg ha^{-1}, Calcium superphosphate 28 kg ha^{-1}, Potassium sulfate 70 kg ha^{-1}) and irrigated with potable water (PWF) as control, complete fertilization and irrigated with untreated wastewater (WWF), complete fertilization and irrigated with aerobic tank effluent (AEF), complete fertilization and irrigated with treated effluent (TEF). In addition, there were 2 other controls that one was just irrigated with potable water without planting (CK-PW) and the other just untreated wastewater (CK-WW). For all the planting experiments, each irrigation treatment was replicated for 4 times.

Characteristic analysis of the soil

The characteristic analysis was performed for the original soil and the soil irrigated with the untreated wastewater without planting. The parameters of pH and electrical conductivity (EC) were detected with DZB-718 portable multi-parameter meter (REX Instrument Factory, Shanghai) in a 1:5 soil-to-water ratio. Organic matter (OM) was measured using the potassium dichromate volumetric method. Other analysis included assessment of NH_4^+-N, NO_3^--N, available nitrogen, available phosphorus and cations of Cu, Pb, Zn, Cd were performed according to Chinese

standards (GB: 5749-2006 and GB: 15618-1995) by Pony Testing International Group (Beijing, China).

For the 6 soil samples of different irrigation treatments, the parameters of pH, EC and OM were determined as above. Besides, the urease and alkaline phosphatase were monitored based on the methods reported by Tabatabai et al. and Bielinska et al., respectively.

Growth analysis of the plants

After the plants grew for 8 weeks, for each pot 4 plants were selected randomly, washed thoroughly and measured for the physicochemical growth indicators (A. Mark Ibekwe et al., 2009). The plant above the soil surface were used to detect the plant height, the fresh weight and the nitrate concentration, which were determined with the electric sensitive balance and by Salicylic acid method (Cataldo et al., 1975), respectively. And the amount of water soluble sugars and water soluble proteins in the fresh leaves were tested by anthrone colorimetric method (Michel et al., 1956) and folin phenol method (Lowry et al., 1951), respectively.

Pathogenic analysis of the plants

Plant phyllosphere and rhizosphere samples were collected for pathogens analysis. The phyllosphere samples were obtained following Baoguo Zhang's description. In brief, phyllosphere samples were cut above the soil surface with sterile blade, placed in the stomacher bags and weighed. Ten grams of each phyllosphere samples were aseptically transferred into a polypropylene tube containing 100 mL washing solution (0.1 M sodium phosphate buffer, pH 7.0), and sonicated in an ultrasonic cleaning bath (Shanghai Kudos Instrument Co.) at 40 KHz for 10 min. The leaf debris was centrifuged at a slow speed of 500 g at 4℃ for 5 min and removed the suspension into a new tube. The suspension was centrifuged at 7000 g at 4℃ for 15 min and the pellet was used to analyze the pathogens inside. The rhizosphere samples were gained after shaking loosely held soil on the roots into the stomacher bags and weighed.

The method of real-time quantitative PCR was used to assay the phyllosphere

and rhizosphere pathogens. The details of the experimental procedures were described in Section 1.1.3.3 as above.

Data analysis

The software Microsoft Office Excel 2013 was used for calculating the average values and standard deviations. The one-way ANOVA followed by a post hoc Turkey test was used for testing the differences of the plant characteristics among different irrigation treatments. All the statistical analyses were performed using SPSS 20.0 statistical software (SPSS Inc., Chicago, USA).

Results and discussion

MBR operating performance assessment

The physicochemical and microbial quality of the water samples, as well as the reference standards are summarized in Table 2 and Table 3. After the treatment procedures, all the quality parameters of the treated effluent met the discharge standard of pollutants for municipal wastewater treatment plant (GB18918-2002) in China. The physicochemical quality parameters of the water samples from the untreated wastewater, aerobic tank effluent and the treated effluent were fairly accorded with Environmental protection agency (EPA) reuse guidelines (USA, 2012) and standards for irrigation water quality (GB 5084-2005, China.) except for COD of the untreated wastewater. The treated effluent was fairly consistent throughout the study with low average levels of chemical oxygen demand around 35 mg/L which removal efficiency was up to 89.9 % and concentrations of total N, total P were not decreased significantly in the treated effluent. Concentrations of heavy metals such as Cu, Zn, Pb, Cd were undetectable in the treated effluent, respectively.

Total bacteria, *E. coli*, total coliforms, and fecal coliforms were detected in all wastewater samples. The geometric mean value of total bacteria in the untreated wastewater decreased from $(1.24\pm0.045)\times10^{6}$ CFU/mL to $(2.80\pm0.2)\times10^{3}$ CFU/mL before and after the treatment. The total coliforms and *E. coli* were up to

$(6.05\pm1.75)\times10^5$ CFU/mL and $(8.9\pm0.19)\times10^4$ CFU/mL in the untreated wastewater, respectively, while the treated effluent was not detected at all. Helminths were enumerated using sedimentation method, the corresponding values for untreated wastewater and the effluent were in the range from 34 ind./L to null. The results of investigations of full scale wastewater treatment plant showed that the removal efficiency of bacterial depends on the technological individual process applied.

Impact of wastewater on soils physicochemical properties

Comparing the original soil and the soil which irrigated with untreated wastewater, some parameters such as pH, EC, OM, available N, total Zn, Cu, Cd and Pb were slightly reduced or increased. For the other parameters, NO_3-N varied within a large range from 8.56 mg/kg to 104mg/kg, NH_3-N increased from 0.063 mg/kg to 4.43mg/kg, available P increased from 3.1 mg/kg to 10.8 mg/kg, which increased significantly, the results were summarized in Table 4. (Environmental quality standards for Soils, GB 15618-2008, China).

No significant changes in soil pH, EC, organic matter were recorded throughout the study in any of the pots receiving different types of treatment (Table 5). There was little or no change in the treatments during the study period, only urease and alkaline phosphatase increased in the pots irrigated with the untreated wastewater. The wastewater containing organic and natural fertilization plays a positive important role in enzyme activities in soils. Previously studies showed that wastewater was able to improve the physicochemical properties of soils, increased soil enzymatic activities (Chevremont et al., 2013) and induced the conversion of sparingly soluble N, P, and K states into more plant-available.

The wastewaters used for irrigation contained considerably higher levels of some chemicals, including nutrients than the potable water. However, Most parameters of the soil irrigated with the wastewater did not showed much difference from potable water irrigation except for somewhat increasing of average NO_3-N and NH_3-N levels The concentrations of heavy metals (Zn, Cu, Pb, Cd) were found to be far below the recommended maximum values for soil (GB 15618-2008). Many studies also

confirmed the similar results that soil properties slightly are affected among three types water including concentrations of several heavy metals (Zinc, Cadmium and Nickel) and metal concentration did not increasing with time were observed over the five-year period (Richard et al., 1987). The other study was focused on reuse of graywater for irrigation, which was inconsistent with portable water irrigation throughout the study except for saturation point, SAR (sodium adsorption ratio) and concentration of sodium (Maya et al., 2013). Since wastewater has a high nutritive value that may supply plant growth, reduce fertilizer application rates and increase productivity of poor fertility soils.

Effect of watering on vegetable growth

As shown in Table 6, compared with the control (PWF), there was no obvious difference from the plant height, the fresh weight and the soluble protein of the plants irrigated with the untreated wastewater (WWF), aerobic tank effluent (AEF) and treated effluent (TEF). However, the soluble sugar of the plants irrigated with untreated wastewater (WWF) was significantly higher than other treatments ($p<0.05$). In addition, the nitrate content of the plants of WWF also increased, which is below the maximum limitation for nitrate in lettuce established by GB 18406. 1-2001 (China) and the European Commission (2005), although no significant difference among the PWF, AEF and TEF treatments were observed.

In comparison, irrigation with untreated wastewater led to a little increase of nitrate in vegetables, while soluble sugar was much higher than potable water. A study of seven crops was carried out at Loess Plateau in China to test the feasibility of using secondary treatment sewage effluent. It was suggested that the quality of crops was not significant differences by the irrigation of sewage and fresh water. But for yields, the former was much higher than the latter (Wang Jun-feng, *et al*. 2007). Tomato planting showed that irrigation with treated wastewater did not affect fruit pH, but fruit size and weight increased and their SSC (soluble solids content) and firmness decreased. (O. Al-Lahham et al., 2003).

Table 2: Summary of the water quality parameters and the comparison with the standards

Water quality parameters	Water types		
	Untreated wastewater	Aerobic tank effluent	Treated effluent
pH	7.60	7.44	7.59
EC (μs/cm)	754	514	376
COD (mg/L)	346	150	35
TSS (mg/L)	26	5385	21
TN (mg/L)	54.6	85.2	26.2
TP (mg/L)	4.97	19.9	7.66
Total Cu (mg/L)	ND	0.07	ND
Total Pb (mg/L)	0.003	0.029	ND
Total Zn (mg/L)	0.153	0.815	ND
Total Cd (mg/L)	0.0002	0.0009	0.0002
Total heterotrophic bacteria (CFU/mL)	$(1.24\pm0.045)\times10^{6}$	$(2.60\pm0.4)\times10^{5}$	$(2.80\pm2)\times10^{3}$
E.coli (CFU/mL)	$(8.9\pm0.19)\times10^{4}$	$(6.1\pm0.51)\times10^{3}$	ND
Total coliforms (CFU/mL)	$(6.05\pm1.75)\times10^{5}$	$(5.70\pm0.35)\times10^{3}$	ND
Fecal coliforms (MPN/L)	240000	3900	ND
Helminth (ind./mL)	34	60	ND

ND. Not detectable; **NM.** Not mentioned.

Table 3: Limits value of water quality parameters in different standards

Water quality parameters	Standards		
	GB18918-2002[a]	GB 5084-2005[b]	EPA (2012)[c]
pH	6-9	5.5-8.5	6.5-8.4
EC (μs/cm)	NM	NM	＜0.7ds/m
COD (mg/L)	50-120	60-100	NM
TSS (mg/L)	10-50	15-60	NM
TN (mg/L)	15-20	NM	NM
TP (mg/L)	0.5-5.0	NM	NM
Total Cu (mg/L)	0.5	1.0	0.2
Total Pb (mg/L)	0.1	0.2	5.0
Total Zn (mg/L)	1.0	2.0	2.0
Total Cd (mg/L)	0.01	0.01	0.01
Total heterotrophic bacteria (CFU/mL)	NM	NM	NM
E.coli (CFU/mL)	NM	NM	NM
Total coliforms (CFU/mL)	NM	NM	NM
Fecal coliforms (MPN/L)	1000-10000	10000-40000	
Helminth (ind./mL)	NM	2	NM

a. Discharge standard of pollutants for municipal wastewater treatment plant (China); **b.** Standards for irrigation water quality (China); **c.** Guidelines for Water Reuse, Environmental protection agency (US).

Detection of pathogenic bacteria by RT PCR

The pathogens of *Aeromonas hydrophila*, *Arcobacter* sp., *Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli*, *Hartmannella vermiformis*, and *Legionella* sp. were detected by real-time quantitative PCR in the whole process of the

wastewater treatment plant (Fig. 2). The frequencies of the pathogenic bacteria were higher in the untreated wastewater in which the concentrations of *A. hydrophila* and *Arcobacter* sp. were up to 9.74 and 11.00 log copies·L^{-1}, respectively. At each subsequent step, almost the identical trends were observed in terms of the presence of the potential pathogenic bacteria. Compared with the untreated wastewater, *A. hydrophila*, *Arcobacter* sp., *C. perfringens*, *E. coli*, and *Hartmannella vermiformis* decreased by 2.15 log copies·L^{-1}, 2.54 log copies·L^{-1}, 1.46 log copies·L^{-1}, 2.33 log copies·L^{-1}, 1.62 log copies·L^{-1} respectively, and *C. difficile*, *Legionella* sp. slightly decreased in the treated effluent.

Table 4: Comparison of major physical and chemical properties in the original soil and the irrigated soil

Parameters	Soil types and values[a]		Limiting values
	Original soil	CK-WW[b]	GB 15618-2008 (China)[c]
pH	7.96	7.75	6.5-7.5
EC (μs/cm)	571	868	NM
Organic matter (OM, %)	1.58	2.43	NM
NH_4^+-N (mg/kg)	0.063	4.43	NM
NO_3^--N (mg/kg)	8.56	104	NM
Available N (mg/kg)	107.72	109	NM
Available P (mg/kg)	3.1	10.8	NM
Total Cu (mg/kg)	20.302	22.3	35-400
Total Pb (mg/kg)	36.26	26.1	35-500
Total Zn (mg/kg)	67.309	64.3	100-500
Total Cd (mg/kg)	0.023	0.10	0.2-1.0

a, the average values; b, CK-WW: irrigated with untreated wastewater as control**; c,** Environmental quality standards for soils (China)**. NM,** Not mentioned. Average relative deviations of Cu, Pb, Zn, Cd ≤10%.

Table 5: Comparison of soil profiles in different treatments at harvest of Lettuce

Parameters	Irrigation treatment					
	PWF	WWF	AEF	TEF	CK-PW	CK-WW
pH	7.90±0.05a	7.95±0.05a	7.90±0.09a	7.82±0.04a	7.69±0.01a	7.75±0.03a
EC (μs/cm)	910±24.5a	1399±26.5ab	995±17ab	1117±4ab	918±26.5a	868±14.5b
Organic matter (%)	2.47a	2.55a	2.93a	2.65a	2.42a	2.43a
Urease (mg/g)	31.31±1.58a	37.17±0.73ab	28.06±7.71b	30.98±0.61a	39.21±0.12ab	39.63±3.46ab
Alkaline phosphatase (mg/g)	18±3.03a	31±10.75b	22±0.79a	27±2.90ab	21±1.45a	22±1.95a

PWF: irrigated with potable water; **WWF:** irrigated with untreated wastewater; **AEF:** irrigated with aerobic tank effluent; **TEF:** irrigated with treated effluent; **CK-PW:** no plant and irrigated with potable water; **CK-WW:** no plant and irrigated with untreated wastewater.

Standard deviation of the mean (n = 3) is shown, different letters demonstrate a significant difference at $P<0.05$.

Table 6: Quality parameters of harvested lettuce following irrigation with PWF (control), WWF, AEF and TEF treatments

Parameters	Treatments			
	PWF	WWF	AEF	TEF
Plant height (cm)	12.10±0.26a	12.03±0.23a	11.50±0.44a	13.03±0.91a
Fresh weight (g)	1.75±0.21a	2.07±0.20a	1.68±0.24a	2.04±0.18a
Soluble sugars (%)	0.77±0.09a	1.27±0.07c	0.62±0.12bc	0.42±0.12ab
Soluble proteins (mg/g)	11.05±0.02a	12.08±1.43a	10.57±1.7a	11.29±0.53a
Nitrate (mg/kg)	195.67±21.31a	265.00±38.79ab	221.33±13.76ab	290±12.10b

PWF: irrigated with potable water; **WWF:** irrigated with untreated wastewater; **AEF:** irrigated with aerobic tank effluent; **TEF:** irrigated with treated effluent. Standard deviation of the mean (n = 3) is shown, different letters demonstrate a significant difference at $P<0.05$.

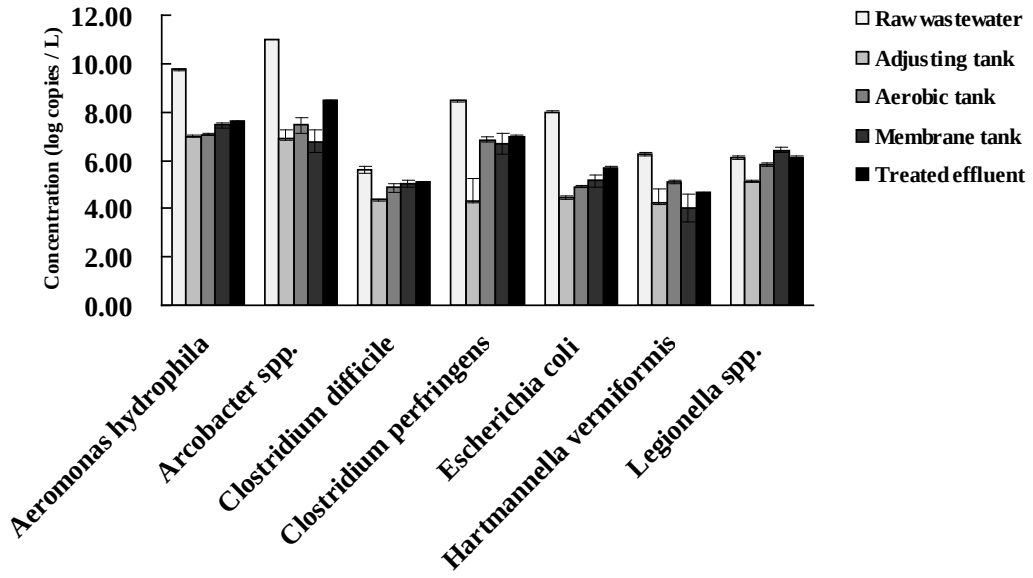

Fig. 2: Real-time quantitative PCR results for 8 kinds of pathogens in each step of the wastewater treatment processes.

Wastewater and fresh water is known to be a source and reservoir of microorganisms, most of them were found to be potentially pathogenic. For each step of wastewater treatment processes, it was found in a high proportion. *E. coli* and *C. perfringens* are both common indicator microorganisms for water and wastewater, which has a reduction in gene copies of 1.46 and 2.33 orders of magnitude from the untreated wastewater stage to the final effluent stage, respectively. *A. hydrophila* was sampled from untreated wastewater to final treated effluent for assay. It is suggested that it maintained in a high level from beginning to end. After processing, the level of *A. hydrophila* decreased 2.15 log. No changes in volume of these microorganisms may be implied that they could multiply or persist in the water niche, this phenomenon that did not commonly occur in treated effluent may be arouse concern. In a sanitary sewage stabilization pond treatment system, the distribution of *Aeromonas* was carried out by colony isolation which utilized blood agar ampicillin. The study demonstrated that 13 species were isolated from wastewater, and 72.4% of

influent samples, 55.2% and 48.3% of effluent from anaerobic and facultative lagoons. Understanding the distribution of pathogens in wastewater treatment processes, attention should be paid due to it represents potential health risks for reclamation (Martone-Rocha S et al., 2010). There is no significant difference for *Legionella* sp. before and after processing, merely reduced 0.32 log. Since typically reclaimed water is examined only for the presence of coliform bacteria, *Legionella* sp. was known to be more resistant to chlorination than common bacteria. Previously, researchers observed no significant reduction in population numbers throughout the treatment process and detected $>10^3$ *Legionella* cells/mL in primary and secondary sewage effluents by using an EnviroAmp *Legionella* PCR kit and direct fluorescent antibody (DFA) staining (Carol et al., 1995). Free-living amoebae are widely ubiquitous in soils and various water body, certain species were recognized as potential health risk for human and animals as they might be opportunistic pathogenicity and harbor potential pathogenic bacterial, in particular, *Acanthamoeba* sp., *Hartmannella* sp., and *Naegleria* sp.. They acted as predators of bacteria, viruses, fungi, and algae, and as reservoirs of pathogenic bacteria such as *Legionella* sp., *Mycobacterium* sp., *Staphylococcus aureus*, and *Vibrio cholerae* (Muchesa P, et al., 2014).

The two other dominant microorganisms that were detected at the whole processes of wastewater treatment were *Arcobacter* sp. and *C. difficile*, two typical of emerging bacterial pathogens. Thus, far little is known about the epidemiology of *Arcobacter* species, among them only *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, and *A. cibarius* have been associated with animal and human infections (Houf et al., 2005), which are recognized as a potential foodborne and waterborne pathogen probably transmitted through a fecal-oral route. The concentration of *Arcobacter* sp. varied from samples between 6.79 log and 11.00 log per liter. *C. difficile* infections are the most common cause of pseudomembranous colitis, and in rare cases this can progress to toxic megacolon, which can be life-threatening. Occurrence of *C. difficile* in the wastewater was determined by real-time PCR and ranged from 4.37 log to 5.61 log per liter. Previously, researchers were able to detect *C. difficile* using molecular beacons when at least 5×10^4 CFU /g were present in feces (Belanger et al., 2003), but rarely study in

water body. With a reduction of 0.5 to 2.5 orders of magnitude which pathogens were monitored between the untreated wastewater and final treated effluent stage. Romano V et al. investigated the occurrence of *Clostridium difficile* in nine wastewater treatment plants employing activated sludge processes in southern Switzerland. The study concerns that toxigenic ribotypes of *C. difficile* involved in human infections that presented both in raw sewage influents and treated effluents, The results showed that 47 characterized *C. difficile* strains belonged to 13 different reference PCR ribotypes, and 85% isolates were toxigenic. In addition, a toxigenic emerging ribotype was isolated from the outgoing effluent of one plant that treated wastewater from hospitals. The findings provided a reference profile for irrigation reuse which might give rise to health risks (Romano V et al., 2012).

Many studies have shown that untreated and treated wastewater is used for irrigation in place of potable water (Birks et al., 2007; Bixio et al., 2005). Sampling from wastewater may contain large numbers of pathogenic bacteria, and then we examined the effects of irrigation on soils and leafy greens using different irrigation schemes. No detection of the presence of *C. difficile*, *C. perfringens*, *Hartmannella vermiformis*, and *Legionella* sp. were found in all samples and gave a negative signal in all real-time PCR assays, instead, *Bacillus cereus* and *Mycobacterium* sp..

The roots and leaves harbor a diverse pathogens due to irrigation by wastewater, the presence of these bacterial pathogens in phyllosphere and rhizosphere was measured after harvesting during the 8-week growth period by real-time quantitative PCR assay. Pathogens densities were measured by four treatments of irrigation corresponding to when the greens reached the size typically harvested for human consumption (approximately 8 week-old). Compared to potable water treated, pathogenic microorganisms in other treatments had pooled more quantities except for *Arcobacter* sp. in the phyllosphere. In the rhizosphere, there had almost identity in each treatment but *Arcobacter* sp. from irrigated with untreated wastewater was predominately presented. *B. cereus* and *Mycobacterium* sp. abundance in the rhizosphere were significantly higher than in the phyllosphere compared to each treatment, whilst other pathogens had slightly differences (Fig. 3).

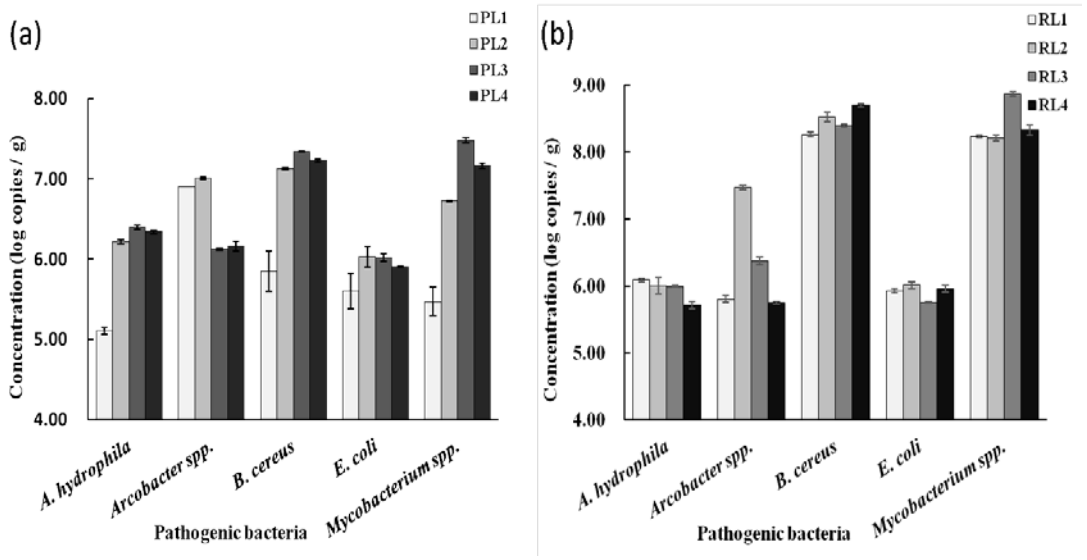

Fig. 3: The results are shown as mean amounts and standard deviations of target genomes in one gram of samples. (a) quantification of phyllosphere genomes with real-time quantitative PCR, and **(b)** quantification of rhizoshpere genomes with real-time PCR.

PL1, phyllosphere irrigated with potable water; **PL2,** phyllosphere irrigated with untreated wastewater; **PL3,** phyllosphere irrigated with aerobic tank effluent; **PL4,** phyllosphere irrigated with treated effluent. **RL1,** rhizosphere irrigated with potable water; **RL2,** rhizosphere irrigated with untreated wastewater; **RL3,** rhizosphere irrigated with aerobic tank effluent; RL4, rhizosphere irrigated with treated effluent.

In this study, we have investigated the occurrence of pathogens to survive in the phyllosphere and rhizosphere environment in order to gain a better understanding of the epidemiological importance and risks associated with the contamination of vegetables. From the results, the greatest numbers of pathogenic bacteria in the phyllosphere irrigated untreated wastewater (PL2) and aerobic tank effluent (PL3) ranged from 6.02 log to 7.48 log copies·g^{-1}. Compared to potable water treatment (PL1), the treated effluent treatment (PL4) has a little increase for all pathogens except for *Arcobacter* sp.. Lettuces from the trial harbored similar numbers of *E. coli*

by different irrigation treatments, while it was obvious that *B. cereus* and *Mycobacterium* sp. has a lower value in PL1 treatment than the other treatments. Comparisons among the rhizosphere microbiota from lettuces through different irrigation waters revealed several significant trends. The rhizosphere environment is a niche for pathogenic bacteria that causes only in debilitated or immune compromised humans occasionally (Rodrigo et al., 2013). In this study, it was shown that pathogenic bacteria were greatly enriched in the rhizosphere of different treatments. The population of *A. hydrophila* and *E. coli* were introduced into the soils 5.72 log -6.09 log copies·g^{-1} and 5.76 log - 6.02 log copies·g^{-1}, respectively, these two bacteria has no greatly obvious differences using different irrigation water. Other studies investigating the risks of using manure as fertilizer on vegetables have found that transmission may occur under experimental conditions in the greenhouse through the internal organs and to the edible portion of the plant with *E. coli* O157:H7 of about 10^6 CFU/g (Natvig et al., 2002). The significance of the occurrence of pathogenic species on fresh vegetables for the epidemiology of food-borne infections is yet unclear.

Additionally, all crops are grown under low or high temperatures, a condition that favors survival of pathogens in the environment. So far, most studies have focused on the role of pathogen infections of edible plant. For example, fruits and vegetables infected with wastewaters and manures led to significant increases in populations of *Salmonella* and *E. coli* O157: H7 (Shane et al., 2011). The microbial data collected from samples suggested that *B. cereus* and *Mycobacterium* sp. are easier to live and multiply in rhizosphere due to root exudates providing variety of nutrient factors. On leaf surface, microbial qualities of phyllosphere are influenced under different environmental conditions, such as UV, humidity, temperature.

Nevertheless, despite these differences, there were also similarities between treatments and among the potting trials. Based on this observation, it is noteworthy that the population sizes of pathogens in the lettuce phyllosphere or rhizosphere appear to be persisted using treated wastewater slightly higher than conventional irrigation.

Conclusions

The ability of pathogenic bacteria to actively colonize a number of agriculturally important plants has fairly recently been recognized as a major public health concern. Most pathogens were thought to result from passive contamination of root and leaf surfaces through spray or drip irrigation from wastewater. Our present studies showed that there was little risk to consumers using treated wastewater for agricultural irrigation, the treated wastewater can be used as an alternative for irrigation of vegetable planting. However, pathogenic monitoring is very necessary and important because wastewater sources and treatment technology are different in rural areas. It is also suggested that there is needs to establish a comprehensive risk assessment including setting appropriate standards and guidelines for wastewater irrigation.

Acknowledgments

This work was supported by the Key Project of Chinese Academy of Sciences (nos. KZZD-EW-09-3/1 and KZZD-EW-11-3) and the National Science and Technology Major Project of China (nos. 2012ZX07203-006 and 2014ZX07204-005).

References

Akponikpe,` PBI., Wima, K., Yacouba, H., Mermoud, A., 2011. Reuse of domestic wastewater treated in macrophyte ponds to irrigate tomato and eggplant in semi-arid West-Africa: benefits and risks. Agric Water Manag. 98 (5), 834–840.

A. Mark Ibekwe, C. M. Grieve, S. K. Papiernik and C. –H. Yang., 2009. Persistence of *Escherichia coli* O157:H7 on the rizhosphere and phyllosphere of lettuce. Letters in Applied Microbiology. 49 (6), 784-790.

Andersson A., Ronner U., Granum PE., 1995. What problems does the food industry have with the spore-forming pathogens Bacillus cereus and Clostridium perfringens? Int J Food Microbiol. 28 (2), 145-155.

Asano, T., and Levine A. D., 1996. Wastewater reclamation, recycling and reuse: past, present and future. Water Science and Technology. 33, 1-14.

Ashbolt, N. J., A. Ball, M., Dorsch, C., Turner, P., Cox, A., Chapman, and S. M. Kirov., 1995. The identification and human health significance of environmental aeromonads. Water Science and Technology. 31 (5-6), 263-270.

Baoguo Zhang, Zhihui Bai, Daniel Hoefel, 2009. The impacts of cypermethrin pesticide application on the non-target microbial community of the pepper plant phyllosphere. Science of the Total Environment. 407 (6), 1915-1922.

Bastyns K., Cartuyvels D., Chapelle S., Vandamme P., Goossens H., and Dewachter R., 1995. Variable 23S rDNA region is a useful discriminating target for genus-specific and species-specific PCR amplification in *Arcobacter* species. Sysem. Appl. Microbiol. 18 (3), 353-356.

Battilani, A., Steiner, M., Andersen, M., Back, SN., Lorenzen, J., Schweitzer, A., Dalsgaard,A.,Forslund, A., Gola, S., Klopmann, W., Plauborg, F., Andersen, MN., 2010. Decentralised water and wastewater treatment technologies to produce functional water for irrigation. Agric Water Manag. 98:385–402.

Baydal, D., 2009. "Municipal Wastewater Recycling Survey." California Water Recycling Funding Program (WRFP).

Belanger, S. D., Boissinot, M., Clairoux, N., Picard, F. J. and Bergeron, M. G., 2003). Rapid detection of *Clostridium. difficile* in feces by real-time PCR. Journal of Clinical Microbiology. 41 (2), 730-734.

Bielinska E.J., Zukowska G., 2002. Protease and urease activity in light soil fertilized with sewage sludge. Acta Agrophysica. 70, 41.

Birks R., Hill S., 2007. Characterization of indicator organisms and pathogens in domestic greywater for recycling. Environ Monit Assess. 129 (1-3), 61-69.

Bixio D., De Koning J., Savic D., Wintegens T., 2005. Wastewater use in Europe. In Integrated Concepts in Water Recycling. 80-92 (University of Wollongong, NSW, Australia).

Carol J. Palmer, G. Fred Bonilla, Bruce Roll, Christine Paszko-Kolva, Louis R. Sangermano, and Roger S. Fujioka., 1995. Detection of *Legionella* species in reclaimed water and air with the EnviroAmp Legionella PCR kit and direct fluorescent antibody staining. Appl Environ Microbiol. 61 (2), 407-412.

Cataldo DA., Haroom M., Schrader LE., Young VL., 1975. Rapid colorimetric determination of nitrate in plant tissues by nitration of salicylic acid. Comm. Soil Sci. Plant Anal. 6 (1), 71-80.

Chevremont AC., Boudenne JL., Coulomb B., Farnet AM., 2013. Impact of watering with UV-LED-treated wastewater on microbial and physico-chemical parameters of soil. Water Res. 47 (6), 1971-1982.

Day, D., 1996. How Australian social policy neglects water environments. Australian Journal of Soil and Water Conservation. 9 (4), 3-9.

do Monte, H. M., and M. S. e Sousa., 1992. Effects on crops of irrigation with facultative pond effluent. Water Science and Technology. 26 (7-8), 1603-1613.

Florida Department of Environmental Protection (FDEP), 2011. 2010 Reuse Inventory Florida Department of Environmental Protection.

Frank, S., Andrew, H., Vincent, V., Daniel, I., 2007. Wastewater reuse: environmental impacts and risk assessment.

Houf K., On SL., Coenye T., Mast J., Van Hoof J., and Vandamme P., 2005. *Arcobacter cibarius* sp. nov., isolated from broiler carcasses. Int J Syst Evol Microbiol. 55 (2), 713-717.

Hamilton AJ., Stagnitti F., Xiong X., Kreidl SL., Benke KK., Maher P., 2007. Wastewater irrigation: the state of play. Vadose Zone Journal. 6 (4), 823–840.

Irrigation with nutrient-rich wastewater, WQPN 22, JULY 2008.

Joseph A. Whelan, Nick B. Russell, Michael A., Whelan. A., 2003. Method for the absolute quantification of cDNA using real-time PCR. Journal of Immunological Methods. 278 (1-2), 261 - 269.

Kingombe CI., Huys G., Tonolla M., Albert MJ., Swings J., Peduzzi R., Jemmi T., 1999. PCR detection, characterization, and distribution of virulence genes in Aeromonas sp. Appl Environ Microbiol. 65(12), 5293-5302.

Kuiper MW, Valster RM, Wullings BA, et al., 2006. Quantitative detection of the free-living Amoeba Hartmannella vermiformis in surface water by using real-time PCR. Applied and Environmental Microbiology. 72(9): 5750-5756

Lowry, O. H., A. L., Farr and J. J. Randally, 1951. Protein measurement with the folin

phenol reagent. J. Biol. Chem. 193, 265-275.

Martone-Rocha S, Piveli R, Matte G, et al., 2010. Dynamics of Aeromonas species isolated from wastewater treatment system. Journal of water and health. 8(4), 703-711.

Maya Benami, Amit Gross, Moshe Herzberg, Ezra Orlofsky, Ahuva Vonshak, Osnat Gillor, 2013. Assessment of pathogenic bacteria in treated graywater and irrigated soils. Science of the Total Environment. 458-460, 298-302.

Michel Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and Fred Smith, 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350-356.

Miyamoto, H., Yamamoto, H., Arima, K., Fujii, J., Maruta, K., Izu, K., Shiomori, T., Yoshida, S., 1997. Development of a new seminested PCR method for detection of Legionella species and its application to surveillance of legionellae in hospital cooling tower water. Appl. Environ. Microbiol. 63 (7), 2489-2494.

Muchesa P, Mwamba O, Barnard T G, et al., 2014. Detection of Free-Living Amoebae Using Amoebal Enrichment in a Wastewater Treatment Plant of Gauteng Province, South Africa. BioMed research international, 2014.

Natvig, E. E., Ingham, S. C., Ingham, B. H., Cooperband, L. R. and Roper, T. R., 2002. Salmonella enterica serovar Typhimurium and Escherichia coli contamination of root and leaf vegetables grown in soils with incorporated bovine manure. Appl Environ Microbiol. 68 (6), 2737-2744.

O. Al-Lahham, N. M. El Assi, M. Fayyad, 2003. Impact of treated wastewater irrigation on quality attributes and contamination of tomato fruit. Agric Water Manag. 61 (1), 51-62.

Pedrero, F., Kalavrouziotis, I., Alarcon, JJ., Koukoulakis, P., Asano, T., 2010. Use of treated municipal wastewater in irrigated agriculture – review of some practices in Spain and Greece. Agric Water Manag. 97 (9), 1233–1241.

Pimentel, D., and M. Pimentel, 2003. “Sustainability of Meat based and Plant-based Diets and the Environment.” American Journal of Clinical Nutrition. 78 (3): 660S-663S.

Poitelon, J. B., Joyeux, M., Welt, B., Duguet, J.P., 2009a. Assessment of phylogenetic diversity of bacterial microflora in drinking water using serial analysis of ribosomal sequence tags. Water Res. 43 (17), 4197-4206.

Priha O., Hallamaa K., Saarela M., Raaska L., 2004. Detection of Bacillus cereus group bacteria from cardboard and paper with real-time PCR. J Ind Microbiol Biotechnol. 31(4), 161-169.

Quilliam RS., Williams AP., Jones DL., 2012. Lettuce cultivar mediates both phyllosphere and rhizosphere activity of *Escherichia coli* O157:H7. PLoS ONE 7 (3), e33842.

Rajni Kaushik, Rajasekhar Balasubramanian, and Armah A. de la Cruz., 2012. Influence of air quality on the composition of microbial pathogens in fresh rainwater. Appl. Environ. Microbiol. 78 (8), 2813-2818.

Richard G. Burau, Bahman Sheikh, Robin P. Cort, Robert C. Cooper, David Ririe, 1987. Reclaimed water for irrigation of vegetables eaten untreated. California Agriculture, July-August.

Rodrigo Mendes, Paolina Garbeva, Jos M. Raaijmakers, 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol Rev. 37 (5), 634-663..

Romano V, Pasquale V, Krovacek K, et al., 2012. Toxigenic *Clostridium difficile* PCR ribotypes from wastewater treatment plants in southern Switzerland. Applied and environmental microbiology. 78(18): 6643-6646.

Sanjay K. Sharma，Rashmi Sanghi, 2013. Wastewater Reuse and Management. DOI 10.1007/978-94-007-4942-9_6

Shainberg I., Oster J D., 1978. Quality of irrigation water [M]. London: Pergamon Press.

Shane W. Rogers, Matthew Donnelly, Lindsay Peed, Catherine A. Kelty, et al., 2011. Decay of bacterial pathogens, fecal indicators, and real-time quantitative PCR genetic markers in manure-amended soils. Appl Environ Microbiol. 77 (14), 4839-4848.

Tabatabai M. A., Bremner J. M., 1969. Use of P-nitrophenyl phosphate for assay of

soil phosphatase activity. Soil Biol. Biochem. 1, 301.

T. Huber, G. Faulkner and P. Hugenholtz, 2004 Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics. 20, 2317-2319

Tom A. Mendum, Ben Z. Chilima, Penny R. Hirsch, 2000. The PCR amplification of non-tuberculous mycobacterial 16S rRNA Sequences from soil. FEMS Microbiol Lett. 185 (2), 189-192.

T. Rinttila, A. Kassinen, E. Malinen, L. Krogius and A. Palva., 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol. 97 (6), 1166-1177.

Wang Junfeng, Wang Genxu, WanYan Hua, 2007. Treated wastewater irrigation effect on soil, crop and environment: Wastewater recycling in the loess area of China. Journal of Environmental Sciences. 19, 1093-1099.

Wesley, I. V., 1996. Helicobacter and Arcobacter species - risks for foods and beverages. Journal of Food Protection. 59: 1127-1132.