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Investigating the bacterial community and amoebae population in rural domestic wastewater reclamation for irrigation

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ABSTRACT

Reclamation of domestic wastewater for agricultural irrigation is viewed as a sustainable option to create an alternative water source and address water scarcity. Free-living amoebae (FLA), which are amphizoic protozoa, are widely distributed in various environmental sources. The FLA could cause considerable environmental and health risks. However, little information is available on the risk of these protozoa. In this study, we evaluated the feasibility using rural domestic wastewater for agricultural irrigation, and analyzed dynamic changes of the microbial community structure and FLA populations in raw and treated wastewater, as well as the phyllosphere and rhizosphere of lettuce production sites that were irrigated with different water sources. The bacterial community dynamics were analyzed by terminal restriction fragment length polymorphism (T-RFLP). The bacterial community structures in the influent were similar to that in the effluent, while in some cases relative abundances varied significantly. The populations of *Acanthamoeba* spp. and *Hartmannella vermiformis* in the anaerobically treated wastewater were significantly higher than in the raw wastewater. The vegetables could harbor diverse amoebae, and the abundances of *Acanthamoeba* spp. and *H. vermiformis* in the rhizosphere were significantly higher than in the phyllosphere. Accordingly, our studies show insight into the distribution and dissemination of amoebae in wastewater treatment and irrigation practices.

Introduction

Domestic wastewater is used as an alternative water source for agricultural irrigation, aquaculture production and industrial processes in water shortage and dry regions over the world. Its environmental and health risks need serious consideration. The untreated domestic wastewater not only contains numerous hazardous agents, but also harbors a range of pathogenic bacteria and amoebae, which pose a potential health risk. Irrigation with wastewater for agriculture may introduce and accumulate chemical and biological contaminants in soils and crops. Many studies have identified the transfer of waterborne pathogens to the phyllosphere and rhizosphere from wastewater and the environment (Al-Lahham et al., 2003; Warriner et al., 2003; Cooley et al., 2003; Heaton and Jones, 2008; Orlofsky et al., 2011; Yang et al., 2015).

Free-living amoebae (FLA) are amphizoic protozoa that can be found commonly in various environments, such as wastewater,

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swimming pools, surface water and soils (Tsvetkova et al., 2004; Rodriguez-Zaragoza, 1994). *Acanthamoeba* spp., *Hartmannella vermiformis*, *Naegleria fowleri*, *Balamuthia mandrillaris* and *Sappinia diploidea* are known as causative agents that have pathogenicity, mainly in immunocompromised groups (Visvesvara et al., 2007; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004; Grün et al., 2014; Qvarnstrom et al., 2013; Khan, 2009). The amoebae genera *Acanthamoeba* and *Hartmannella* were determined to be potential carriers of pathogenic bacteria, especially *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Mycobacterium* spp., *Vibrio cholera*, *Listeria monocytogenes* and *Escherichia coli*, which are widely distributed in water systems (Loret and Greub, 2010; Berger et al., 2006; Thom et al., 1992; Lorenzo-Morales et al., 2007). Additionally, scientists found that *Acanthamoeba* genotypes and *Hartmannella* showed great thermotolerance and osmotolerance, and their cysts had high resistance to disinfection practices (Lu et al., 2015). In a previous study, the FLA population was determined in the wastewater samples from five Spanish wastewater treatment plants. It was observed that treatment with sodium hypochlorite showed no significant reduction in the number of amoebae at concentrations of 0–100 mg/L (García et al., 2011). The FLA and amoebae-resisting bacteria were investigated at various stages of a drinking water plant fed with river water. Amoebae were identified positively with a quantitative method, though not isolated after chlorination (Thomas et al., 2008). Another concern with FLA is their colonization in water systems that allows the survival of waterborne pathogenic bacteria, as they have the ability to recolonize in crops after wastewater reclaimation. Most of the published studies on biological contamination and health risks, as well as wastewater reuse, have been based on certain indicator bacteria, such as *E. coli*, *Salmonella* sp., *Staphylococcus aureus* and Coliform (Zhang et al., 2015). However, few investigations have reported the occurrence of pathogenic amoebae, even though they are widely present in near all ecosystems. Wastewater that is commonly used for agricultural irrigation contains a variety of bacteria and protists, while the transfer of pathogenic microorganisms from wastewater to the phyllosphere and rhizosphere has seldom been discussed. The treated effluent may pose potential health risks by disseminating pathogenicity when discharged into the receiving rivers or reused for agricultural irrigation. Considering the potential environmental and health risks, the pathogenic amoebae community and population in not only wastewater, but also on leafy greens and root zones, need to be investigated.

This study aims to characterize dynamic changes in the bacterial community and amoebae population in raw and treated domestic wastewater from an on-site anaerobic biofilm reactor treatment process. The treated wastewater was intended for use in local agricultural irrigation. We also investigated the amoebae population on the phyllosphere and rhizosphere of lettuce irrigated with domestic wastewater. The sedimentation and culture methods were used for counting helminth eggs. A culture-independent real-time PCR was used for detection of the protozoan parasites. This study highlighted the occurrence of two amoebae in raw and treated wastewater, and on the phyllosphere and rhizosphere of lettuces. Moreover, further research is needed to elucidate the microecological behavior of amoebae and amoebae-associated pathogens in both sewage systems and agricultural fields, which may better assess the potential health risks, as some FLA are pathogenic and involved in the dissemination of pathogenic bacteria.

1. Materials and methods

1.1. Design and operation of pilot anaerobic biofilm bioreactor process

The pilot-scale domestic wastewater treatment reactor in this study was operated with three anaerobic biofilm bioreactor processes. The bioreactor was made of polyethylene materials and had a working volume of 360 L. The reactor temperature was maintained at 20–28°C, and had a 72 hr. hydraulic retention time (HRT). As sketched in Fig. 1, the pilot plant was installed with a wastewater pooling tank and a set of anaerobic biofilm processes, followed by an effluent storage tank. No disinfection facility was set up with the pilot plant.

The plot experiment was performed in a vegetable garden located in Huairou District, Beijing, China. The lettuce (*Lactuca sativa* L.) was used as a model vegetable to estimate microbial contamination on crops irrigated with the different water sources in this study. The lettuce seeds, purchased from Chinese Academy of Agricultural Sciences (CAAS.), were pretreated before seeding as described previously (Quilliam et al., 2012). Briefly, seeds were surface sterilized using 3% sodium hypochlorite solution for 15 min, followed by several rinses with sterile distilled water. The lettuce seeds were planted in 2 × 2 m plots in an open local field. Experimental sites were designed and operated with 3 irrigation patterns using raw wastewater (RW), treated effluent (TE), and potable water (PW) as a controlled trial. Each operation site was applied in four replicates with a randomized block design.

1.2. Samples collection and DNA extraction

Water samples were collected in June–October at monthly intervals. All samples were pooled in 1 L sterile polyethylene bottles, and transported immediately to the laboratory for physicochemical analysis and molecular assays. The pH and electrical conductivity were tested on site using a portable multi-parameter meter (HACH HQ40d, USA). For analyzing microbial communities and pathogens, 100 mL of each water sample was filtered with 0.22 μm mixed cellulose membranes (47 mm diameter, Millipore, USA) to obtain microbial cells in a centrifuge tube, and then stored at −80°C until required.

Lettuces were harvested at the mature stage (about 8 weeks), and the leaf samples were collected aseptically using sterilized scissors and placed in homogeneous bags. Microbiological pellets were obtained as described previously (Zhang et al., 2009). Rhizosphere soil samples were collected after shaking off soil loosely adhered to the roots, and sieved and homogenized through a 0.9 mm sieve after vacuum freeze drying for further analysis.

Genomic DNA of the aforementioned samples was extracted in Lysing Matrix tubes using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions, except for the following: the tubes were shaken in a FastPrep® Instrument for 45 sec at a speed of 5.0 m/sec, and DNA

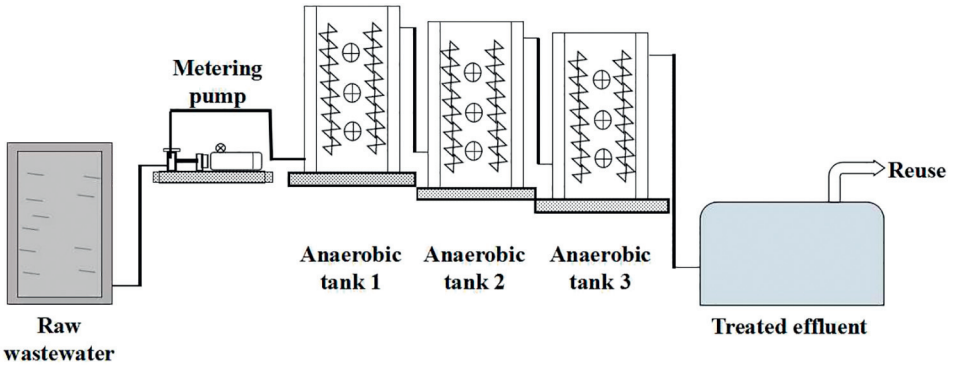

Fig. 1 – Schematic drawing of anaerobic biofilm reactor.

was dissolved in 80 μL of DNase/Pyrogen-free water, and stored at –80°C.

1.3. T-RFLP analysis

For T-RFLP analysis, bacterial 16S rRNA genes were amplified from genomic DNA using universal primer pair 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1392R (5′-ACG GGC GGT GTG TRC-3′) (Liu et al., 1997), while the 8F primer was fluorescently labeled with the dye 5-carboxyfluorescein (FAM). PCR amplification was performed in a 50 μL reaction volume containing 25 μL of 2 × PCR PreMix, 1 μL of each primer (10 μmol/L), 1 μL template DNA and 22 μL nuclease-free water. PCR were performed using a EDC-810 PCR instrument (EASTWIN, China.) under the following conditions: initial denaturation step of 5 min at 95°C followed by 35 cycles of 45 sec at 95°C, 45 sec at 50°C, and 60 sec at 72°C, followed by 10 min final extension at 72°C, and then held at 4°C. The PCR reaction was run in duplicate and the products were pooled together to reduce errors and biases. Then the PCR products were validated by electrophoresis on 1% (W/V) agarose gels and stained with GelRed (Biotium, USA), followed by purification with the E.Z.N.A™ Gel Extraction Kit (Omega Bio-tek, USA) according to the manufacturer's instruction.

The purified PCR products were digested with the tetrameric restriction endonuclease *Hha*I (NEW ENGLAND Biolabs® Inc. USA) at 37°C for 2 hr. The restriction-digestion mixture consisted of 2 μL of 10 × CutSmart Buffer, 0.5 μL of restriction endonuclease (10 U/μL), and 8 μL of purified DNA. FAM-labeled terminal restriction fragments (T-RFs) were analyzed by a commercial service (Ruibo Biotechnology Inc., China) using the ABI 3730xl DNA analyzer (Applied Biosystems, USA) in the GeneMapper 50_POP7_1 Module. Their size and fluorescence intensities (peak height or area) were calculated using GeneMarker software V1.80 (Applied Biosystems, USA). To avoid detection of primers and uncertainties of size determination, T-RF fragments smaller than 50 bp were excluded from further analysis. T-RFs with relative abundance below 1% were regarded as background noise and excluded from analysis (Casamayor et al., 2002). Because the PCR products were nearly 900 bp, the 1200 LIZ GeneMarker Size Standard (Applied Biosystems, USA) was used as the internal label to measure the size of labeled T-RFs by capillary electrophoresis in each sample. T-RFs with a peak height of 100 fluorescence units were considered to have positive peaks. Each T-RF represented an operational taxonomic unit (OTU), and the relative abundance of each OTU was signified by the size and fluorescence intensity. The phylogenetic positions representing predominant T-RFs were presumably identified using a database (Microbial Community Analysis (MiCA) III, http://mica.ibest.uidaho.edu), a deviation of ±2 bp was allowed.

1.4. Real-time quantitative PCR analysis

Detection and quantification of pathogenic amoebae from wastewater, phyllosphere, and rhizosphere samples using qPCR targeted the 18S rRNA gene of *Acanthamoeba* spp. and *H. vermiformis*. The primers were detected as follows: AcantF900 (5′-CCC AGA TCG TTT ACC GTG AA-3′), AcantR1100 (5′-TAA ATA TTA ATG CCC CCA ACT ATC C-3′); Hv1227 (5′-TTA CGA GGT CAG GAC ACT GT-3′), Hv1728R (5′-GAC CAT CCG GAG TTC TCG-3′) (Qvarnstrom et al., 2006; Kuiper et al., 2006). Conventional PCR was conducted to identify the presence of these two amoebae, and PCR products were confirmed with 1% (W/V) agarose gel electrophoresis.

The obtained PCR products were gel-purified and ligated into the pGEM-T Easy Vector (Promega, USA), then transformed into competent *Escherichia coli* DH5α (Biomed, China). The positive clones were screened on X-Gal-IPTG-Ampicillin-indicator plates by color-based recombinant selection, and inserted fragments were further confirmed by PCR amplification with T7 and SP6 primers and sequenced by a commercial company (Ruibo Biotechnology Inc., China). The nucleotide sequences were submitted and aligned by BLASTn in NCBI. After determination, the positive clones were selected to extract plasmid DNA using the E.Z.N.A.® Plasmid Mini Kit Spin Kit (Omega Bio-tek, USA) according to the manufacturer's instructions. The concentration of the plasmid DNA was detected with a Nanodrop 2000 UV–Vis Spectrophotometer (Thermo Scientific, USA), and used as pathogenic gene standards. The gene copy number was calculated directly from the concentration of the extracted plasmid DNA (Whelan et al., 2003). Amoeba concentrations in wastewater, phyllosphere, and rhizosphere samples were confirmed quantitatively by assaying 10-fold serial dilutions of plasmid DNA.

The real-time PCR assay was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA) in a 25 μL volume containing 2 × SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Biotechnology, China), 0.4 μL of each primer (10 μmol/L),

and 2 μL of template DNA. Thermal cycling conditions were as follows: initial denaturation at 95°C for 30 sec, 45 cycles of 95°C for 5 sec, 60°C for 30 sec, 72°C for 30 sec, followed by a melt curve stage: from 65°C to 95°C with increments of 0.5°C. All experiments were tested in triplicate, and the corresponding negative (DNase/RNase-free distilled water) control was included.

1.5. Data analysis

The evaluation of diversity indices was based on the T-RF areas. The profiles of microbial community diversity were evaluated by the following indices: the Shannon–Weiner diversity index (*H*), Simpson index (*D*), Margalef richness index (*R*), and Pielou's evenness index (*J*) were calculated by the *PAST* 2.14 software package (Hammer et al., 2001). Other statistical analyses were performed with Microsoft Office Excel 2007 and Origin 8.0.

2. Results and discussion

2.1. Assessment of anaerobic biofilm reactor pilot plant

The performance results from operating the anaerobic biofilm reactor pilot plant are summarized in Table 1. 75.2%–91.9% COD removals were performed during the five-month operation. Compared to the raw wastewater, the total nitrogen (TN), ammonia nitrogen (NH_3-N), and total phosphorus (TP) concentrations of the treated effluent increased slightly. The reason may be that removal of the nitrogen and phosphorus did not take place in the anaerobic environment, so that nitrogenous compounds were reduced to ammonia nitrogen and the soluble phosphate was anaerobically released. Although most quality parameters of the treated effluent did not meet the discharge standard of pollutants of a municipal wastewater treatment plant (GB 18918-2002, China), the water quality of the treated effluent could meet guideline standards for irrigation water quality (GB 5084-2005, China) from the perspective of farmland reuse.

2.2. Bacterial community structure dynamics

The T-RF profiles of 16S rRNA genes can provide useful information indicating the relative diversity of bacterial communities (Dunbar et al., 2000). To investigate bacterial community structures over time, samples taken from the anaerobic biofilm treatment system throughout the study period were analyzed by T-RFLP based on the 16S rRNA genes. Fig. 2 illustrates that the bacterial community dynamics and the relative abundances (*Pi* values) of T-RFs changed significantly with the temporal pattern. T-RFs of 58 bp, 88 bp, 91 bp, 98 bp, 201 bp, 203 bp and 209 bp were commonly present in all of the raw wastewater and treated effluent. T-RFs of 91 bp, 98 bp, 201 bp, 203 bp and 209 bp were commonly present in the raw wastewater. The relative abundances of those T-RFs were 0.026–0.086, 0.010–0.181, 0.011–0.098, 0.018–0.181 and 0.011–0.22, respectively. The T-RF of 91 bp increased along with time and reached a maximum of 0.086 in September. The T-RF of 98 bp was predominant in June with a relative abundance of 0.18, while declining to a minimum of 0.01 from July to October. T-RFs of 201 bp, 203 bp and 209 bp varied irregularly throughout the period. Based on the MiCA database search, T-RFs of 91 bp, 98 bp, 201 bp, 203 bp and 209 bp were assumed to be derived from *δ-Proteobacteria, Bacteroidales, Actinobacteria, β-Proteobacteria* or *Firmicutes* and *Acidobacteria*, respectively. McLellan et al. analyzed the bacteria community compositions in untreated sewage samples collected from two WWTPs in the United States, and they found that *Proteobacteria* (mainly *β-* and *γ-Proteobacteria*) dominated in the untreated sewage (McLellan et al., 2010). Although T-RFs of 54 bp and 77 bp were predominant in the raw wastewater with a maximum of 0.16 and 0.11, respectively, none of these were detected in June and July. T-RFs of 58 bp, 88 bp, 91 bp, 98 bp, 201 bp, 209 bp and 509 bp were predominantly presented in the treated effluent, and these relative abundances of T-RFs exhibited temporal variability. The MiCA database search suggested that T-RFs of 58 bp, 88 bp and 509 bp may represent *Planctomycetes*, *Bacteroidetes* and uncultured bacterium. In contrast, predominant T-RFs (54 bp, 73 bp, 203 bp and 562 bp) in the raw wastewater were undetectable or minor in the treated effluent. The treated effluent had specific

Table 1 – Performance of raw wastewater and treated effluent.

Parameters	Raw wastewater	Treated wastewater	Criteria of irrigation water
pH	6.75–7.41	6.36–7.86	5.5–8.5
EC (μS/cm)	556–814	382–866	≤1000
COD (mg/L)	135–218	11–54	≤100[a] or 60[b]
TN (mg/L)	11.8–22.5	13.2–16.4	–
NH_3-N (mg/L)	8.4–17.7	6.7–17.6	–
TP (mg/L)	2.5–5.2	2.0–7.9	–
Total bacteria (CFU/mL)	4.3×10^4–4.3×10^6	1.7×10^4–3.4×10^6	–
Total coliforms (CFU/mL)	1.0×10^3–6.0×10^5	1.0×10^3–7.2×10^4	–
Fecal coliforms (CFU/mL)	2.4×10^5	No detectable (<3)	≤20[a] or 10[b]
Escherichia coli (CFU/mL)	1.0×10^3–1.1×10^4	0–1.0×10^3	–

–: no data.
[a] Vegetables needing processing, cooking or peeling.
[b] Rabbit food, melons and fruit.

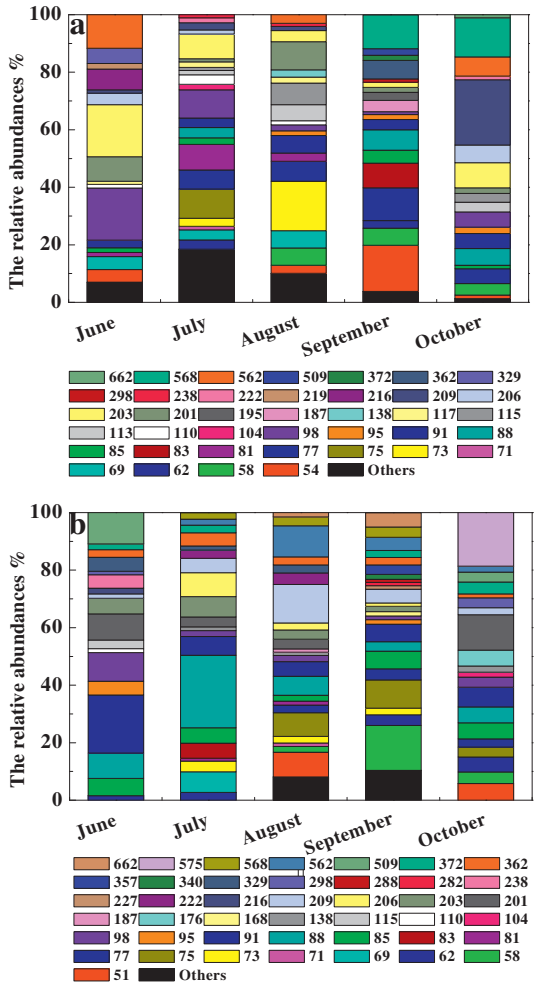

Fig. 2 – Histograms of terminal restriction fragment (T-RF) relative abundances in (a) raw wastewater and (b) treated effluent.

T-RF profiles (51 bp, 168 bp, 176 bp, 216 bp, 222 bp, 227 bp, 282 bp, 288 bp, 340 bp, 357 bp and 575 bp) and most of them were undetected in RW samples. In this study, although accurate representation of the detected T-RFs was not achieved, some common predominant bacteria were deduced based on the specific database. The T-RF of 203 bp was assumed to be derived from *Firmicutes* or *Proteobacteria* (mainly *Enterobacteriaceae* and *Enterococcaceae*), which accounted for a large proportion of bacteria in human feces; this specific T-RF dominated in the raw wastewater but decreased gradually in the treated effluent. The T-RF profiles were unable to provide reliable measures of phylotype richness and community structure. However, this method was shown to be very effective in elucidating differences between communities for microbial ecology research (Dunbar et al., 2000).

The numbers of T-RFs and biodiversity indices were obtained from raw wastewater and treated effluent, which are shown in Table 2. The numbers of T-RFs detected in RW and TE samples were 17–23 and 18–26, respectively. The T-RF profiles of the RW samples were similar to each other throughout the monitoring period. These findings suggested

Table 2 – Biodiversity indices of raw wastewater and treated effluent.

	Sample site	T-RF numbers	*H*	*D*	*R*	*J*
June	RW	17	2.52	0.90	1.50	0.69
	TE	18	2.65	0.91	1.56	0.74
July	RW	23	3.05	0.94	2.16	0.78
	TE	20	2.83	0.91	1.95	0.70
August	RW	20	2.90	0.93	1.81	0.79
	TE	24	3.20	0.95	2.56	0.77
September	RW	20	2.90	0.92	1.87	0.73
	TE	26	3.09	0.94	2.33	0.76
October	RW	20	2.89	0.92	2.04	0.69
	TE	20	3.04	0.94	2.08	0.83

RW: Raw wastewater; TE: Treated effluent; *H*: Shannon–Weiner index; *D*: Simpson index; *R*: Margalef richness index; *J*: Pielou's evenness index.

that the bacterial community in the raw wastewater was generally stable against temporal changes. T-RF numbers in TE were higher than in RW samples, but the relative abundances of T-RFs were lower. The Shannon–Weiner index calculated in terms of T-RFLP profiles varied between 2.52–3.05 (RW) and 2.65–3.20 (TE), and the Simpson index was 0.90–0.94 (RW) and 0.91–0.95 (TE). Biodiversity from a certain region was depicted by the Shannon-Weiner and Simpson indices, for which a greater index indicates a higher diversity but a lower dominance. The Margalef richness index and Pielou's evenness index showed consistency in RW and TE. The structure and diversity of the microbial community could reflect the functional stability of a bioreactor. In addition, microbial community structure could go through a certain degree of dynamic changes, with specific species introduced without affecting the stability of system operation (Hallin et al., 2005). Thus, our results appeared to be partly in accordance with the previous study (Hashimoto et al., 2014). Some previous studies have demonstrated that bacterial communities were highly variable in lab-scale and pilot-scale systems with the temporal fluctuation of environmental parameters (Wang et al., 2011; Wittebolle et al., 2008; Zumstein et al., 2000).

To investigate dynamic changes in the bacterial community during the irrigation period, we analyzed bacterial communities in the phyllosphere and rhizosphere with different irrigation patterns. The biodiversity of bacterial communities was estimated by determining the numbers of unique T-RFs and their peak areas. Fig. 3 shows the community profiles of phyllosphere and rhizosphere samples irrigated with potable water (L-PW), raw wastewater (L-RW), and treated effluent (L-TE). 51 bp, 55 bp, 77 bp, 85 bp, 88 bp, 91 bp, 201 bp, 203 bp, 216 bp, 220 bp, 237 bp, 340 bp, 506 bp and 512 bp T-RFs were presented commonly in the phyllosphere. As the predominant T-RFs, the relative abundances of 77 bp and 91 bp showed little change in all samples. T-RFs of 51 bp, 203 bp, 216 bp and 512 bp in the L-PW were considerably different from those in the L-WW and L-TE samples, indicating that the abundance of certain species was increased by wastewater irrigation. T-RFs of 51 bp, 55 bp, 77 bp, 85 bp, 88 bp, 91 bp, 216 bp and 237 bp were presented predominantly in the rhizosphere. Although the T-RF profiles in all the samples from the phyllosphere and rhizosphere were relatively similar to one another, with common dominant T-RFs

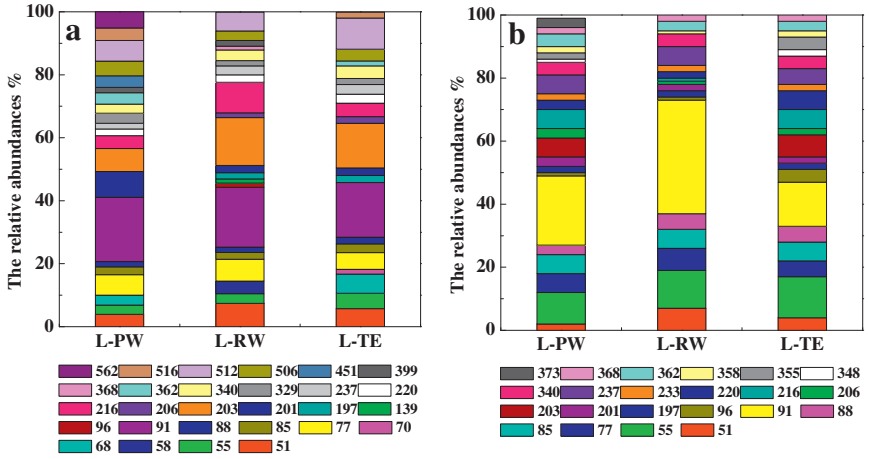

Fig. 3 – Histograms of T-RF relative abundances in (a) phyllosphere and (b) rhizosphere samples. L-PW: irrigated with potable water; L-RW: irrigated with raw wastewater; L-TE: irrigated with treated effluent.

of 55 bp, 77 bp, 85 bp, 91 bp and 237 bp, the relative abundances typically fluctuated.

The biodiversity indices of the phyllosphere and rhizosphere are shown in Table 3. T-RF numbers detected in the phyllosphere and rhizosphere samples were 21–22 and 19–22, respectively. The T-RF numbers were almost identical among phyllosphere samples, as well as the Shannon-Weiner and Simpson index, indicating relatively less variability. A uniform distribution of the bacterial community was found in all the phyllosphere samples, although Pielou's index was slightly low. The phyllosphere could support large and diverse naturally occurring microbial communities, of which bacteria, living both epiphytically and endophytically, are the most numerous and diverse (Hunter et al., 2010). The T-RF numbers and Shannon–Weiner index in the rhizosphere samples irrigated with TE reached maximum values of 22 and 3.14, respectively. The bacterial community structures in rhizosphere samples irrigated with RW and TE were different from those irrigated with potable water, suggesting that the wastewater could affect the bacterial community in the rhizosphere, which may be attributable to the accumulation of untreated pollutants from wastewater.

Table 3 – Biodiversity indices of phyllosphere and rhizosphere.

	Sample site	T-RF numbers	*H*	*D*	*R*	*J*
Phyllosphere	L-PW	21	2.93	0.92	1.97	0.75
	L-RW	22	2.87	0.92	2.17	0.68
	L-TE	21	2.87	0.92	1.97	0.74
Rhizosphere	L-PW	22	2.97	0.92	2.27	0.70
	L-RW	19	2.57	0.85	2.03	0.52
	L-TE	22	3.14	0.94	2.45	0.77

L-PW: irrigated with potable water; L-RW: irrigated with raw wastewater; L-TE: irrigated with treated effluent; *H*: Shannon–Weiner index; *D*: Simpson index; *R*: Margalef richness index; *J*: Pielou's evenness index.

2.3. *Amoebae qPCR analysis*

In this study, a real-time quantitative PCR assay with specific primers was used to detect and quantify *Acanthamoeba* spp. and *H. vermiformis* in wastewater, phyllosphere and rhizosphere. The concentrations of the samples were calculated from the linear regression of standard curves generated by plotting *Ct* versus log10 of starting DNA quantities (Appendix A Fig. S1). The efficiencies of each assay were calculated from the slope of the standard curve. The slopes of −3.1314 and −3.4134 corresponding to the PCR efficiencies of 107.0% (R^2 = 0.9916) and 96.2% (R^2 = 0.9904) for amoebae 18S rRNA genes, respectively, indicated a good linear relationship according to quantitative requirements.

As shown in Fig. 4, the concentrations of *Acanthamoeba* spp. and *H. vermiformis* in RW and TE were calculated based on standard curves. *Acanthamoeba* spp. ranged from 4.8 to 8.2 log copies/L in RW and TE samples, and *H. vermiformis* ranged from 2.6 to 6.3 log copies/L. Furthermore, the concentrations of these two amoebae in the treated effluent were higher than in the raw wastewater. These results were consistent with the previous study (Cui et al., 2015). The wastewater used for agricultural irrigation is typically pretreated with an anaerobic process that easily leads to deposition of amoebae cysts. Due to the properties of resistance to adverse pH, osmotic pressure, salinity and temperature conditions, the amoebae cysts can persist in distilled water, tissue cultures, mammal body fluids and soils, where they play an important role in the maintenance of microbial communities, and can act as reservoir of known or emerging pathogenic bacteria, enabling their survival, reproduction, and dissemination, and thus could pose a health risk (Greub and Raoult, 2004).

In this investigation, the anaerobic biofilm treatment process was used to treat domestic wastewater and evaluate the occurrence of free-living amoebae. This processing facility was operated independently without disinfection devices. Amoebae could phagocytize organic matter and bacteria attached to the biofilm for growth-reproduction. *Acanthamoebae* spp. and *Hartmannella* spp. could remain viable after treatment with chlorine and ozone, as well as high temperature (80°C),

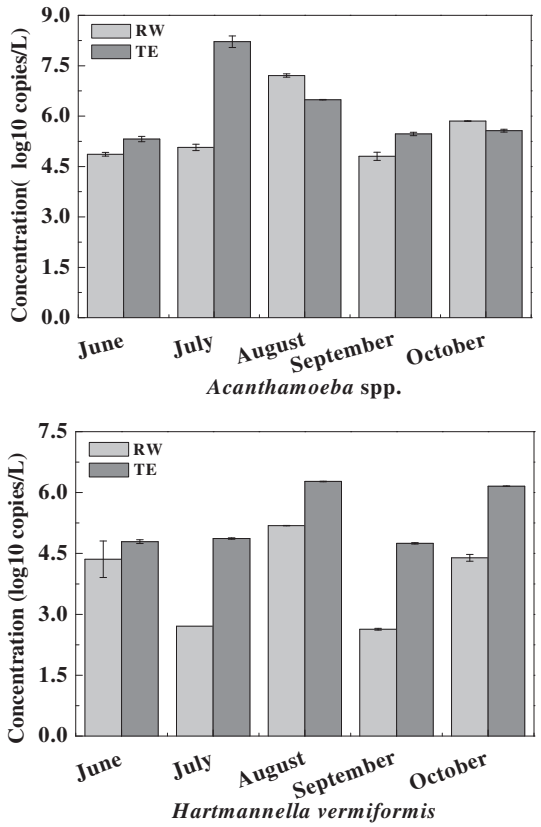

Fig. 4 – *Acanthamoeba* spp. and *Hartmannella vermiformis* gene copy numbers in wastewater at different sampling stages. RW: raw wastewater; TE: treated effluent.

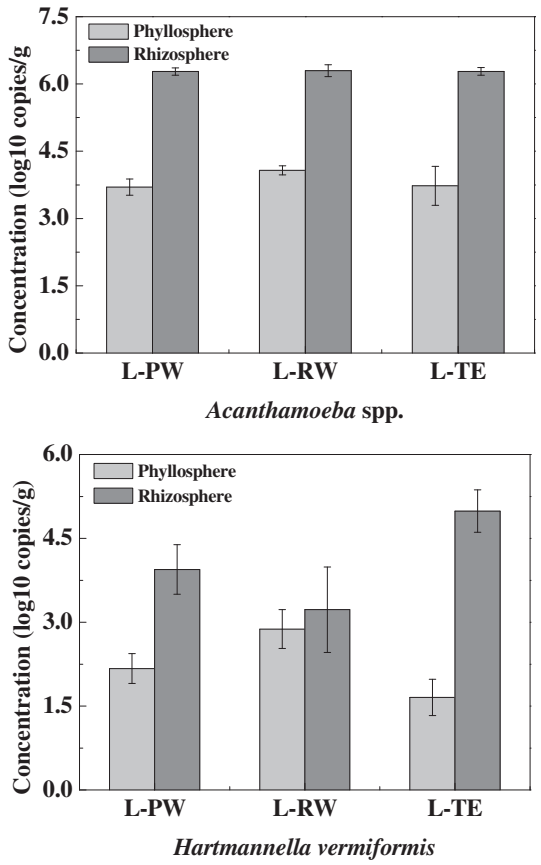

Fig. 5 – *Acanthamoeba* spp. and *Hartmannella vermiformis* gene copy numbers in phyllosphere and rhizosphere. L-PW: irrigated with potable water; L-RW: irrigated with raw wastewater; L-TE: irrigated with treated effluent.

implying that conventional disinfection and sterilization methods might be insufficient for long-term control of these amoebae in water distribution systems (Storey et al., 2004; Thomas et al., 2004). A ten-month investigation on the presence of pathogenic amoebae and bacterial indicators in influent and effluent samples was performed, and results showed that thirty-two species of amoebae were isolated from a constructed wetland treating domestic wastewater, and the frequency of *Acanthamoeba* and *Hartmannella* genera in influent and effluent samples was 59% and 10%, respectively (Ramirez et al., 2005), suggesting that wastewater is an important resource for distribution and transmission of amoebae, especially if it is discharged into surface water or as reclaimed water for agricultural irrigation.

The pathogenic amoebae *Acanthamoeba* spp. and *H. vermiformis* were detected in the phyllosphere and rhizosphere of lettuce exposed to the different irrigation waters. *Acanthamoeba* spp. and *H. vermiformis* were found to be the most frequent protozoa in domestic wastewater, and their populations were still very high after treatment. The concentrations of *Acanthamoeba* spp. in the phyllosphere and rhizosphere were similar to the control group. As described by Fig. 5, the abundances of amoebae in the rhizosphere were significantly higher than in the phyllosphere. This phenomenon could imply that the phyllosphere niche was affected greatly by UV, temperature and humidity. However, it is noteworthy that the concentrations of *Acanthamoeba* spp. and *H. vermiformis* detected on the surface of lettuce plants irrigated with raw wastewater and treated effluent were not significantly different from the control. To date, research on amoebae on fresh produce with wastewater irrigation is scarce. The transferability of *Acanthamoeba castellanii* and *Acanthamoeba polphaga* from irrigation water to fresh produce surfaces was firstly determined on the basis of the culture inoculation method, and results indicated that the surface texture of fresh produce and the use of contaminated water might play an important role in the transfer of free-living amoebae to fresh produce, as well as being able to act as potential sources for transmission of enteric viruses associated with amoebae (Hsueh and Gibson, 2015). The quantification of *Acanthamoeba* and *Vannella* on eight cultivars of vegetable sprouts was determined by enrichment and cultivation, showing that fresh produce harbored abundant and diverse amoebae, and results found no significant relationship between protozoan community composition and bacterial load under seasonal and local circumstances, but significantly differed between the sprout types (Chavatte et al., 2016). The microbial load of crops might be affected by the types of pathogenic microorganisms, while the effect of treated effluent enriched with high concentrations of amoebae and indicator bacteria is not recognized. The reuse of treated effluent for irrigation with minimal levels of potential microbiological contaminants is

proposed as a precondition to achieve sustainable wastewater reuse; further studies are being planned to determine the long-term effects in the field.

3. Conclusions

In general, the present study supports the premise that anaerobic biofilm processes provide a cost-effective way for domestic wastewater treatment. Although most of the pathogens are removed, the treated wastewater retains large amounts of amoebae. In our study, we found relatively high levels of amoebae on the phyllosphere and rhizosphere of lettuce irrigated with wastewater. Treated domestic wastewater, as a reusable and valuable water source, could meet the current standards or guidelines for agricultural irrigation. However, neither fecal indicator levels nor pathogen concentrations are adequate for assessing exposure health risk. Indeed, it is shown that vegetables harbor abundant and diverse amoebae, and further investigation should highlight the transferability of pathogenic amoebae on leafy greens, and then support improving water and land resource management to decrease potential health risks.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2017.11.018.

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