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Impact of treated wastewater for irrigation on soil microbial communities

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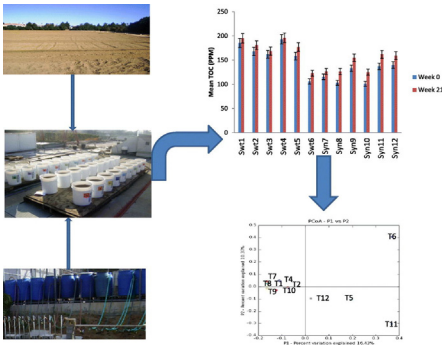
HIGHLIGHTS

- No significant differences in microbial diversity between soils with TWW and SFW
- Greater number of nitrifying bacteria in TWW than from soils treated with SFW
- TWW effluent contained higher potential pathogens than SFW.
- *Bacillus and Mycobacterium* in all soils but *Clostridium* and *Mycobacterium* in TWW soils
- Higher levels of *Acinetobacter*, *Legionella*, and *Pseudomonas* in TWW irrigated soil than in SFW

GRAPHICAL ABSTRACT



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ABSTRACT

The use of treated wastewater (TWW) for irrigation has been suggested as an alternative to use of fresh water because of the increasing scarcity of fresh water in arid and semiarid regions of the world. However, significant barriers exist to widespread adoption due to some potential contaminants that may have adverse effects on soil quality and or public health. In this study, we investigated the abundance and diversity of bacterial communities and the presence of potential pathogenic bacterial sequences in TWW in comparison to synthetic fresh water (SFW) using pyrosequencing. The results were analyzed using UniFrac coupled with principal coordinate analysis (PCoA) to compare diversity and abundance of different bacterial groups in TWW irrigated soils to soils treated with SFW. Shannon diversity index values (H') suggest that microbial diversity was not significantly different ($P < 0.086$) between soils with TWW and SFW. Pyrosequencing detected sequences of 17 bacterial phyla with *Proteobacteria* (32.1%) followed by *Firmicutes* (26.5%) and *Actinobacteria* (14.3%). Most of the sequences associated with nitrifying bacteria, nitrogen-fixing bacteria, carbon degraders, denitrifying bacteria, potential pathogens, and fecal indicator bacteria were more abundant in TWW than in SFW. Therefore, TWW effluent may contain bacterial that may be very active in many soil functions as well as some potential pathogens.
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1. Introduction

Water scarcity problems are well known in the arid and semiarid regions of the world which include the highly populated regions of the southwestern United States and most of the Middle East. This is applicable to other rapid population growth areas across the globe, where

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increases in per capita water consumption, coupled in part with global climate change have resulted in increased demands on available freshwater resources (Jueschke et al., 2008; Maimon et al., 2010; Navon et al., 2011).The current freshwater use in arid and semiarid lands is not sustainable, as use exceeds replenishment and demand for water continue to increase. The primary use of fresh water in arid and semiarid regions is for irrigation, and in California, approximately 70% of water use is for agriculture (Suarez, 2013). The irrigated acreage in the western U.S. is already decreasing due to water availability and diversion of water to other uses. Agriculture will either need to reduce acreage under irrigation, which is undesirable since it will reduce food supply, or irrigate with alternative water sources and more effectively utilize existing water supplies as suggested by Suarez et al., 2008. In 1992, the U.S. Environmental Protection Agency (U.S. EPA) developed guidelines for the reuse of treated wastewater (US-EPA, 1992) intended for the irrigation of residential landscapes, parks, school yards, highway medians, fodder, and fiber crops, as well as for environmental purposes such as creating artificial wetlands, and sustaining stream flows. The guideline was modified in 2004 (US-EPA, 2004) and in 2012 (US-EPA, 2012). In California, the production of recycled water is governed by the United States Environmental Protection Agency Guidelines for Water Reuse (US-EPA, 2004, 2012), WHO (WHO, 2006) and by the State of California Department of Health Services, Title 22 of the California Code of Regulations (State of California, 2000). The recommendations on wastewater reuse established by these entities provided most of the legal guidelines proposed in countries such as the USA, Portugal, Spain, Italy, Cyprus, France, Australia, Israel, Jordan, Kuwait, Oman, Saudi Arabia, and China (Becerra-Castro et al., 2015). A summary of the main parameters considered in the different guidelines and policies in different countries are provided by Becerra-Castro et al., 2015. Recycled water is defined by these entities (EPA, WHO, and CA Department of Health Services) as reclaimed wastewater which has undergone primary, secondary, and tertiary treatment and disinfection, allowing it to be used for landscape irrigation and other non-potable uses. In short, this definition was highlighted because EPA acknowledged that technologies are now advanced enough to treat wastewater to the water quality required for the intended use, the concept of "fit for purpose" is highlighted to emphasize the efficiencies realized by designing reuse for specific end applications. In California, the "fit for purpose" requirement allows only treated wastewater to be applied in most instances after tertiary treatment. Also, in Israel, the "fit for purpose" requirement allows for >50% of the total water used for irrigation of crops to be recycled wastewater (Israel Water Authority, 2015). The concept illustrates that water treatment technologies (combined with disinfection) offer a ladder of increasing water quality, and choosing the right level of treatment should be dictated by the end application of the reclaimed water for achieving economic efficiency and environmental sustainability. Therefore, treatment levels are different from state to state and from one country to another, and data should be evaluated based on the regulations from each state or nation.

Use of treated wastewater is possible, but sustained use requires consideration of the impacts of these waters on both crop production and maintenance of good soil physical properties (Suarez and Gonzalez-Rubio, 2017; Suarez et al., 2006) as well as public health. In many instances waters previously considered not useable or impractical for irrigation can be used with careful management. Freshwater scarcity and regulations on wastewater disposal have necessitated the reuse of treated wastewater for soil irrigation, which has several environmental and economic benefits (Wafula et al., 2015). For instance, wastewater irrigation provides water, minerals, and nutrients such as nitrogen and phosphorus as well as other organic matter to soil (Minz et al., 2011). These factors may have beneficial effects on soil microbial communities and in turn soil fertility (Gans et al., 2005). In addition, the potential to transfer heavy metals (Khan et al., 2008), pharmaceuticals (Conkle et al., 2008), and even pathogens (Salgot et al., 2003) in the environment and into the food chain (Broszat et al., 2014), may directly affect soil microbial diversity and activity as well as public health (Minz et al., 2011). Furthermore, irrigation with treated wastewater may alter soil physicochemical and microbiological properties and introduced different kinds of contaminants into soil. The potential for treated wastewater use is enormous, but the impacts of these waters on plant production, soil physical and hydraulic properties, and microbial composition, soil quality, and subsequently public health require considerable investigation.

In this study, we examined the effects of sodium adsorption ratio (SAR) and pH of TWW used for irrigation on soil microbial community and composition using pyrosequencing in comparison to SFW irrigation. One major restriction on the use of TWW for irrigation is the large concentrations of sodium relative to calcium and magnesium (Ayers and Westcot, 1985; Suarez et al., 2006; Suarez et al., 2008). Therefore, wastewater irrigation may promote soil salinization (an increase of soluble salts concentration) or sodification. Soil salinization is associated with the increase of electrical conductivity, hyperosmotic, oxidative stress and ion toxicity, constituting a limiting factor for plant growth, development and productivity (Levy and Tai, 2013; Ngara et al., 2012), while sodification affects negatively the stability of soil aggregates and soil structure, leading to an increase of soil compaction, loss of soil permeability and reduction of hydraulic conductivity (Sparks, 2003; Becerra-Castro et al., 2015; Suarez et al., 2006). In a recent study, there was a decrease in infiltration with time for all treatments both with SFW and those made up from TWW (Suarez and Gonzalez-Rubio, 2017). Their data indicate that SAR 4 and pH 7.0 had a reduction in infiltration from an initial value of 65 cm/d to a final value of 50 cm/d. In contrast, the SAR 10 pH 8 irrigation water resulted in a final infiltration rate of only 25 cm/d. Increasing SAR and elevated pH both had a marked adverse impact on infiltration with differences becoming more pronounced according to these authors. They attributed this to the adverse effects of elevated SAR and pH resulting in clay dispersion and subsequent sealing of pores. The results of this study indicate that short-term studies with one or several infiltration events do not represent the long-term consequences of irrigating with degraded water. Salinization and/or sodification are common problems under irrigated agriculture, especially in low rainfall and high evaporative demand areas of southwestern United States and other semi-arid regions around the world. While the effects of irrigation-induced salinization and sodification on soil chemical and physical properties and plant growth are well studied, their effects on soil microbial communities have received little attention from a few studies.

Pyrosequencing data on bacterial 16S rRNA had previously been used to compare genes from soils irrigated with treated wastewater and those from soils irrigated with freshwater (Frenk et al., 2013). Compared to earlier microbial ecology techniques such as denaturing gradient gel electrophoresis (Ibekwe et al., 2002), pyrosequencing protocols have many advantages such as higher throughput, better coverage, and greater resolution, which enable researchers to identify and study microbial groups with relatively lower abundance (<1%) (Shendure and Ji, 2008). However, the influence of SAR and pH on bacterial soil communities after irrigation with TWW has not been studied in detail. Elevated SAR and pH may have an adverse impact on infiltration, and reduction in infiltration has been observed as a result of irrigation with treated wastewater. Low infiltration will ultimately result in higher runoff and subsequent contamination of nearby surface waters. Our main objective was to examine the effects of treated wastewaters used for irrigation on soil microbial community and composition, and the presence of gene sequences associated with potential pathogenic organisms.

2. Materials and methods

2.1. Soil preparation

Surface samples (0–10 cm) of Arlington sandy loam (fine clay, mixed, active, Thermic Haplic Durixeralf) were collected from the Agricultural Experiment Station on the campus of the University of

California, Riverside, CA (Fig. S1). Soils were crushed and passed through a 5-mm screen, air dried, and analyzed for texture and chemical characteristics. Physical and chemical properties were analyzed before the initiation of irrigation treatment (Table S1; Suarez and Gonzalez-Rubio, 2017) as well as microbiological properties (Ma et al., 2012). Soil columns were prepared in plastic containers (35.5 cm tall and 26 cm diameter at the base and 28.5 cm at the top), fitted with two ceramic extractors (2 by 7 cm) at the bottom of the containers in a layer of 7 cm of No. 90 fine quartz sand (Fig.S1; Suarez and Gonzalez-Rubio, 2017). Tap water (EC = 0.6 dS m^{-1} and SAR < 0.4 $mmol^{1/2}$ $L^{-1/2}$), was applied to enable soil settling before the initiation of the irrigation treatments. Wastewaters used for this study consisted of tertiary treated municipal wastewater with a dissolved organic carbon (DOC) of 13 mg L^{-1}. Portions of the wastewater were adjusted to three levels of SAR, 4, 7 and 10 and pH values of 7.0 and 8.0. Table S1 shows the initial wastewater had a SAR of 4. ($mmol^{0.5}$ $L^{-0.5}$). Fig. S2 shows total organic carbon of both waste water and synthetic waste water before initiation of irrigation treatment. The adjustment and balance of these SAR and pH values were obtained by using the Extract Chem. v. 2.0 Program) (Suarez and Taber, 2012). Synthetic freshwater of the same macro ion chemical composition, electrical conductivity (EC) and pH were prepared by adding various salts, constrained by the solubility of calcium carbonate (up to 3 fold supersaturation can be maintained without precipitation (Suarez and Rhoades, 1982). The initial water had a SAR of 4 and an EC of 1.7 dS m^{-1}. The experiment was a randomized design with three (3) levels of SAR, two (2) pH values, two (2) types of water and three (3) replications, for a total of (36) experimental units. The irrigation waters were applied as flood irrigation events. Infiltration rates were calculated for each event and container. After watering, the soil was left to dry to −50 kPa (−0.5 bar), at which time a new irrigation was applied. Drying was monitored using tensiometers installed in containers. The experiment was conducted for 153 days. The EC of all irrigation waters was relatively constant, at EC 2.4 dS m^{-1}.

At the end of the experiment, soil samples from the containers were collected to conduct analytical determinations. Saturation paste and extracts were obtained for each depth. Electrical conductivity (EC), pH, SAR = $(Na/(Ca + Mg)^{1/2}$, solute concentrations ($molm^{-3}$), were determined. Soluble Na, Mg, and Ca concentration for calculation of SAR were determined using inductively coupled plasma (ICP) emission spectrometry. Total alkalinity was determined by titrating of a measured amount of sample with 1.00 mM HCL (standardized against potassium biiodate) to a pH of 4.40. A digital chloridometer (coulometric titrator) was used to determine the chloride ion concentrations. In general, soil properties characterized included clay, silt, and sand contents, pH, electrical conductivity (EC), bulk density, water content, water-holding capacity (WHC), total organic carbon (OC), and total nitrogen (T-N) (Klute, 1996). Soil microbial biomass carbon (MBC) was determined by the chloroform-fumigation-extraction method (Vance et al., 1987). The texture and physiochemical properties of the soils were characterized as previously described (Ma et al., 2012).

2.2. DNA extraction, pyrosequencing, and statistical analysis

Total bacterial DNA was extracted from 500 mg of each soil sample collected from the beginning and at the end of the study using Power Soil DNA kits (MO BIO, Inc., Solana Beach, CA), according to the manufacturer's protocol. Extracted DNA (2 μL) was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington DE), and run on 1.0% agarose gel and used for pyrosequencing. The soil DNA samples (15.0 μL) were then submitted to Research and Testing Laboratories (Lubbock, TX) for PCR optimization and pyrosequencing analysis. Bacterial tag-encoded FLX amplicon pyrosequencing was carried out as previously described (Acosta-Martinez et al., 2008 and Dowd et al., 2008). The 16S universal Eubacterial primers 530F (5′-GTG CCA GCM GCN GCG G) and 1100R (5′-GGG TTN CGN TCG TTG) were used for amplifying the ~600 bp hypervariable region of 16S rRNA genes. Primer and PCR optimizations were done at the Research and Testing Laboratories (Lubbock, TX) according to protocols described previously (Acosta-Martinez et al., 2008). All FLX-related procedures were performed following Genome Sequencer FLX System manufacturer's instructions (Roche, NJ, USA). Thus, moderate diversity pyrosequencing analysis (≥3000 reads per sample) was performed at the Research and Testing Laboratory (Lubbock, TX, USA). Tags which did not have 100% homology to the original sample tag designation were filtered from the data set. Sequences which were <200 bp after quality trimming also were not considered.

Bacterial pyrosequencing population data were further analyzed by performing multiple sequence alignment techniques using the dist.seqs function in MOTHUR, version 1.33.3 (Schloss et al., 2009). All the raw reads were treated with the Pyrosequencing Pipeline Initial Process (Cole et al., 2009) of the Ribosomal Database Project (RDP), (1) to sort those exactly matching the specific barcodes into different samples, (2) to trim off the adapters, barcodes and primers using the default parameters, and (3) to remove sequences containing ambiguous 'N' (Claesson et al., 2009). Given that a number of diversity and richness estimators tend to suffer from sample size bias (Champely and Chessel, 2002), we "re-sampled" our sequence libraries so that they contained similar numbers of sequences. The subsample function in MOTHUR was used to randomly select a subsample of sequences from each library and these equally sized. The reduced data sets were used in all subsequent analyses, including detrended correspondence analysis (DCA), canonical correspondence analysis (CCA), variation partition analysis (VPA), and dissimilarity indices analysis.

Following chimera detection, and the re-sampling of the larger sequence libraries, the RDP Classifier function was used to assign identities to the bacterial pyrotag sequence data (Wang and Qian, 2009). MOTHUR was used to align the re-sampled data set and create an all-sample distance matrix, as well as assign sequences to operational taxonomic units (OTU = 97 similarity, using the h-cluster function), calculate diversity indices and richness estimates, and determine the degree of overlap shared among the soil communities. Overlap was calculated using the Yue-Clayton similarity estimator (θ_{YC}), a metric that is scored on a scale of 0 to 1, where 0 represents complete dissimilarity and 1 represents identity (Yue and Clayton, 2005 and Schloss et al., 2009). When comparing any given set of communities, θ_{YC} considers the distribution of OTUs between the communities, as well as their relative abundances. DCA, CCA, VPA, and dissimilarity indices analysis were performed using R package v3.1.0.

3. Results and discussion

3.1. Characteristics of recycled wastewater and synthetic freshwater

The composition of the original TWW, adjusted TWW and SFW were previously determined and as shown in Table S1 (Suarez and Gonzalez-

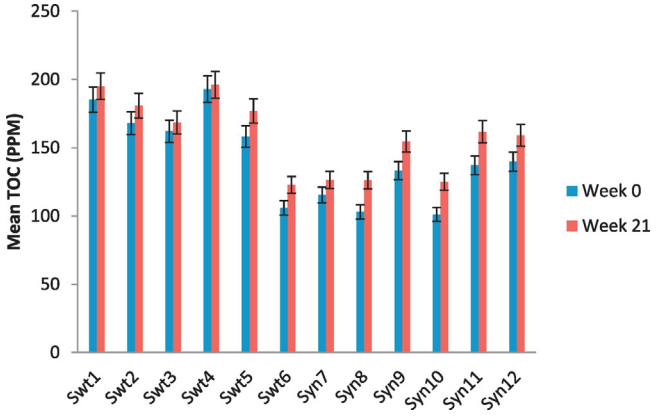

Fig. 1. Mean total organic carbon between samples collected at the end of the study (week 21) and the beginning of the study (week 0).

Table 1
Summary statistics of diversity indices from different treatments based on normalized data.

Treatments	SAR	pH	# Seqs	OTUs	Chao	Invsimpson	Shannon (H')	Simpson	Coverage
Wastewater	4	7	4692	543	1029.54	8.98	3.94	0.112	0.933
Wastewater	4	8	4692	935	1911.03	58.73	5.57	0.017	0.885
Wastewater	7	7	ND	ND	ND	ND	ND	ND	ND
Wastewater	7	8	4692	589	1039.10	15.97	4.49	0.062	0.936
Wastewater	10	7	4692	1160	2753.36	71.39	5.88	0.014	0.841
Wastewater	10	8	4692	1030	2310.58	60.96	5.70	0.016	0.869
Synthetic water	4	7	4692	1132	2612.71	85.29	5.93	0.011	0.852
Synthetic water	4	8	4692	1173	2574.09	74.87	5.92	0.013	0.843
Synthetic water	7	7	4692	1064	2342.01	67.57	5.78	0.014	0.863
Synthetic water	7	8	4692	1072	2152.62	85.44	5.87	0.011	0.868
Synthetic water	10	7	4692	431	600.31	16.37	4.41	0.061	0.968
Synthetic water	10	8	4692	838	1574.23	48.50	5.37	0.020	0.904

*T3 was not determined due to PCR amplification problems. T3 = SAR 7, pH 7.

Rubio, 2017). After 153 days, soil samples were collected and the results were compared to data collected from day 0. Mean soil total organic carbon showed that there were no significant differences between samples collected at the end of the study (day 153) and the beginning of the study (Fig.1), although TOC was greater for all treatments at the end of the study. Furthermore, there were no significant differences in TOC ($P < 0.086$) between soils treated with SFW and TWW, but for the corresponding SAR and pH treatments, TWW had higher TOC than SFW (Fig. S2). The wastewater had a greater negative effect on soil water infiltration than synthetic water with the same level of pH and SAR, indicating that the elevated DOC of the waste water adversely impacts infiltration (Suarez and Gonzalez-Rubio, 2017), thus it may likely lead to high microbial activity depending upon the treatment level of the water and plugging of pores. The effect of this wastewater was not severe- in that, it caused an approximate 10% reduction in infiltration across all treatments (relative to the synthetic water at the same EC, SAR, and pH). This resulted in most instances no significant impact at the higher level (Phylum) microbiological analysis in comparison to results at the lower level (genus and species) that showed some impacts. The net effect, therefore, on soil physical property may be the potential increase in the overland flow of the irrigation water to adjacent land or surface water. This may result in the transport of contaminants such as pathogens, pharmaceutically active compounds or other contaminants from the irrigated land to adjacent land or waterbodies. Therefore, SAR, DOC, and pH of typical wastewaters, in some instances, may be high enough to have negative impacts on soil physical properties as well as the surrounding landscape.

3.2. Community composition, diversity, and estimated richness

A total of 169,227 16S rRNA sequences were generated through 454 pyrosequencing, with an average read length of about 400 bp. The 454 sequence libraries ranged in size from 4692 sequences and 431 operational taxonomic units (OTUs) with SFW (SAR 10 and pH 7.0) to 31,410 sequences and 3583 OTUs (Table 1) with SFW (SAR 4 and pH 7.0). This suggests a negative impact of higher SAR on sequencing size. Shannon diversity index values (H) suggest that microbial diversity was not significantly different ($P < 0.086$) between soils with TWW and SFW (Table 1). Our study is in agreement with other studies that wastewater irrigation may have no significant impact on the overall diversity and richness of bacteria depending on the quality of the TWW (Elifantz et al., 2011; Frenk et al., 2013; Broszat et al., 2014). Although we determined thousands of sequence tags per sample, rarefaction curves of OTUs were far from the plateau, indicating that there were more undetermined sequence tags either from real rare species or artificial sequences produced by PCR and sequencing mistakes (Fig. S3). Chao richness estimates confirmed no significant higher sequences in soils treated with SFW than TWW. Overall, the 454 libraries detected 17 bacterial phyla (Table 2) with *Proteobacteria* (32.1%) followed by *Firmicutes* (26.5%) and *Actinobacteria* (14.3%). The communities in soils treated with SFW were the most evenly distributed, while communities in soils irrigated with TWW were most biased with a high ratio of bacterial community variabilities. In general, there were some increases in *Actinobacteria*, *Firmicutes*, *Acidobacteria*, *Nitrospira*, and unclassified bacteria in soils treated with wastewater (TWW) in comparison to the

Table 2
Relative bacterial percent concentration at the phylum level between soils treated with secondary treated wastewater and soils treated with synthetic fresh water irrigation.

Phyla	Total	T1	T2	T4	T5	T6	T7	T8	T9	T10	T11	T12	T0*
Acidobacteria	3.15	0.66	3.27	1.99	4.28	3.71	4.04	3.80	4.47	3.44	1.6	2.6	0.82
Actinobacteria	14.3	9.64	16.8	11.1	14.9	14.1	15.8	15.0	14.2	18.3	23	15	11.4
Armatimonadetes	0.14	0.02	0.03	0.17	0.17	0.10	0.13	0.25	0.16	0.16	0.2	0.1	0.35
Bacteroidetes	1.62	2.13	0.75	1.72	2.36	1.90	1.17	1.78	1.13	1.47	0.5	0.5	2.87
BRC1	0.01	0.00	0.03	0.00	0.00	0.00	0.00	0.01	0.02	0.00	0.0	0.0	0.00
Deinococcus-Thermus	0.02	0.05	0.04	0.00	0.00	0.00	0.03	0.02	0.00	0.03	0.0	0.1	0.19
Firmicutes	26.5	33.3	25.3	21.9	30.2	28.9	23.2	22.3	27.0	22.0	17	33	14.6
Fusobacteria	0.01	0.00	0.00	0.01	0.00	0.00	0.03	0.01	0.01	0.00	0.0	0.0	0.00
Gemmatimonadetes	0.07	0.02	0.08	0.04	0.13	0.18	0.08	0.05	0.09	0.03	0.1	0.6	0.06
Nitrospira	0.24	0.07	0.25	0.15	0.29	0.34	0.33	0.26	0.32	0.34	0.0	0.3	0.00
OD1	0.06	0.00	0.06	0.06	0.01	0.06	0.12	0.09	0.11	0.11	0.0	0.0	0.03
Planctomycetes	0.86	0.16	0.93	0.52	1.09	1.01	1.14	1.13	0.99	1.34	0.4	0.6	0.32
Proteobacteria	32.1	48.72	30.03	49.05	22.54	25.56	26.67	28.72	23.50	27.19	42	26	60.2
TM7	0.15	0.21	0.22	0.01	0.21	0.11	0.08	0.19	0.10	0.11	0.00	0.29	0.66
unclassified bacteria	20.5	4.92	22.0	13.0	23.4	23.8	26.8	25.8	27.6	25.3	14.0	19.6	8.02
Verrucomicrobia	0.19	0.03	0.13	0.08	0.33	0.12	0.23	0.36	0.24	0.13	0.00	0.05	0.41

T3 was not determined due to PCR amplification problems: T0 is the baseline analysis without the addition of wastewater or synthetic waste water (control). Wastewater (T1), SAR 4 and pH 7, Wastewater (T2), SAR 4 and pH 8, Wastewater (T3), SAR 7 and pH 7, Wastewater (T4), SAR 7 and pH 8, Wastewater (T5), SAR 10 and pH 7, Wastewater (T6), SAR 10 and pH 8, Synthetic water (T7) SAR 4 and pH 7, Synthetic water (T8), SAR 4 and pH 8, Synthetic water (T9), SAR 7 and pH 7, Synthetic water (T10), SAR 7 and pH 8, Synthetic water (T11), SAR 10 and pH 7, Synthetic water (T12), SAR 10 and pH 8. Treatment 3 is missing. Total is the average percent concentrations from all the treatments in comparison to samples without any treatment added.

background soil sample that did not receive treatment with either TWW or SFW (T0). The reverse was the case with *Proteobacteria* and *Bacteroidetes* as the background soil sample had a higher relative percent population than the treated samples. Closer examination also showed that there was a general decrease in *Actinobacteria* in soils treated with wastewater in comparison to synthetic water.

In the current study, we observed a relative decrease in percent composition of *Actinobacteria* and increase in *Alphaproteobacteria* in soils irrigated with TWW. *Beta- Gamma-* and *Deltaproteobacteria* were about the same in percent relative composition in both the TWW irrigated soils and soils irrigated with SFW (Fig. S4). Our study is in agreement with what was previously reported by Frenk et al., 2013, that showed a decrease in relative abundance of *Actinobacteria* in a semiarid Mediterranean soil irrigated with TWW. They also pointed out that decreases in the relative abundance of the members of *Actinobacteria* and *Firmicutes* have been previously associated with amendments in soil organic matter, as Elifantz et al., 2011 reported a higher increase in organic matter content in treated wastewater irrigated soils relative to soils irrigated with fresh water. Furthermore, higher levels of relative abundances of *Acinetobacter*, *Legionella*, and *Pseudomonas*, respectively, were detected in TWW- irrigated soil than in SFW irrigated soil during our study. Species within these genuses, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* and *Enterococcus* species are organisms that frequently cause nosocomial infections (Boucher et al., 2009; Rice, 2008; Broszat et al., 2014).

3.2.1. Phylogenetic structure of bacterial community

The distributions of bacterial phylogenetic similarity were sorted into different groups by applying PCoA, and hierarchical clustering analysis to a matrix of UniFrac distances using the UniFrac web interface in MOTHUR. SAR was not a significant factor ($R^2 = 0.0588$, $P < 0.21$) separating bacterial assemblages in TWW irrigated soils as well as in synthetic water (Fig. 2A). Soil DOC was also observed to be not significant ($R^2 = 0.067$, $P < 0.19$) separating bacterial assemblages (Fig. 2B)

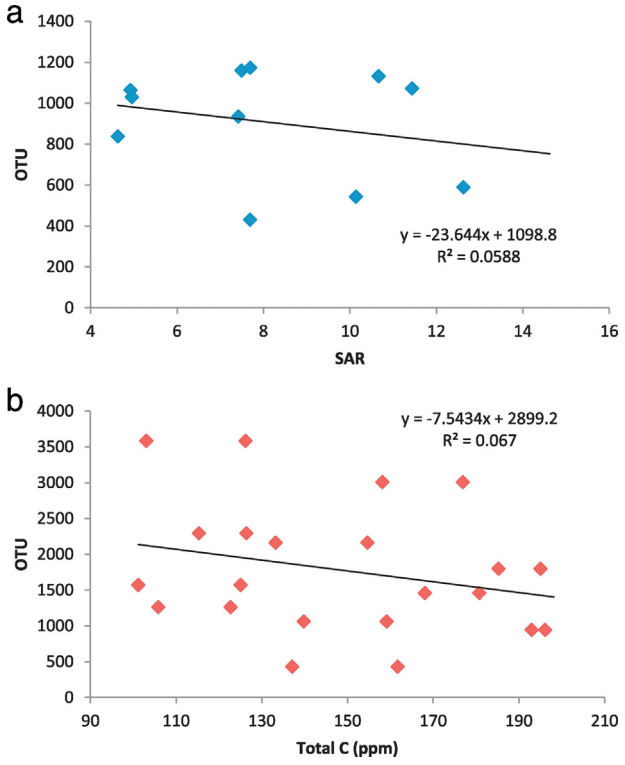

Fig. 2. Effects of SAR on bacterial assemblages (A) Effects of SAR on bacterial assemblages in TWW irrigated soils and in synthetic water; (B) Effect of total carbon on bacterial assemblages between TWW and SFW.

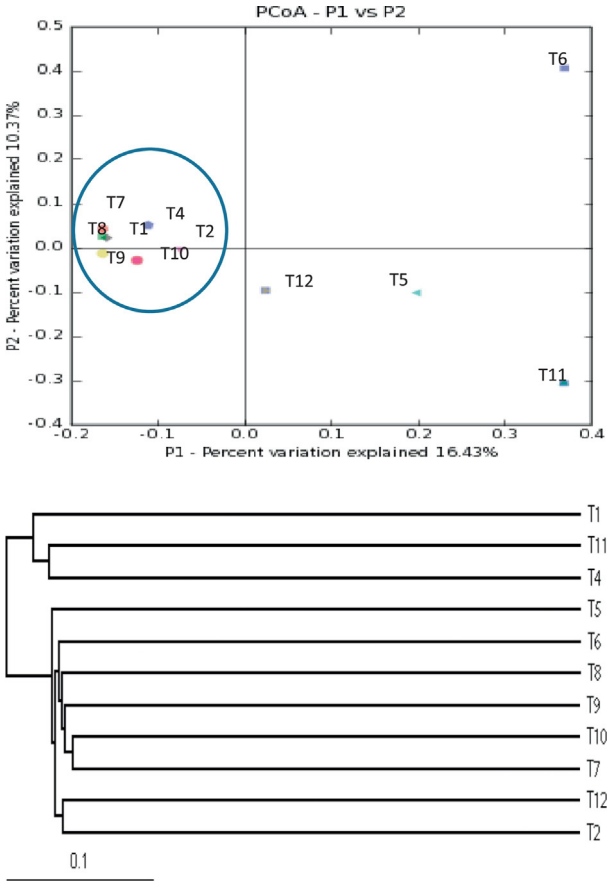

Fig. 3. Bacterial phylogenetic similarity sorted by PCoA (Fig. 3A), and the UPGMA hierarchical clustering analysis (Fig. 3B) using a matrix of UniFrac distance on the UniFrac web interface. Wastewater (T1), SAR 4 and pH 7, Wastewater (T2), SAR 4 and pH 8, Wastewater (T3), SAR 7 and pH 7, Wastewater (T4), SAR 7 and pH 8, Wastewater (T5), SAR 10 and pH 7, Wastewater (T6), SAR 10 and pH 8, Synthetic water (T7) SAR 4 and pH 7, Synthetic water (T8), SAR 4 and pH 8, Synthetic water (T9), SAR 7 and pH 7, Synthetic water (T10), SAR 7 and pH 8, Synthetic water (T11), SAR 10 and pH 7, Synthetic water (T12), SAR 10 and pH 8.

between TWW and SFW. Further analysis of the distributions of bacterial phylogenetic similarity after sorting into distinct groups by applying PCoA (Fig.3A), and the UPGMA hierarchical clustering analysis (Fig. 3B)

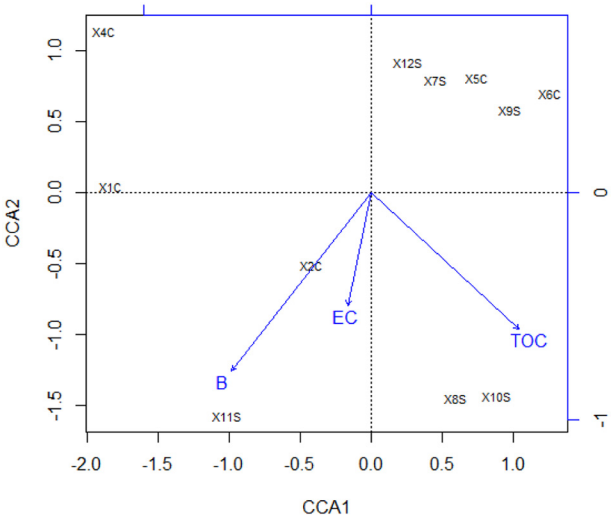

Fig. 4. CCA (canonical correspondence analysis) showed that soil properties, including EC (electrical conductivity), B (boron), and TOC (total organic carbon) had minimal effects on soil microbial community.

to a matrix of UniFrac distances using the UniFrac web interface in MOTHUR showed that soil samples with SAR of 4 and 7 were grouped together irrespective of whether from TWW or from SFW. However, soil samples with SAR of 10 were distributed outside this group (Fig. 3A). Further analysis of the data by detrended corresponding analysis (DCA) confirmed the results from PCoA (data not shown). In order to identify which factors influenced bacterial community structure in soils, CCA was performed. CCA results showed that soil properties, including EC, boron, and DOC had minimal effects on soil microbial community (Fig. 4).

3.3. Functional affiliations of bacterial taxonomic groups at the genus and species levels

Using RDP Classifier and greengene database to identify bacterial sequences at the genus and species levels, respectively, (Table 3) we showed their relative concentrations (sequence tags) in soils treated with TWW and SFW. Species identified in Table 3 are those with the highest percent similarities to the bacteria in the greengene database. It should be noted that this technique mainly contained pyrosequencing and bioinformatic analysis, which showed a comprehensive profile of microbial sequences from TWW and SFW. It serves as a powerful and promising approach to monitoring changes in microbial communities in TWW and SFW during the 153 days of this study. It is worthy to note that this technique does not quantify the members in the community in terms of cell number in the soil samples, nor does it imply viability of the organism, but may demonstrate the presence of potential bacterial DNA sequences as shown in our previous study (Ibekwe et al., 2013).

The pyrosequencing data from this study showed that the techniques could detect a greater number of OTUs of *Nitrosococcus*, *Nitrosovibro*, and *Nitrospira* which are all nitrifying bacteria (NT) from soils treated with wastewater (T1 – T6) than from soils treated with synthetic water (Table 3). Higher numbers of OTUs for nitrogen-fixing bacteria (NF) such as *Bradyrhizobium* and *Agrobacterium* were also observed in soils treated with wastewater than synthetic water, all indicating the potential usefulness for this water for soil improvement. Also, our data included a large number of genera from *Betaproteobacteria* that were closely associated with ammonia oxidizing bacteria that are part of the nitrification (NT) process. It has been reported that *Rhizobiales* thrived at high-DOC sites probably fueled by metabolism of one-C compounds (Bastida et al., 2016). The DOC in their study was reported as between 65 and 1700 mg C per kg soil. Correcting their DOC to a 1:1 soil water extract corresponds to about 15–320 mg L^{-1}, compared to DOC in TWW water of 13 mg L^{-1} and DOC in soil extract of 95–195 mg L^{-1}in our study. The DOC concentration in their study is thus higher and over a larger range of values than determined in our study. *Nitrospira*-classified OTUs were in high abundance from TWW-irrigated soils. The high abundance of nitrite-oxidizing *Nitrospira* was detected in almost all samples in this study (Table 3). Other studies

Table 3
Bacterial taxonomic and functional affiliations with relative distribution at the genus level from TWW and synthetic water irrigated soils.

Genus	% similarity	Functions*	species§	% similarity	Total	T1	T2	T4	T5	T6	T7	T8	T9	T11	T12
Acinetobacter	92–100	PP	*johnsonii*	96.3	14	2	0	4	3	0	0	1	0	2	2
Arcobacter	98.8–99.7	DN	*cryaerophilus*	99.2	1	0	0	0	0	0	0	1	0	0	0
Bacillus	82.3–100	PP	*amyloliquefaciens*	98.6	1177	135	88	73	203	120	125	176	143	32	82
Haemophilus	98.6–100	PP	*parainfluenza*	99.9	2	0	0	0	2	0	0	0	0	0	0
Legionella	80.5–92.8	PP			1	1	0	0	0	0	0	0	0	0	0
Clostridium	91.3–99.9	PP	*bowmanii*	98.8	45	20	4	3	8	1	3	2	4	0	0
Comamonas	91.2–92.5	PP	*aquatica*	92.1	5	2	0	3	0	0	0	0	0	0	0
Bacteroides	81.2–100	FB	*intestinalis*	85.4	12	7	0	1	1	0	0	1	1	0	1
Corynebacterium	98.3–99.8	PP	*kroppenstedtii*	98.7	11	1	1	2	0	1	2	0	2	1	1
Coxiella	86.2–92.1	PP	*burnetii*	90.5	9	0	1	1	1	1	1	1	1	1	1
Paenibacillus	96.5–98.8	PP	*chondroitinus*	99	1	0	0	0	0	0	0	0	0	1	0
Enterococcus	95–96.2	PP			2	1	0	0	1	0	0	0	0	0	0
Escherichia/Shigella	97.6–98.7	PP			5	1	1	0	1	1	0	0	0	0	1
Flavobacterium	94–99.2	DN			5	2	0	0	1	0	0	2	0	0	0
Helicobacter	98–100	PP	*pylori*	100	3	1	0	1	0	0	0	0	0	1	0
Lactobacillus	89.3–100	FB	*ruminis*	100	3	2	0	0	1	0	0	0	0	0	0
Prevotella	98.2–100	PP	*corporis*	100	1	0	0	1	0	0	0	0	0	0	0
Methylobacterium	90.1–92.8	PP	*komagatae*	93	5	0	3	0	0	1	0	0	1	0	0
Bradyrhizobium	98.4–99.7	NF	*elkanii*	98.4	10	1	1	1	1	1	1	1	1	1	1
Mycobacterium	89.7–98.6	PP	*flavescens*	86.4	54	4	4	7	9	5	7	7	7	3	1
Nitrosococcus	83.5–98.9	NT			10	1	3	1	2	1	0	1	1	0	0
Nitrosovibrio	85.3–99.1	NT	*tenuis*	98.9	21	1	1	0	5	2	3	2	4	2	1
Nitrospira	83.2–94	NT			112	20	9	8	17	12	13	11	13	0	9
Nocardia	94.1–99.8	PP	*fluminea*	99.7	20	2	2	2	3	3	2	2	2	1	1
Rubrobacter	95.8–100	CD			13	11	0	0	1	0	1	0	0	0	0
Ralstonia	85.8	PP			1	0	0	0	0	0	0	1	0	0	0
Propionibacterium	82.1–98.9	pp	*acne*	98.9	4	0	0	0	3	1	0	0	0	0	0
Pseudomonas	98.9–100	PP	*umsongensis*		20	4	0	4	2	1	3	2	2	1	1
Agrobacterium	94.0–97.2	NF	*rhizobium* sp.IRBG7	88.4	3	0	0	1	1	0	0	1	0	0	0
Steroidobacter	98.7–99.8	DN			3	1	0	0	0	0	0	2	0	0	0
Sphingomonas	82.1–92.2	PP	*wittichii*	85.5	12	2	1	0	2	1	2	1	1	1	1
Solibacillus	99.6–100	CD	*silvestris*	100	1	0	0	0	1	0	0	0	0	0	0
Staphylococcus	99.6–100	PP	*epidermidis*	96.8	5	2	1	2	0	0	0	0	0	0	0
Stenotrophomonas	98.8–100	PP	*geniculata*	95.7	6	2	0	3	0	0	0	1	0	0	0
Streptococcus	99.8–99.9	PP	*oralis*	94.7	14	4	0	2	1	1	1	2	1	2	0
Treponema	83.2–85.8	PP			3	3	0	0	0	0	0	0	0	0	0
Vibrio	95.2–99.4	PP	*harveyi*	97.9	2	2	0	0	0	0	0	0	0	0	0
Total					1541	230	120	120	270	153	164	218	184	49	103
Percent					0.95%	0.14%	0.07%	0.07%	0.16%	0.09%	0.10%	0.14%	0.11%	0.03%	0.06%

*Abbreviations: NT: nitrifying bacteria, NF: nitrogen fixing bacteria, CD: carbon degraders, DN: denitrifying bacteria, FB: fecal indicator bacteria, PP: potential pathogens. Wastewater (T1), SAR 4 and pH 7, Wastewater (T2), SAR 4 and pH 8, Wastewater (T3), SAR 7 and pH 7, Wastewater (T4), SAR 7 and pH 8, Wastewater (T5), SAR 10 and pH 7, Wastewater (T6), SAR 10 and pH 8, Synthetic water (T7) SAR 4 and pH 7, Synthetic water (T8), SAR 4 and pH 8, Synthetic water (T9), SAR 7 and pH 7, Synthetic water (T10), SAR 7 and pH 8, Synthetic water (T11), SAR 10 and pH 7, Synthetic water (T12), SAR 10 and pH 8. Treatment 3 is missing. Genus and species sequence similarities were based on greengene database (http://greengenes.lbl.gov).

with measured nitrification activities (Elifantz et al., 2011) indicated that nitrite-oxidizing bacteria are present and active in TWW-irrigated soils. Due to the higher organic matter content in TWW-irrigated water and consequently, the abundance of heterotrophic bacteria, it could be assumed that these populations outnumber the ammonia oxidizing bacteria. Moreover, Oved et al., 2001 reported that soils irrigated with TWW are enriched with many different *Nitrosomonas*-like species. However, more *Nitrospira*-like populations were found in soil irrigated with TWW water in this study than SFW. It was very surprising that we did not detect *Nitrosomonas*-like sequences in our study because other studies have shown that *Nitrosomonas*-like populations were dominant in TWW effluent-irrigated soils. It has been shown that *Nitrosomonas* strains are the most common type of ammonia oxidizers found in wastewaters (Watson et al., 1989; Wagner et al., 1995). *Nitrosomonas*-like populations were not detected in this study, probably due to the limitations of the methods used here. Furthermore, we did not detect *Nitrosospira* sequences in our study. *Nitrosospira* and *Nitrosomonas* are involved in the first step of nitrification that converts ammonium to nitrite. This is not surprising because the presence of easily metabolisable nutrients from TWW may contribute first to the selection of bacterial groups with high growth rates, such as members of the classes *Alpha-* and *Betaproteobacteria* (Smit et al., 2001), resulting in limited nutrients for other members of the community. Furthermore, Gelsomino et al. (2006) reported that the increase of total N observed in soils flooded with waste water was concomitant with a decrease in the genetic diversity of ammonia-oxidizing bacteria when compared to controls. It has been reported that high concentrations of ammonium could cause a decrease in the relative abundance of *Nitrosospira* species due to competition with other ammonia-oxidizing bacteria (Avrahami and Bohannan, 2007). Irrigation with treated wastewater has been shown to result in an increase in the activity of several microbial processes, including nitrification, during the irrigation season (Minz et al., 2011). Oved et al., 2001 also showed the effect of treated wastewater irrigation on community composition and function of ammonia-oxidizing bacteria (AOB). Few OTUs of carbon degraders (CD) such as *Rubrobacter*, and denitrifying bacteria (DN), such as *Arcobacter* showed higher OTUs in soil treated with TWW than soil with SFW in this study. Another important bacterial group found more in soil treated with TWW than SFW was *Flavobacterium*, and these bacteria are known to be closely related to nitrifying bacteria (Dong and Reddy, 2010).

The relative abundance of 25 sequences identified as sequences for potential pathogens showed that *Bacillus*, *Mycobacterium*, and *Nocardia* occurred in all the soils (T1 –T12). In addition, different OTUs associated with *Clostridium* and *Mycobacterium* were present in all the soils treated with TWW. Therefore, application of treated waste water to soil for irrigation has many advantages and disadvantages. *Clostridium* is in the *Firmicutes* phylum, and it is also associated with FIB. Other OTUs associated *Firmicutes* phylum were *Lactobacillus*, *Bacillus*, and others. Some are capable of surviving in extreme conditions due to their ability to form spores, and the ability to increase soil P availability (Han and Lee, 2005). Although most of them are fecal indicator bacteria, some of them, such as *Clostridium*, were found in all samples associated with TWW. This indicates the wide distribution of *Clostridium* in TWW samples and with a reduced concentration in fresh water as shown in this study with lower percent concentration in SFW. Further analysis with the greengene database (http://greengenes.lbl.gov) showed the detection of four sequences associated with *Clostridium* species all from soils with treated wastewater and these were *Clostridium perfringens*, a potential human pathogen, *Clostridium bowmanii*, *Clostridium bifermentan*, and *Clostridium spiroform*. Therefore, treated wastewater effluent has the potential for pathogen release into local surface water, which can be hazardous to human health.

Treated wastewater effluent can add large numbers of potential pathogens to soils (Santamaria and Toranzos, 2003; Gerba and Smith, 2005). These may include pathogenic bacteria such as *Escherichia coli* 0157:H7, *Salmonella* spp., *Listeria monocytogenes*, *Campylobater* spp., *Clostridiun botulinum*, *Clostridiun perfringens*, *Shigella* spp. and *Streptococcus*; parasites as *Giardia* sp. and *Cryptosporidium parvum* and *Entamoeba histolytica* and several human pathogenic viruses (Santamaria and Toranzos, 2003; Gerba and Smith, 2005; Ibekwe et al., 2013; Ibekwe et al., 2016). These pathogens could be a potential health hazard to humans (Wachtel et al., 2002; Warriner et al., 2003; Steele and Odumeru, 2004), and in this study we identify about 25 sequences that may contain potential human pathogens such as *Mycobacterium*, *Nocardia*, and *Clostridium*. Steele and Odumeru, 2004 suggested that contaminated irrigation water might be a source of foodborne pathogens on fruit and vegetables. They added that epidemiological investigations of food poisoning outbreaks, experimental studies examining *E. coli* O157:H7 contamination of lettuce, and observations of increased incidence of disease in areas practicing wastewater irrigation with little or no wastewater treatment to be a major public health concern. It should be noted for example that irrigation water was implicated as a source of *E. coli* contamination on cabbage seedlings irrigated with sewage-contaminated water as compared to seedlings in an adjacent field irrigated with municipal water (Wachtel et al., 2002). It is because of the potential presence of pathogens that different countries and states have different rules for land application of treated wastewater (USEPA, 2004, 2012; State of California, 2000).

In conclusion, TWW may contain a higher abundance of bacteria sequences for nitrification and other bacteria that may help in different soil processes than SFW. It may also contain bacterial sequences that are derived from pathogenic organisms. In general, we detected higher percent concentration of potentially harmful bacteria in TWW than in SFW, which might result in health risks for people that come in contact with the contaminated soil. This indicates that TWW has both advantages and disadvantages, and care must be taken in applying TWW to land for cropping of vegetables or tree crops due to the presence of potential contaminants. Therefore, more investigations are needed on the impacts of TWW irrigation on the potential transmission of pathogens and other contaminants.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.10.039.

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