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Soil Properties and Habitats Determine the Response of Bacterial Communities to Agricultural Wastewater Irrigation

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ABSTRACT

The objective of this study was to characterize the importance of soil properties and habitats for the response of soil bacteria and archaea to irrigation with secondary treated wastewater (TWW). Two agricultural soils with contrasting textures (loamy sand and silt loam), and for each, three variants differing in soil organic carbon and nitrogen, as generated by long-term fertilization, were analyzed. For each of these six soils, prokaryotic communities from two habitats, *i.e.*, root-free bulk soil and the rhizosphere of developing cucumber plants in the greenhouse, were characterized. Communities were analyzed by the quantity and diversity of their polymerase chain reaction (PCR)-amplified 16S rRNA genes. To account for TWW associated nutrient effects, potable water (PW) served as a control treatment. Amplicon sequence analysis showed that prokaryotic communities mainly consisted of bacteria (99.8%). Upon irrigation, regardless of the water quality, prokaryotic diversity declined, pH increased, and no bacterial growth was detected in bulk soil. In contrast, the growth of cucumbers was stimulated by TWW, indicating that plants were the main beneficiaries. Moreover, strong responses were seen in the rhizosphere, suggesting an indirect effect of TWW by altered rhizodepositions. The main bacterial responders to TWW were Proteobacteria, Bacteroidetes, Actinobacteria, and Planctomycetes. Changes in bacterial communities due to TWW were less pronounced in all variants of the silt loam, indicating the importance of clay and soil organic carbon for buffering effects of TWW on soil bacterial communities. Hence, soil organic carbon and soil texture are important parameters that need to be considered when applying TWW in agriculture.

Key Words: 16S rRNA gene sequences, buffering effect, cucumber rhizosphere, microbial community dynamics, rhizodeposition, soil texture, treated wastewater irrigation

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INTRODUCTION

Increasing temperatures and variability of precipitation events due to climate change will lead in the future to higher irrigation demands in agroecosystems (IPCC, 2013). Secondary treated wastewater (TWW), *i.e.*, the water from the efflux of municipal wastewater treatment plants, represents an economically and ecologically attractive alternative source of irrigation water compared to more purified waters. In addition, the nutrient load has the potential to save on fertilizers. However, the use of TWW could have consequences for the receiving soil environment and its resident microbial communities.

Treated wastewater applications can alter soil pH, increase salinity, and change other physicochemical soil characteristics, thereby modifying the living conditions of the resident soil microbial communities (Becerra-Castro *et al*., 2015; Jaramillo and Restrepo, 2017). It was previously demonstrated that TWW application increased soil salinity and thereby triggered changes in the microbial community composition while reducing microbial activity and abundance (Yan *et al*., 2015). Treated wastewater may contain human-associated microbial pathogens, which bear a health hazard if established within the soil or rhizosphere (Ibekwe *et al*., 2018). It has been further suggested that TWW irrigation can add heavy metals, which may impair microbial growth and activity (Becerra-Castro *et al*., 2015; Jaramillo and Restrepo, 2017). Recent studies using high throughput sequencing of polymerase chain reaction (PCR)-amplified rRNA gene amplicons demonstrated strong responses of specific bacterial lineages, such as Actinobacteria and Gammaproteobacteria, to TWW irrigations (Frenk *et al*., 2014; Ibekwe *et al*., 2018). Such changes in bacterial communities composition may have consequences for soil fertility and plant nutrition (Jacoby *et al*., 2017).

While responses of soil microorganisms, including bacteria, archaea, and fungi, to TWW can be expected, it is still unclear to what extent the input of water and the introduction of nutrients modifies these main microbial groups and the taxa within them. It is well known that the application of organic and mineral fertilizers changes soil microbial communities structure, activity, and abundance (Allison and Martiny, 2008; Geisseler and Scow, 2014; Francioli *et al*., 2016). Nutrient availability through different fertilization regimes can promote taxon-specific shifts in soil microbial communities that can influence the productivity and stability of agroecosystems (Hartmann *et al*., 2015). While these and other studies have evaluated the effect of TWW on specific soil microbial communities, there is a gap regarding a more systematic understanding of the importance of soil types and habitat conditions and its effects on the responsiveness of the soil microbial communities to irrigation and nutrient additions. In order to gain a better understanding of TWW applications, it is therefore important to identify the most influential factors that affect the habitat conditions of soil microorganisms.

Besides soil salinity and pH, there is a potential influence of soil texture and soil organic carbon (SOC) on microbial communities. As recently demonstrated, the majority of the dominant soil bacterial taxa may show a preference for specific soil particle size fractions, *i.e.*, sand, silt, or clay (Hemkemeyer *et al*., 2015). This underlines the importance of heterogeneous soil surface properties in habitats for structuring the soil bacterial community at a microscale. For instance, bacteria associated with clay particles were found to be less susceptible to nutrient additions by long-term agricultural fertilization than those associated with larger particle sizes (Neumann *et al*., 2013). Thus, soil texture may have a strong effect on the responsiveness of soil microbial communities to TWW irrigation. Moreover, a strong influence of SOC on the soil bacterial community structure was recently demonstrated in a survey across European soils under different land use, including arable soil (Szoboszlay *et al*., 2017). Soil organic carbon can act as a nutrient and energy source for microbial communities, but it is also part of the organo-mineral complex, and thus, it modifies surface properties of soil particles with consequences for bacterial diversity.

The objective of this study was to characterize the impact of TWW irrigation on the prokaryotic community structure and bacterial abundance in two habitats, *i.e.*, bulk soil and the rhizosphere of cucumber plants. To that end, two soil types differing in texture were compared, including a loamy sand

and a silt loam. For each soil type, three variants differing in soil total organic carbon (TOC) and total nitrogen (TN) levels generated by different long-term fertilization regimes over more than 100 years were available. In order to understand the importance of nutrient additions by TWW, a control with potable water (PW) was included. The studies were conducted in soil microcosms under greenhouse conditions.

We hypothesized that i) soil irrigated with TWW would have a stronger effect on bacterial diversity than soil irrigated with PW due to the additional nutrient input, which should enhance the growth of the typically nutrient limited soil bacteria; ii) this nutrient effect will be less pronounced in the rhizosphere due to presumably more abundant and alternative nutrients provided by rhizodepositions; and iii) bacteria in soils with coarser textures, *i.e.*, more sand and less clay, will be more responsive to irrigation with TWW and PW because clay-associated bacteria were previously found to be more protected and thus less receptive to environmental-mediated changes (Neumann *et al*., 2013).

MATERIALS AND METHODS

Soil origin and sampling

Soil variants used in this study were obtained from the Lermarken site of the Askov long-term experiments on animal manure and mineral fertilizers, initiated in the year 1894 at the Askov experimental station, Denmark (AS) (Christensen *et al*., 2006) and from the static fertilization experiment in Bad Lauchstädt, Germany (BL), which started in the year 1902 (Körschens and Müller, 1996). The soil variants from the loamy sand of AS (silt 26%, clay 10%, weight/weight) and the silt loam of BL (silt 68%, clay 21%, weight/weight) have previously been described in more detail (Altermann *et al*., 2005; Neumann *et al*., 2013; Hemkemeyer *et al*., 2015). Despite site-specific fertilization practices, the same abbreviations were used in this study for simplicity. In AS, soils were sampled from 0--18 cm depths from the unfertilized (NIL), mineral fertilized (NPK), and manure fertilized (ORG) plots. In BL, soils were sampled from 0--30 cm depths from the NIL, NPK, and ORG plots. Soil samples were sieved through 2 mm and stored in the dark at 4 °C. Soils were rewetted to 50%--55% saturation of their water holding capacity and pre-incubated for two weeks at room temperature in the dark prior to laboratory irrigation experiments.

Sources of irrigation water

Secondary TWW was collected from a local municipal wastewater treatment facility in Braunschweig Steinhof, Germany. Samples were taken at the effluent, which further deviated partially onto agricultural fields for irrigation and partially after passage through a sewage farm to the Oker river. The TWW was centrifuged at 11 800 × *g* for 30 min and filtered through 2 µm pore size cellulose acetate filter (Sartorius, Germany). In addition, PW was collected from a tap at our research institute.

The chemical analyses of the TWW showed the following values (mg L^{-1}): TOC 12.7; total bound N 7.0; organic N 2.4; ammonium N 4.6; nitrite N 0.12, nitrate N 1.14; phosphate phosphorus 0.17; magnesium 6; potassium 23.3; calcium 54.8; sodium 103.0; chloride 127.0; and sulfate 100.0. The electric conductivity was 1 000 µS cm^{-1} and the pH 7.4.

For each type of water (TWW, PW), reservoirs of 10 L were prepared to ensure the same water stock throughout the experiment. Water was stored in 5 L glass bottles at 4 °C in the dark with constant, slow magnetic stirring.

Dissolved organic carbon (DOC) was measured from each sample with three replicates using a DimaTOC 2000 (Dimatec Analysentechnik GmbH, Germany). The DOC was calculated as the difference between total carbon (TC) (combustion at 850 °C) and total inorganic carbon (TIC) (combustion at 165 °C after acidification).

Soil irrigation experiment

Four replicates were set up for both soil types (AS, BL) and each soil variant (NIL, NPK, ORG), which were then subjected to different irrigation treatments (TWW, PW), thus resulting in 12

independent treatments.

For each treatment, a total of 200 g of dry weight soil were incubated in 0.5 L glass laboratory bottles (GL 45 Duran® clear glass, DWK Life Sciences, Germany) at 20 °C in the dark, and irrigated once a week to 43% saturation of the water holding capacity, corresponding to soil moisture of 12.5% for AS and 14.7% for BL. Before the first irrigation treatments, soils were adjusted to a soil moisture of 10% and subsequently irrigated with TWW or PW by careful addition with a pipette, corresponding to 18.6 mL for the AS soils and 24.6 mL for the BL soils.

Soil samples were collected before irrigation (t_0) and after 4 (t_1), 8 (t_2), 12 (t_3), and 16 (t_4) weeks. Three aliquots of each soil sample were air dried, dried at 105 °C, and frozen at -80 °C, respectively, depending on the different proceeding analytical methods.

Cucumber rhizosphere irrigation experiment

At the end of the soil irrigation experiment, a total of *ca*. 46 g dry weight soil were left for the subsequent irrigation study with cucumber (*Cucumis sativus* L.) for rhizosphere analyses. For this purpose, the two soils (AS, BL) from the different treatments (NIL, NLP, and ORG), including all replicates (n = 48), were transferred to flowerpots (black plastic, diameter 5.5 cm, height 5 cm). Each pot received two cucumber seeds (*C. sativus* var. Derby, kindly provided by Menahem Edelstein, Department of Vegetable Crops, Newe Ya'ar Research Center, Agricultural Research Organization, Ramat Yishay, Israel). The pot soils were then irrigated to reach a moisture level of 20% for seed germination. During plant development, the amount of water was adjusted to support healthy plant growth, while the same water types (TWW and PW) were used as before. Only one plant was allowed to grow in each pot, while the second was removed immediately after germination. Plants were grown in a greenhouse under 12 h day/night cycles. Temperatures had a minimum of 19 °C during the night and a maximum of 26 °C during day time. Relative humidity ranged 70%--80%. Four weeks after sowing, cucumber plants were sampled in a destructive harvest.

The individual plants with adherent soil were removed from the pots, and the roots were separated from the aerial part of the plant. Then, fresh weights of above and below ground plant parts were determined. Soil adhering to the roots was removed by shaking. Bacterial cells adhering to the roots were detached by suspending the collected root material in 30 mL of sterile saline (0.85% NaCl (weight/volume)) for 30 min at 4 °C in an overhead shaker (Model 3040, GFL, Germany) at 10 r min^{-1}. Microbial cells from the washing solution were collected by centrifugation at 4 000 × g and 4 °C for 30 min. The supernatant was aspirated, and the pellet was suspended in 1 mL sterile saline (0.85 % NaCl (weight/volume)), transferred to a 2 mL polypropylene screw cap tube, and centrifuged at 10 000 × g for 10 min. Cell pellets were stored at -80 °C for subsequent molecular analyses.

Soil physicochemical parameters

Soil pH was measured in soil suspensions (one part of air-dried soil suspended in two parts of 0.01 mol L^{-1} $CaCl_2$) using a Professional Meter PP-25 (Sartorius, Germany) and an electrode PY-P21 (Sartorius, Germany). Electric conductivity was measured in a suspension of 10 g air-dried soil in 20 mL distilled water with a Multi 340i measuring instrument (WTW, Germany) and a TetraCon 325 measuring cell (WTW, Germany). Total carbon and TN were measured *via* dry combustion with LECO TruMac analyzer (LECO Instrumente GmbH, Germany) from dried (at 105 °C) and ground (Pulverisette, Fritsch GmbH, Germany) soil material.

DNA extraction

DNA was extracted from 0.5 g of soil or from microbial cell pellets using the FastDNA SPIN kit for soil (MP Biomedicals, France). The extraction included two bead beating steps of 45 s at 6.5 m s^{-1} on a FastPrep-24 system (MP Biomedicals, France) and additional washing steps of the binding matrix with 1 mL 5.5 mol L^{-1} guanidine thiocyanate (Carl Roth, Germany) until the matrix regained its original color.

In addition, DNA was extracted from 1 L TWW and 5 L PW by centrifugation at 11 800 × g for 30

min and filtering through a sterile 0.2 µm pore size cellulose acetate filter of 9 cm diameter (Sartorius, Germany). Pellets and filters were stored at -80 °C until further processing. Filters were cut into small pieces with sterile scissors and used for DNA extraction with the FastDNA SPIN kit for soil using 15 mL Lysing Matrix E Tubes (MP Biomedicals, France). The extraction included two bead beating steps of 45 s at 6.5 m s^{-1} on a FastPrep-24 system with the 12 × 15 mL TeenPrep™ Adapter (MP Biomedicals, France).

DNA extracts were quantified with a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Thermo Fisher Scientific, USA) in a Mithras LB 940 fluorimeter (Berthold Technologies, Germany).

Quantitative PCR (qPCR) of the 16S rRNA gene

Bacterial abundances were determined by real-time qPCR assays using the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA) and Maxima Probe qPCR Master Mix (2×) (Thermo Fisher Scientific, USA) with primers BAC338F, BAC516F, and BAC805R (Yu *et al*., 2005). Reactions for qPCR and PCR for amplicon sequencing were performed as described earlier (Hemkemeyer *et al*., 2015), and PCR efficiencies were 98.9% (R^2 = 0.9969).

Terminal restriction fragment length polymorphism (T-RFLP)

The T-RFLP technique was applied to profile the temporal dynamics of the soil bacterial community composition induced by irrigation as a tool to select the best point for in-depth community analyses by high-throughput DNA sequencing of PCR amplicons. The analyses were restricted to randomly chosen three out of the four replicates for each soil type and variant. The PCR, purification, and digestion conditions have been described before (Hemkemeyer *et al*., 2015)

The T-RFLP profiles were generated by capillary electrophoresis on a CEQ 8800 genetic analysis system (Beckman Coulter GmbH, Germany). The chromatograms were analyzed with CEQ 8800 specific software applying a maximum bin width of 2 bp. Fragments representing less than 0.25% of the total peak heights were considered to be noise and therefore removed. Terminal restriction fragments (T-RFs) that appeared in only one or two samples of all samples analyzed (including all replicates) were considered outliers and excluded from the analysis.

Illumina MiSeq DNA sequencing

Guided by the results of the above described T-RFLP analyses, the Illumina sequencing approach was used to characterize the diversity of bacterial and archaeal 16S rRNA encoding gene PCR amplicons. These originated in the irrigation experiment from samples collected at t_0 and t_4 of the soil irrigation experiment. In addition, the prokaryotic communities were analyzed at the end of the greenhouse incubation, *i.e.*, after 4-week cultivation of cucumber. The prokaryotic community composition of TWW and PW samples were also analyzed. All samples were amplified and sequenced by StarSEQ GmbH (Mainz, Germany) applying MiSeq Illumina technology and 2 × 300 nucleotides (nt) paired-end sequencing with V3 chemistry. Targeted bacterial and archaeal V4--V5 regions in the 16S rRNA gene were amplified using the primers 515F (5′-GTGYCAGCMGCCGCGGTA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′) (Li *et al*., 2014). All samples were sequenced twice. Reads were split into six groups to account for the three habitat soils, rhizosphere and water, and the two sequencing reactions. Reads were merged with USEARCH (v10.0.240_i86linux32) asking for a minimum length of the overlap of 100 nt and a minimum length of the merged read of 370 nt and a maximum of 380 nt (Edgar, 2010). This was achieved for 15 699 971 (87.4%) of all paired-end reads. Sequences with total expected errors E > 1 were discarded with the fastq_filter command of VSEARCH (v2.4.3_linux_x86_64) and good quality sequences were retained (Rognes *et al*., 2016). Sequences from the two sequencing reactions were combined, with separate files kept for each habitat. Then, VSEARCH derep_fulllength was applied to remove sequences that appeared only once (singletons). Chimeric sequences were removed with VSEARCH uchime_denovo (Edgar *et al*., 2011). Sequences were classified using the SILVA reference file release 128 (Yilmaz *et al*., 2014) in MOTHUR version 1.38.1 (Schloss *et al*., 2009). Sequences classified as chloroplast, mitochondria, Eukaryota, or

unclassified sequences were removed from the datasets. Sequences were clustered in zero-radius operational taxonomic units (zOTUs) with USEARCH at a threshold of 100% sequence identity (Edgar, 2010). Then, zOTU tables were created for each habitat with USEARCH and combined to give a single zOTU table. The zOTUs approach was chosen because it is widely applied, whereas the classic identity threshold of 97% is no longer suitable based on currently available information on high-quality 16S rRNA sequencing data (Edgar, 2018).

Before the analyses, zOTUs were collapsed to genus level, *i.e.*, each remaining zOTU represented a collection of zOTUs with the same taxonomic affiliation. The respective lower taxonomic classification was used in case the genus level could not be classified. In addition, zOTUs that did not have at least ten sequences across all samples were excluded from the analyses. The resulting data matrix contained 11 912 604 sequences (20 121--195 385 sequences per sample) in 841 zOTUs with 44% sparsity.

The 16S rRNA gene sequences were deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB30512.

Statistical analyses

Physicochemical parameters and 16S rRNA gene numbers were compared using analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post-hoc test in JMP 13.0.0 (SAS Institute, USA). Plots were created with SigmaPlot for Windows Version 11.0 (Systat Software GmbH, Germany).

The T-RFLP profiles were analyzed by non-metric multidimensional scaling (NMDS) based on Bray Curtis distances, and environmental fitting was done using package vegan (version 2.5-2) in the R environment for statistical computing (version 3.5.1) (Oksanen *et al*., 2016; Team, 2018).

Soil and root bacterial communities based on Illumina sequencing were analyzed using tools for compositional data analysis, which have been shown to be more statistically sound and appropriate for analyzing high throughput sequencing data (Gloor *et al*., 2016, 2017). First, Bayesian-multiplicative replacement of count zeros were performed with the R package zCompositions (Palarea-Albaladejo and Martín-Fernández, 2015) to treat zeros in compositional count data, followed by applying the centered log-ratio (CLR) transformation in the R package compositions in order to correct for differences in sequencing depth between samples (Gloor *et al*., 2016). Then, differences in community composition were visualized by using principal component analysis (PCA) plots based on Aitchison distance implemented in the R package stats. The R package ggplot2 was used for more advanced graphics (Wickham, 2009). Shannon diversity indices were calculated with R package vegan (Oksanen *et al*., 2016). Significant differences were calculated using ANVOA and Tukey's HSD post hoc test as implemented in the R package stats. Variation in community structure was assessed with permutational multivariate ANVOA (PERMANOVA) implemented in the R package vegan.

The R package ALDEx2 (Fernandes *et al*., 2014) was used to identify differently abundant zOTUs between the two compared groups applying Welch's t-test with correction for multiple testing with a false discovery rate (FDR) of 5% and an effect size > 1.

RESULTS

Soil physicochemical parameters and cucumber plant growth

Both soils, the loamy sand (AS) and the silt loam (BL), showed similar electric conductivities before irrigation, with lower values in NIL, higher values in NPK, and the highest values in the respective ORG soil variants (Fig. 1a). Irrigation caused higher conductivities in all soil variants, with a significantly greater ($P < 0.05$) increase in soils irrigated with TWW compared to PW.

Fig. 1

Fig. 1 Electric conductivity (a), pH (b), total organic carbon (TOC) (c), and total nitrogen (TN) (d) of the loamy sand from Askov, Denmark (AS) and the silt loam from Bad Lauchstädt, Germany (BL) at the start of irrigation (t_0) and after 16 weeks of irrigation (t_4) with potable water (PW) or secondary treated wastewater

(TWW). Both soils included three variants generated by long-term fertilization treatments, *i.e.*, no fertilization (NIL), application of mineral fertilizer (NPK), and additional application of organic fertilizers (ORG). The whiskers indicate standard deviations (n = 4). Different letters indicate significant differences ($P < 0.05$) based on Tukey's honestly significant difference (HSD) post-hoc test.

The soil pH was 5.7--6.0 in AS and 6.2--7.3 in BL before irrigation (Fig. 1b). In both soil types and all variants, pH increased upon irrigation; for AS, the average increase was 0.4, while for BL it was 0.5.

As expected, the respective long-term fertilization variants of both soil types differed in their TOC (Fig. 1c) and TN contents (Fig. 1d). Compared to the non-fertilized control (NIL), TOC and TN were high in ORG and NPK, especially in ORG. Irrigation with PW did not increase the contents of TOC and TN. Surprisingly, both parameters were also not elevated by TWW irrigation, even though the DOC content of TWW was significantly higher than that of PW (Table S1, see Supplementary Material for Table S1).

Independent of whether TWW or PW was applied for irrigation, the cucumber plants displayed a normal phenotype in all three soil variants of both AS and BL. When the incubation was terminated after four weeks, 90% of the plants were at the second leaf-stage. The above ground plant biomass of cucumber was greater in BL compared to in AS, and in both, there was a trend showing that the TWW application resulted in higher plant biomass compared to PW, indicating plant uptake of the nutrients provided by TWW (Fig. S1a, see Supplementary Material for Fig. S1a).

The root biomass development was not affected in BL, neither by the soil variants (NIL, NPK, and ORG) nor by the water quality (TWW and PW) (Fig. S1b, see Supplementary Material for Fig. S1b). In contrast, in AS, root biomass was more developed in ORG than in NPK and NIL, independent of the water quality (Fig. S1b).

Soil bacterial abundances and prokaryotic diversity indices

Total bacterial abundance, as indicated by 16S rRNA gene copy number, was slightly higher in the untreated loamy sand AS than in the silt loam BL at t_0 (Fig. 2). After 16 weeks of TWW or PW irrigation, the loamy sand bacterial population sizes declined significantly ($P < 0.05$) in the NPK and ORG variants, while this was not the case for the silt loam. Thus, despite the introduction of additional nutrients by TWW, there was no increase in the overall bacterial abundance in the investigated soils.

Fig. 2

Fig. 2 Abundances of bacterial 16S rRNA genes in the loamy sand from Askov, Denmark (AS) and the silt loam from Bad Lauchstädt, Germany (BL) at the start of irrigation (t_0) and after 16 weeks of irrigation (t_4) with potable water (PW) or secondary treated wastewater (TWW). Both soils included three variants generated by long-term fertilization treatments, *i.e.*, no fertilization (NIL), application of mineral fertilizer (NPK), and additional application of organic fertilizers (ORG). The whiskers indicate standard deviations (n = 4). Different letters indicate significant differences ($P < 0.05$) based on Tukey's honestly significant difference (HSD) post-hoc test.

Shannon diversity index estimates derived from PCR amplicon sequencing data showed a significantly lower prokaryotic diversity in irrigated soil samples than in samples before irrigation. This effect was stronger in the loamy sand than in silt loam soil (Table S2, see Supplementary Material for Table S2). Rhizosphere samples did not show any significant differences in diversity estimates, except for the PW irrigated NPK variant in BL soils (Table S3, see Supplementary Material for Table S3). No differences were recorded for the diversity indices between the irrigation sources TWW and PW (Table S4, see Supplementary Material for Table S4).

Prokaryotic community structures in bulk soil

The T-RFLP profiles were chosen to estimate the time-dependent changes of the bacterial community structures after irrigation. The profiles from soil DNA consisted of a total of 141 different

T-RFs, with an average of 68 ± 7 different T-RFs identified per sample. The NMDS ordinations from loamy sand and silt loam T-RFLP profiles demonstrated that the bacterial communities changed in response to irrigation mainly during the first eight weeks (Fig. S2, see Supplementary Material for Fig. S2). Thus, the 16S rRNA amplicon sequencing approach was applied to samples taken immediately before and after 16 weeks of incubation.

Principal component analysis of PCR amplicon sequencing data depicted distinct community structures for both soil types and their three soil variants (NIL, NPK, and ORG) (Fig. S3, see Supplementary Material for Fig. S3). Permutational multivariate ANOVA identified a strong effect of soil type (AS *vs*. BL) on the prokaryotic community structure (R^2 = 0.55, P < 0.001) (Fig. S3). Applying such analyses separately for each soil type showed that both analytical techniques revealed an additional effect of the respective soil variants NIL, NPK, and ORG, respectively (AS: R^2 = 0.43, P < 0.001; BL: R^2 = 0.52, P < 0.001), as also indicated by PCA (Fig. 3).

Fig. 3

Fig. 3 Principal component ordination plot based on zero radiance operational taxonomical units (zOTUs) for the loamy sand soils from Askov, Denmark (AS) (a) and the silt loam soils from Bad Lauchstädt, Germany (BL) (b) at the start of irrigation (t_0) and after 16 weeks of irrigation (t_4) with potable water (PW) or secondary treated wastewater (TWW). Both soils included three variants generated by long-term fertilization treatments, *i.e.*, no fertilization (NIL), application of mineral fertilizer (NPK), and additional application of organic fertilizers (ORG).

Irrigation with either PW or TWW resulted in clearly distinct bacterial community structures independent of the AS soil variants (R^2 = 0.19, P < 0.001) (Fig. 3a), yet there was no significant effect of irrigation water type on soil prokaryotic communities (AS: R^2 = 0.03, P < 0.693; BL: R^2 = 0.03, P < 0.786). Amplicon sequence analyses revealed that 99.2% of zOTUs were assigned to the domain bacteria, only 0.8% of zOTUs to the domain Archaea (File S1, see Supplementary Material for File S1). The zOTUs from AS were dominated by members of the phyla Proteobacteria (20% in NIL, 23% in NPK, and 22% in ORG), Acidobacteria (21% in NIL, 18% in NPK, and 17% in ORG), Actinobacteria (19% in NIL, 21% in NPK, and 23% in ORG), and Bacteroidetes (10% in NIL, 8% in NPK, and 8% in ORG) (File S1).

For BL, the effect of irrigation was less pronounced than for AS (R^2 = 0.07, P < 0.031) (Fig. 3b). Most zOTUs found in BL were affiliated with Actinobacteria (20% in NIL, 21% in NPK, and 19% in ORG), Acidobacteria (21% in NIL and 18% in NPK and ORG), Proteobacteria (16% in NIL, NPK, and ORG), and Bacteroidetes (7% in NIL and 6% in NPK and ORG). Water quality (PW or TWW) had no significant effect (R^2 = 0.03, P < 0.803) (Fig. 3) on the phyla to which the zOTUs were assigned (File S1).

The analyses of the prokaryotic community in PW and TWW revealed compositional differences (File S1). However, these differences were not seen in the overall soil prokaryotic communities measured after irrigation.

Prokaryotic community structures in the rhizosphere

Distinct prokaryotic community compositions, as assessed by 16S rRNA amplicon sequencing, were detected in the cucumber rhizosphere cultivated in the two different soil types, AS and BL, as revealed by PCA and PERMANOVA (R^2 = 0.55, P < 0.001) (Fig. 4). Significant effects of both, the three soil variants (AS: R^2 = 0.34, P < 0.001; BL: R^2 = 0.41, P < 0.001) and water quality (AS: R^2 = 0.16, P < 0.001; BL: R^2 = 0.11, P < 0.019), were also detected. In contrast to the bulk soil results, irrigation with TWW and PW depicted distinct rhizosphere bacterial community structures (AS: R^2 = 0.16, P < 0.001; BL: R^2 = 0.11, P < 0.019) (Fig. 4). In rhizospheres from the loamy sand (AS) variants, most zOTUs were mainly affiliated with the same four phyla as found for bulk soil, which represented on average 86% of all zOTUs. In more detail, Proteobacteria accounted for 48% in NIL, 42% in NPK, and 38% in ORG; Bacteroidetes for 24% in NIL, 25% in NPK, and 23% in ORG; Actinobacteria for 11% in NIL and NPK and 16% in ORG; and Acidobacteria for 6% in NIL and NPK and 7% in ORG. The dominance of these phyla did not differ between PW and TWW irrigation (File S1). Likewise,

zOTUs from the rhizosphere of the silt loam (BL) explained on average 86% of all zOTUs. They belonged to Proteobacteria (45% in NIL and NPK and 44% in ORG), Bacteroidetes (26% in NIL, 28% in NPK, and 22% in ORG), Actinobacteria (9% in NIL, 11% in NPK, and 14% in ORG), and Acidobacteria (6% in NIL, 4% in NPK, and 5% in ORG). As found for AS, the dominance of these phyla was not affected by the irrigation practices (File S1).

Fig. 4

Fig. 4 Principal component ordination plot based on zero radiance operational taxonomical units (zOTUs) for cucumber rhizosphere microbial communities in the loamy sand soil from Askov, Denmark (AS) and the silt loam soil from Bad Lauchstädt, Germany (BL) after 20 weeks of irrigation with potable water (PW) or secondary treated wastewater (TWW). Both soils included three variants generated by long-term fertilization treatments, *i.e.*, no fertilization (NIL), application of mineral fertilizer (NPK), and additional application of organic fertilizers (ORG). The cucumber plants were 4-week old when sampled.

Differential abundance analysis of zOTUs from bulk soil and rhizosphere as two distinct habitats

The Aldex2 algorithm was used to identify zOTUs which were differentially abundant between irrigated and non-irrigated soil samples and different soil variants (Table I and Files S2--S4, see Supplementary Material for Files S2--S4). For bulk soil, due to the indistinguishable effects of TWW and PW indicated by PCA and PERMONOVA and PCA analyses (see above), the two treatments were combined into one variable named "irrigation treatment." In the bulk AS, irrigation caused on average 145 zOTUs to change in relative abundances (NIL, NPK, and ORG combined) while only 44 changed in the bulk BL (Table I and File S2). For AS, the number of responsive zOTUs was not affected by soil variants, but for BL, the number strongly changed: a total of 61 zOTUs responded to irrigation in NIL, 47 in NPK, and only 23 in the ORG soil variant (Table I and File S2). Thus, there was a negative correlation between the number of responsive taxa and soil TOC and TN.

TABLE I

Number of prokaryotic taxa with significant differences in relative abundance based on the Aldex2 differential abundance analysis. The prokaryotic taxa were identified in the bulk soil and rhizosphere of cucumber plants grown in the loamy sand from Askov, Denmark (AS) and the silt loam from Bad Lauchstädt, Germany (BL), both of which included three variants generated by long-term fertilization treatments, *i.e.*, no fertilization (NIL), application of mineral fertilizer (NPK), and additional application of organic fertilizers (ORG). Both soils had been irrigated with potable water (PW) or secondary treated wastewater (TWW) for 20 weeks before and during cucumber planting, and the cucumber plants were 4-week old when sampled. The significance test was based on Welch's t test with Benjamin-Hochberg correction ($P < 0.05$)

Soil		Irrigation effect				Soil variant effect		
		NIL	NPK	ORG	PW *vs*. TWW	NIL *vs*. NPK	NIL *vs*. ORG	NPK *vs*. ORG
AS	Bulk soil	149	142	145		109	134	172
	Rhizosphere				17	95	111	141
BL	Bulk soil	61	47	23		163	198	123
	Rhizosphere				9	173	186	84

In AS, zOTUs responding to irrigation were mainly affiliated to Proteobacteria (29% ± 0.01%), Bacteroidetes (14% ± 0.01%), and Firmicutes (9% ± 0.03%). In response to irrigation, zOTUs affiliated from the phylum Acidobacteria were more abundant in the NIL (13%) than in the NPK and ORG variants (10% ± 0.003%), while zOTUs from the phylum Actinobacteria were more

predominant in the NPK and ORG (17% ± 0.002%) than in the NIL variant (11%). For the BL, most responding zOTUs in the NIL variant were affiliated with Proteobacteria (28%), Actinobacteria (18%), and Acidobacteria (15%). While those values were similar for the NPK soil variant for Proteobacteria (28%) and Acidobacteria (15%), a total of 13% responding zOTUs to irrigation were members of the phylum Planctomycetes. In the case of the ORG variant: following Proteobacteria with 35% of all responding zOTUs, responsive zOTUs were mainly affiliated with the phyla Bacteroidetes (13%) and Planctomycetes (13%) (File S2). Independent of the soil type, soil variants (NIL, NPK, and ORG), or the irrigation practice (PW or TWW), zOTUs from the order Solirubrobacterales (Actinobacteria) increased 4-fold to 1.3% in AS and 2-fold to 0.6% in BL. The archaeal phylum Thaumarchaeota increased in its relative abundances in AS 1.6-fold to 2.6% and in BL 1.4-fold to 5.9% (File S2).

For the rhizosphere, TWW and PW treatments were separately analyzed, since both PERMANOVA and PCA indicated differences between them (see above). It was tested whether the rhizosphere harbors distinct zOTUs responding to irrigation type regardless of different soil variants (File S4). Irrigation caused 17 zOTUs to change in relative abundances in AS rhizosphere samples, but only nine zOTUs in BL rhizosphere samples (Table I). The zOTUs from the genus *Bradyrhizobium* decreased with TWW irrigation, while those of the genus *Stenotrophomonas* increased in both soils (File S4).

In addition, the bacterial communities that developed in the cucumber rhizosphere were affected by the soil variant (Table I). Similarly, the three soil variants from each soil type also differed in their bacterial composition encompassing approximately 100 to 200 OTUs that were equally present in both soil types, BL and AS (Table I).

DISCUSSION

The application of TWW changes the physicochemical conditions of soils. Both water saturation and nutrient limitations are highly important for selecting and supporting soil microbial communities with consequences for their diversity and metabolic activities (Filip *et al*., 2000; Hidri *et al*., 2010; Adrover *et al*., 2012). In order to distinguish the water effect from the nutrient effects, controls with PW for irrigation were included in this study. Our first hypothesis, stating that soil irrigated with TWW would have a stronger effect on the prokaryotic community than PW irrigations due to the additional nutrient input, was in fact confirmed for rhizosphere as a habitat, but it was not confirmed for bulk soil. Consequently, our second hypothesis, stating that nutrient effects were less pronounced in rhizospheres, could not be confirmed. The additional nutrient load of TWW mainly increased plant biomass in both soil types, indicating that plants were the main beneficiaries of the incoming nutrients from TWW. This further suggests that the distinct response of the prokaryotic community structure to TWW in the rhizosphere was mainly a result of modified rhizodepositions rather than the direct nutrient effects of TWW. The strong impact of rhizodepositions on the structure of microbial communities in the rhizosphere is well known (Berg and Smalla, 2009; Dennis *et al*., 2010). The multivariate statistics applied in this study demonstrated the importance of distinct soils and confirmed that soil properties changed by long-term fertilization, *i.e.*, in addition to the immediate impact of fertilizers, TOC and TN also affect the microbial community structure (Hai *et al*., 2009; Chaparro *et al*., 2012).

In addition to the increased water saturation and introduction of nutrients, TWW and PW irrigations also caused an increase in pH by 0.4 to 0.5 units in both soil types. Such pH changes upon irrigation are typical to TWW irrigations because of the continuous addition of exchangeable cations such as sodium to the soil (Becerra-Castro *et al*., 2015). The alteration of the soil pH may explain the decline in bacterial populations induced by irrigation, yet it was significant only for the loamy sand. In silt loam, the decline was not equally pronounced, suggesting that the bacterial populations were stabilized by clay, as the silt loam contained 26% but the loamy sand only 10% clay. Previous studies have demonstrated that the majority of soil bacteria are associated with the clay fraction, which provides, due to its smaller particle size, higher surface areas compared to sand or silt (Neumann *et al*., 2013; Hemkemeyer *et al*., 2018). In contrast to the data presented here, other studies reported that microbial abundance increased upon TWW irrigation (Hidri *et al*., 2010; Frenk *et al*., 2014; Wafula *et al*., 2015). Whether nutrients introduced with TWW may affect soil microbial communities' abundance and diversity will depend on their mobility, their availability as a result of sorption and

desorption, as well as on a potential competition between microbial cells and plant roots. Abundant clay may limit nutrient availability, but it may also protect bacteria against environmental changes better than coarser soil particles like silt and sand (Neumann *et al*., 2013; Hemkemeyer *et al*., 2015).

A prominent impact of pH on soil microbial communities was demonstrated in several previous studies (Lauber *et al*., 2009; Rousk *et al*., 2010). In this study, the addition of TWW or PW changed the pH equally in the loamy sand and the silt loam. Generally, sandy soils would be more responsive, but at the field site of the loamy sand variant used in this study, changes in pH were ameliorated by frequent additions of magnesium-enriched lime (Hemkemeyer *et al*., 2015). Despite similar irrigation-induced pH shifts in both soils and their variants, the bacterial communities responded stronger in the loamy sand, suggesting that the prokaryotes were more protected in the silt loam.

Considering the third hypothesis of this study, it was expected that the presumably lower buffering capacity of the loamy sand compared to the silt loam would result in stronger responses of the bacterial communities to PW and TWW additions. Multivariate statistics of bacterial community structures from both soils and the three respective variants support this hypothesis. Differences in the bacterial community structure caused by irrigation of either PW or TWW were more pronounced in the loamy sand compared to the silt loam. More bacterial genera in both bulk soil and rhizosphere responded to irrigation in the loamy sand compared to the silt loam, regardless of the soil variants. This underlined the importance of clay in mitigating environmental changes to the microbial community. For the silt loam, the comparison of the three soil variants revealed a negative correlation between SOC and the number of genera responding to irrigation. This may indicate that SOC has considerable buffering capacity to mitigate the impact of PW or TWW irrigations on soil prokaryotic communities. Soil organic carbon is a mixture of different organic fractions, some of them more recalcitrant and others more biologically active (Poeplau and Don, 2013; Szoboszlay *et al*., 2017). In this study, carbon fractions were not analyzed, but it appeared that none of these fractions enhanced the growth of the resident soil prokaryotic communities. Thus, the impact of SOC was more likely a result of buffering abiotic effects, *e.g*., by sorption, rather than directly affecting the bacterial metabolism.

Both PW and TW irrigations caused an increase in electric conductivity for both soil types and their respective variants. This increase was more pronounced with TWW, as expected by the introduction of additional water-soluble nutrients. Considering that PW and TWW had indistinguishable effects on the soil bacterial community composition, the change of conductivity apparently did not impose selective osmotic pressure on these organisms. While an increase in conductivity from 0.2 to 0.3 dS m^{-1} was relatively strong, it is still at the low end of conductivity values measured for stressful conditions, *e.g*., those found in saline soils with values typically above 4 dS m^{-1} (Canfora *et al*., 2014). Thus, in contrast to the indirect nutrient effect described above, the salinity effect of TWW irrigation for the prokaryotic communities of the two soil types and their variants was negligible.

Despite the differences of both soils in regard to the sites from which they were sampled and their long-term agricultural use, most prominent patterns were found within the domain Bacteria. More specifically, bacterial communities were dominated by the same phyla, *i.e.*, Proteobacteria, Acidobacteria, Actinobacteria, and Bacteroidetes, which together accounted for more than 60% of the total community. Independent of the soil and their variants, the cucumber rhizosphere was dominated by the same phyla found in bulk soil, however, with an increased relative abundance of Proteobacteria and Bacteroidetes and a decreased abundance of Actinobacteria and Acidobacteria. The phylum-level composition of these communities is commonly found in agricultural soils and rhizospheres across the world, indicating an environmentally stable network structure at this phylogenetic resolution (Fierer *et al*., 2007; Liu *et al*., 2014). However, when searching for genera that specifically responded to irrigation, differences between the soils were detected. For instance, in the bulk loamy sand, Firmicutes were 9% among the main responders, while they were not equally detected in the silt loam. Long-term fertilization practices also had an impact. For instance, Planctomycetes, which are commonly poorly represented in cultivated isolates (Wiegand *et al*., 2018), were among the main responders (13% of all zOTUs) to irrigation in the long-term fertilized versions (NPK, ORG) of the silt loam. In the rhizosphere, the number of genera responding specifically to TWW was also affected by soil type (Table I). Thus, even considering the indirect effects that occurred in the rhizosphere, the reduced numbers of responsive genera in the loam, when compared to sand, indicated a higher

buffering capacity, most likely linked to the higher proportion of clay.

In conclusion, the results of this study suggest that irrigation with TWW has a stronger effect on bacterial communities in loamy sand than in silt loam and that the buffering capacity of soils to respond to TWW is primarily linked to their higher clay content. Also, the higher SOC found in the long-term fertilized silt loam probably added to the buffering effect. Thus, for managing TWW applications in agriculture, it could be advisable to adjust the optimum amount according to the soil texture and SOC of the receiving environment. Results further suggest that the main beneficiaries of the additional nutrients introduced by TWW are irrigated plants rather than soil bacteria. The latter, however, can respond indirectly as the stimulated plant growth modulates the rhizosphere more strongly, thereby modifying the composition of the present bacterial community in this particular habitat. Whether such changes translate to adverse or beneficial effects for plant health or microbial ecosystem services remains to be determined.

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