Soil and rhizosphere microbiome response to treated waste water irrigation
Minz, D. Agricultural Research Organization
Sela, N. Agricultural Research Organization
Hadar, Y. The Hebrew University of Jerusalem
Jansson , J. The Regents of the University of California, Berkeley CA
Lindow, S.E. The Regents of the University of California, Berkeley CA
Green, S.J. The Board of Trustees of the Uni. of Illinois, Chicago IL

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Abstract

Research objectives:

1. Identify genetic potential and community structure of soil and rhizosphere microbial community structure as affected by treated wastewater (TWW) irrigation. This objective was achieved through the examination soil and rhizosphere microbial communities of plants irrigated with fresh water (FW) and TWW. Genomic DNA extracted from soil and rhizosphere samples (Minz laboratory) was processed for DNA-based shotgun metagenome sequencing (Green laboratory). High-throughput bioinformatics was performed to compare both taxonomic and functional gene (and pathway) differences between sample types (treatment and location).

2. Identify metabolic pathways induced or repressed by TWW irrigation. To accomplish this objective, shotgun metatranscriptome (RNA-based) sequencing was performed. Expressed genes and pathways were compared to identify significantly differentially expressed features between rhizosphere communities of plants irrigated with FW and TWW.

3. Identify microbial gene functions and pathways affected by TWW irrigation*. To accomplish this objective, we will perform a metaproteome comparison between rhizosphere communities of plants irrigated with FW and TWW and selected soil microbial activities.

4. Integration and evaluation of microbial community function in relation to its structure and genetic potential, and to infer the in situ physiology and function of microbial communities in soil and rhizosphere under FW and TWW irrigation regimes. This objective is ongoing due to the need for extensive bioinformatics analysis. As a result of the capabilities of the new PI, we have also been characterizing the transcriptome of the plant roots as affected by the TWW irrigation and comparing the function of the plants to that of the microbiome.

*This original objective was not achieved in the course of this study due to technical issues, especially the need to replace the American PIs during the project. However, the fact we were able to analyze more than one plant system as a result of the abilities of the new American PI strengthened the power of the conclusions derived from studies for the 1st and 2nd objectives.

Background: As the world population grows, more urban waste is discharged to the environment, and fresh water sources are being polluted. Developing and industrial countries are increasing the use of wastewater and treated wastewater (TWW) for agriculture practice, thus turning the waste product into a valuable resource. Wastewater supplies a year-round reliable source of nutrient-rich water. Despite continuing enhancements in TWW quality, TWW irrigation can still result in unexplained and undesirable effects on crops. In part, these undesirable effects may be attributed to, among other factors, to the effects of TWW on the plant microbiome. Previous studies, including our own, have presented the TWW effect on soil microbial activity and community composition. To the best of our knowledge, however, no comprehensive study yet has been conducted on the microbial population associated...
with plant roots irrigated with TWW – a critical information gap. In this work, we characterize the effect of TWW irrigation on root-associated microbial community structure and function by using the most innovative tools available in analyzing bacterial community - a combination of microbial marker gene amplicon sequencing, microbial shotgun metagenomics (DNA-based total community and gene content characterization), microbial metatranscriptomics (RNA-based total community and gene content characterization), and plant host transcriptome response.

At the core of this research, a mesocosm experiment was conducted to study and characterize the effect of TWW irrigation on tomato and lettuce plants. A focus of this study was on the plant roots, their associated microbial communities, and on the functional activities of plant root-associated microbial communities. We have found that TWW irrigation changes both the soil and root microbial community composition, and that the shift in the plant root microbiome associated with different irrigation was as significant as the changes caused by the plant host or soil type.

The change in microbial community structure was accompanied by changes in the microbial community-wide functional potential (i.e., gene content of the entire microbial community, as determined through shotgun metagenome sequencing). The relative abundance of many genes was significantly different in TWW irrigated root microbiome relative to FW-irrigated root microbial communities. For example, the relative abundance of genes encoding for transporters increased in TWW-irrigated roots increased relative to FW-irrigated roots. Similarly, the relative abundance of genes linked to potassium efflux, respiratory systems and nitrogen metabolism were elevated in TWW irrigated roots when compared to FW-irrigated roots. The increased relative abundance of denitrifying genes in TWW systems relative FW systems, suggests that TWW-irrigated roots are more anaerobic compare to FW irrigated root. These gene functional data are consistent with geochemical measurements made from these systems. Specifically, the TWW irrigated soils had higher pH, total organic compound (TOC), sodium, potassium and electric conductivity values in comparison to FW soils. Thus, the root microbiome genetic functional potential can be correlated with pH, TOC and EC values and these factors must take part in the shaping the root microbiome. The expressed functions, as found by the metatranscriptome analysis, revealed many genes that increase in TWW-irrigated plant root microbial population relative to those in the FW-irrigated plants. The most substantial (and significant) were sodium-proton antiporters and Na(+)‐translocating NADH-quinone oxidoreductase (NQR). The latter protein uses the cell respiratory machinery to harness redox force and convert the energy for efflux of sodium.

As the roots and their microbiomes are exposed to the same environmental conditions, it was previously hypothesized that understanding the soil and rhizosphere microbiome response will shed light on natural processes in these niches. This study demonstrate how newly available tools can better define complex processes and their downstream consequences, such as irrigation with water from different qualities, and to identify primary cues sensed by the plant host irrigated with TWW.

From an agricultural perspective, many common practices are complicated processes with many ‘moving parts’, and are hard to characterize and predict. Multiple edaphic and microbial factors are involved, and these can react to many environmental cues. These complex systems are in turn affected by plant growth and exudation, and associated features such as irrigation, fertilization and use of pesticides. However, the
combination of shotgun metagenomics, microbial shotgun metatranscriptomics, plant transcriptomics, and physical measurement of soil characteristics provides a mechanism for integrating data from highly complex agricultural systems to eventually provide for plant physiological response prediction and monitoring.
Contribution of the collaboration:

This project successfully joined the research teams of Dr. Dror Minz (Israel: Volcani Center) with that of Dr. Stefan J. Green (US: University of Illinois at Chicago, UIC). The Israeli partners carried out the field experiments, sampling, and DNA and RNA extractions. Nucleic acid samples were sent to Dr. Green’s laboratory, where all the library preparation and sequencing were performed, as well as part of the bioinformatics efforts, including high-throughput annotation and tabulation of the raw sequence data. Dr. Green's lab has extensive experience in high-throughput next-generation sequencing and access to state-of-the-art sequencing instrumentation. Dr. Green is also familiar with innovative sequencing techniques and has extensive experience in plant-soil microbial community characterization. Dr. Noa Sela has been instrumental in guiding some of the downstream bioinformatics analysis, and serves as an important link between the Israeli and US partners. Finally, the collaboration with Prof. Hadar has been essential for both project planning and data analysis.
**Achievements:**

*Experiments procedure:* Tomato and lettuce were grown in lysimeters for two consecutive summers (2014 and 2015, respectively). The lysimeters were irrigated with either FW or tertiary TWW during the experiment but were actually irrigated similarly for eight summer seasons prior to these experiments. Two soil types were used for this experiment: Nir Oz (NO), a loamy sand soil, and Ein-Hashlosha (EH), sandy loam soil. From the two soil, two plant and two irrigation treatment, a total of 24 samples were collected. At the end of each irrigation season, soil, plant and roots were sampled. DNA and RNA were extracted from the soil and root samples, while plant leaves and soil were also chemically examined. The bacterial community composition was evaluated initially by microbial small subunit (16S) ribosomal RNA (rRNA) gene amplicon sequencing. We also analyzed the genetic potential of the bacterial population by sequencing 25-50 million sequences per sample (paired-end, correspond to total 300 GB of data). We then assembled the reads and predicted 4.3 million genes. The actual functions, as expressed by the bacterial community (metatranscriptomic), were described by sequencing 50-65 million transcribed sequences per sample. Those results are summarized at the unpublished data section. The first manuscript is in preparation and about to be submitted, and will summarize the community composition results (total community and that of the active community) in roots and soils. The second manuscript, summarizing the community potential and active functions of the microbial community in TWW irrigated roots, is currently being processed.
Changes to original research plan:

Metaproteome comparison between rhizosphere communities of plants irrigated with FW and TWW was not achieved in the course of this study due to technical issues, especially the need to replace the American PIs during the project. However, the fact we were able to analyze more than one plant system as a result of the abilities of the new American PI strengthened the power of the conclusions derived from studies for the 1st and 2nd objectives. As a result of the capabilities of the new PI, we have also been able to characterize the transcriptome of the plant roots as affected by the TWW irrigation and comparing the function of the plants to that of the microbiome. This was not a part of the original plan and is an important aspect of the system analysis.
### Publications for Project US-4662-13

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Appendix

Unpublished data:

Both soil type and irrigation changed significantly the lettuce (two-way ANOVA- F=42.12, p-value= 0.0029, F=20.17, p- value=0.0065, for soil type and irrigation, respectively) and tomato (F=12.31 p-value=0.017, F=6.659 p-value=0.0289 for stem and leaves, and F=5.687, p-value= 0.0397, F= 18.1, p-value=0.008 for plant fruit weight) yield (Fig. 1), with yield decrease under TWW irrigation regimes.

We examined the effects of TWW irrigation on rhizosphere and root surface (rhizoplane) microbial communities composition from resident and active microbial population. Microbial community structure was significantly different by plant type, total (DNA-based) and active (RNA-based) assay, by niche (i.e., rhizosphere and rhizoplane) and by treatment type (i.e., FW and TW) (Fig. 2). Plant host and irrigation type were the main factors shaping the rhizoplane (ANOSIM R statistic 0.653, with p value<0.0001, and R statistic 0.595 and p value < 0.0001 for plant and irrigation respectively) and rhizosphere (ANOSIM R statistic 0.252, with p value=0.0015, and R statistic 0.448 and p value < 0.0001 for plant and irrigation respectively) bacterial community, while soil-type played role only for the rhizosphere microbiome (ANOSIM R statistic 0.5088, with p value<0.0001). TWW irrigation resulted in a similar number of significantly affected OTUs, compared to plants at the rhizoplane, and soil in the rhizosphere. The active rhizosphere population were less affected by the soil type, and more affected by the plant host. Importantly, the TWW effect was as prominent as those factors for both rhizosphere and rhizoplane (Fig. 3). The most obvious change in microbial community structure was that Actinobacteria relative abundance decreased, while Gamaproteobacteria population increased on TWW irrigated roots (Fig. 4).

We examined functional gene content from shotgun metagenome sequence data, and identified those genes enriched by the microbial community in rhizoplane of TWW irrigated plants (lettuce and tomato), grown in EH soil.

Clustering genes into orthologous groups revealed significant changes between microbial population from TWW and FW irrigated plants (Fig. 5). A higher number of gene orthologue groups were found to be associated with TWW irrigation. The main gene categories affected by the TWW irrigation in both plant systems belonged to membrane transporters, especially sodium efflux membrane protein genes, respiration, and nitrogen metabolism. The increase in the relative abundance of genes putative involved in sodium efflux membrane may indicate an osmotic stress on the microbial community at the root surface in TWW systems.

In addition, an increase in relative abundance of genes involved in denitrification and nitrogen fixation was observed in TWW-irrigated roots of both plant types across the two years of this experiment. An increase in relative abundance of genes from both of these processes strongly suggests that this environment experienced low oxygen levels compared to FW irrigated roots, and this may be an indicated of elevated biological oxygen demand in TWW.

To correlate the microbial community potential function to plant environment, we measured several plant and soil parameters. Under TWW irrigation, soil pH decreased, total organic carbon (TOC), potassium and sodium concentration - as well as the general electric conductivity (EC), increased dramatically. No significant trend related to ammonium and nitrate concentration was seen. We found significant correlation between the rhizoplane gene pool and soil pH, TOC and EC (Fig. 6). These edaphic variables are suspected to participate, either directly or indirectly, in the processes that shape the root microbial community structure and function.
The root surface microbial functions, as revealed from the metatranscriptome analysis, also varied between roots irrigated with TWW and FW. Multiple genes were found, belong to many cellular processes, with significantly higher gene expression in plant systems treated with either TWW or FW. The most substantially enriched (and significant) in TWW systems were sodium protons antiporters and Na(+) - translocating NADH-quinone oxidoreductases (NQR). The NQR protein uses the cell respiratory machinery to harness redox force and convert the energy for efflux of sodium. These expression patterns strongly suggest that osmotic stress is a major concern for microorganisms at the plant root surface during TWW and not FW irrigation.

Currently, we are also analyzing the plant transcriptome, to identify links between host gene expression patterns and environmental variables, and microbial gene expression patterns. This part was an addition to the objectives of the original proposal, and was only possible as a result of the addition of Dr. Green to the project. The ability to compare the TWW effects on the plant gene expression in addition to its microbiome adds an additional power for our understanding of the environmental impact of TWW irrigation.

**Continuing work and expected publications**

Currently we are finalizing the analysis of the metatranscriptome data and conducting a bioinformatic analysis of the plant root transcriptome as affected by TWW irrigation. We are submitting a manuscript on the community composition (total community and that of the active community) in roots and soils of the lyzimeter experiments. The manuscript is to be submitted by the end of January. In addition, we are preparing a manuscript that will combine the metagenome, metatranscriptome and plant transcriptome.
Figure 1: Plant yield decreased under TWW irrigation. Plant yield presented for FW (blue) and TWW (red) irrigated plants. (A) Tomato yield was measured as average fruit weight (gr) per plant, and as total shoot weight for lettuce (B).

NO: Nir- Oz sandy loam soil. EH: Ein Hashlosha- loamy sand soil.
Figure 2: Effect of plant species, water irrigation condition, proximity to root and microbial activity on microbial community structure. Non-metric multi-dimensional scaling (nMDS) plots based on Bray-Curtis dissimilarity of microbial operational taxonomic units (OTU; clustered at 97% sequence similarity) of total (DNA-based) rhizoplane communities (A), active (RNA-based) rhizoplane communities (B), total rhizosphere communities (C) and active rhizosphere communities (D). Microbial community composition was analyzed by 16S rRNA or 16S RNA gene amplicon sequencing (RNA was reverse transcribed to cDNA prior to targeted 16S rRNA amplification and sequencing). The red symbols represent microbial communities associated with tomato samples and green symbols represent microbial communities associated with lettuce samples. Triangles represent communities derived from plants in NO soil samples while circles represent communities derived from plants grown in EH soil. Filled symbols represent TWW-irrigated samples, and empty symbols represent FW-irrigated samples.
Figure 3: The significance of plant, soil and irrigation effect on plant rhizoplane: from the 16S rRNA amplicon sequencing: percentage of OTUs that change significantly ($\alpha=0.05$, Kruskal-Wallis non-parametric test, the P value was corrected by the Benjamini-Hochberg FDR procedure for multiple comparisons) at each environmental parameters, for the resident community. The plant green colored column represent OTU significantly increased at lettuce samples, compare to red- OTU significantly increased in tomato samples. Soil brown color- for EH samples, yellow- NO samples. Irrigation- red for TWW, blue- FW samples.
**Figure 4:** Relative abundance of rhizoplane bacterial groups (class level from the 16S rRNA survey). The red color represents tomato samples and the green lettuce. Triangles mark NO soil samples, circles EH soil. Filled symbols for TWW irrigated samples and empty symbols for FW irrigated samples.
**Figure 5: Microbial community functional gene content.** Microbial communities were characterized through shotgun metagenome sequencing and high-throughput gene annotation. Each horizontal row represents a single gene category. The categories are grouped to higher hierarchical clusters by similar function (colored bars from the left, and their definition in the chart legend). The heatmap colors mark the category normalized gene abundance (z-score; red is higher while blue is lower relative abundance). Genes were annotated and grouped to functional categories by the SEED database. The samples are coded at the bottom of the figure by closed and open colored circles. Red circles represent tomato samples and green circles represent lettuce. All samples were from the EH soil lysimeters. Filled symbols represent TWW-irrigated samples and empty symbols represent FW-irrigated samples.
Figure 6: Canonical correspondence analysis (CCA) to correlate environmental parameters and gene functionality. Three environmental parameters (measured from the soil samples) were significantly correlated to the gene abundance pattern (as defined by the metagenomics analysis). Those parameters are represented by a vector in a bi-plot CCA ordination. Red symbols represent tomato samples; Green symbols represent lettuce samples. All samples were from the EH soil lysimeters. Filled symbols represent TWW-irrigated samples, and open symbols represent FW-irrigated samples.