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Conventional wastewater treatment and reuse site practices modify bacterial community structure but do not eliminate some opportunistic pathogens in reclaimed water

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Abstract

Water recycling continues to expand across the United States, from areas that have access to advanced, potable-level treated reclaimed water, to those having access only to reclaimed water treated at conventional municipal wastewater treatment plants. This expansion makes it important to further characterize the microbial quality of these conventionally-treated water sources. Therefore, we used 16S rRNA gene sequencing to characterize total bacterial communities present in differentially-treated wastewater and reclaimed water (n=67 samples) from four U.S. wastewater treatment plants and one associated spray irrigation site conducting on-site ultraviolet treatment and open-air storage. The number of observed operational taxonomic units was significantly lower ($p < 0.01$) in effluent, compared to influent, after conventional treatment. Effluent community structure was influenced more by treatment method than by influent

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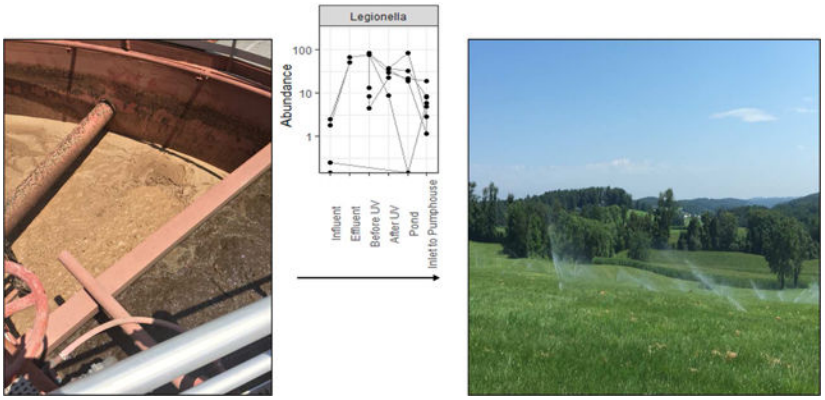
community structure. The abundance of *Legionella* spp. increased as treatment progressed in one treatment plant that performed chlorination and in another that seasonally chlorinated. Overall, the alpha-diversity of bacterial communities in reclaimed water decreased ($p < 0.01$) during wastewater treatment and spray irrigation site ultraviolet treatment ($p < 0.01$), but increased ($p < 0.01$) after open-air storage at the spray irrigation site. The abundance of *Legionella* spp. was higher at the sprinkler system pumphouse at the spray irrigation site than in the influent from the treatment plant supplying the site. *Legionella pneumophila* was detected in conventionally treated effluent samples and in samples collected after ultraviolet treatment at the spray irrigation site, while *Legionella feeleii* persisted throughout on-site treatment at the spray irrigation site, and, along with *Mycobacterium gordonae,* was also detected at the sprinkler system pumphouse at the spray irrigation site. These data could inform the development of future treatment technologies and reuse guidelines that address a broader assemblage of the bacterial community of reclaimed water, resulting in reuse practices that may be more protective of public health.

Graphical Abstract



Keywords

16S rRNA gene sequencing; opportunistic pathogens; wastewater treatment; reclaimed water; spray irrigation; public health

1. Introduction

Reclaimed water use is rapidly expanding in the United States (Asano, 2007; United States Environmental Protection Agency (EPA), 2012), from historically high-use areas such as California—where water users have access to treated wastewater that has undergone chlorination, dual-media filtration, coagulation and flocculation (California Department of Public Health (CA DPH), 2009)—to areas that may only have access to reclaimed water released from conventional wastewater treatment plants (WWTPs). Since the U.S. currently has no legally-binding federal regulations governing reclaimed water use, regulations vary from state to state (EPA, 2012). Moreover, not all states specify the exact type of processes required in order to obtain the proper level of treatment mandated within their particular regional guidelines or regulations. Even though most state regulations focus on the

microbiological quality of wastewater treatment plant effluent EPA, 2012), not all states require reuse site monitoring and reporting (Asano, 2007).

Most regulations and guidelines regarding bacterial pathogens in wastewater and reclaimed water are based on the use of indicator microorganisms (e.g. *E. coli* and *Enterococci*) (EPA, 2012), as well as research utilizing culture-based methods analyzing single species of bacteria in nutrient rich environments (Marcus et al., 2013; Sheikh et al., 1990). However, these approaches do not provide a comprehensive analysis of microbial water quality since indicator microorganisms have been shown to be poorly correlated with the presence of pathogens in reclaimed water (Harwood et al., 2005; Jjemba et al., 2010), and pathogens exist as members of complex microbial communities (Marcus et al., 2013). These microbial communities within wastewater and reclaimed water may be impacted by wastewater treatment processes, operational parameters, organic and inorganic wastewater constituents, and water reuse site practices.

Although most state regulations require the use of chlorine residuals in reclaimed water distribution systems, declines in the microbiological quality of reclaimed water by the time it reaches reuse sites have been previously documented (Jjemba et al., 2010). Opportunistic pathogens (e.g. *Aeromonas* spp., *Mycobacterium* spp. and *Legionella* spp.) have been observed to regrow in disinfected reclaimed water distribution systems due to biofilm development (Narasimhan et al., 2005) and disinfectant dissipation (Jjemba et al., 2010), and have also been detected more often than routinely tested indicator microorganisms (Jjemba et al., 2010). Thus, in order to provide a comprehensive analysis of the potential public health impacts associated with the use of reclaimed water originating from conventional wastewater treatment plants, it is important to characterize the impact of conventional treatment and reuse site practices on total bacterial communities present in water throughout the treatment train and at reuse sites.

In this study, we used 16S rRNA gene sequencing to explore the total bacterial community structure of differentially treated wastewater from four conventional wastewater treatment plants that provide treated effluent for reuse in two distinct geographic regions (the U.S. Mid-Atlantic and Midwest) with differing treatment and reuse regulations (Asano, 2007; EPA, 2012). We also analyzed samples from a spray irrigation site that performs on-site ultraviolet (UV) treatment and open-air storage of treated effluent that it receives from one of the aforementioned wastewater treatment plants. By advancing current knowledge of bacterial community structure of conventionally treated wastewater and resulting reclaimed water, our findings provide insights into wastewater treatment processes and reuse site practices that may be necessary in order to protect public health.

2. Material and methods

2.1. Sampling Sites

Wastewater and reclaimed water samples (n=67) were collected from four wastewater treatment plants previously described as Mid-Atlantic WWTP1(Rosenberg Goldstein et al., 2012), Mid-Atlantic WWTP2, Midwest WWTP1 and Midwest WWTP2 (Rosenberg Goldstein et al., 2012) and a landscape spray irrigation site, previously described as Mid-

Atlantic SI1 (Carey et al., 2016) that receives treated effluent from Mid-Atlantic WWTP1. All sites were chosen based on the willingness of the site operator to participate. A brief description of the treatment steps at each sampling site (Carey et al., 2016; Rosenberg Goldstein et al., 2012) is included in Table 1. A detailed description of the treatment processes utilized at each of the four WWTPs and the spray irrigation site is provided as a part of the Supplementary Material.

2.2. Sample Collection

Grab samples were collected throughout the treatment process at all wastewater treatment plants and the Mid-Atlantic SI1. Timing of sampling events was dependent on the availability of the wastewater treatment plant and spray irrigation site managers. Sampling location schematics have been described previously (Carey et al., 2016; Rosenberg Goldstein et al., 2012). Sterile one-liter polyethylene Nalgene® Wide Mouth Environmental Sampling Bottles (Nalgene, Lima, OH, USA) were used to collect samples which were transported to the laboratory at 4 °C and stored at −80 °C until additional funding could be secured to complete filtrations and DNA extractions. A total of 67 samples were included in this analysis: 37 wastewater treatment plant samples and 30 spray irrigation site samples. The wastewater treatment plant samples included 11 from Mid-Atlantic WWTP1, seven from Mid-Atlantic WWTP2, 10 from Midwest WWTP1 and nine from Midwest WWTP2. There were 11 influent, four activated sludge, two post aeration, six secondary clarifier, four lagoon (cell B), and 10 effluent samples included. The 30 samples from Mid-Atlantic SI1 included seven collected before UV treatment, eight collected after UV treatment, seven recovered from the inlet to the open-air storage pond, and eight recovered from the inlet to the pumphouse that supplied the spray irrigation heads.

2.3. DNA Extraction

Samples were thawed completely and 500 mL of each sample was vacuum filtered through a 0.2 μm, 47mm hydrophilic polyethersulfone (PES) filter (Pall Corporation, Port Washington, NY, USA). Molecular biology grade water (MoBio Laboratories, Carlsbad, CA, USA) was similarly filtered to serve as a negative control. Total genomic DNA was extracted from the filters by adapting previously published procedures (Jackson et al., 2014; Zupancic et al., 2012) utilizing both enzymatic as well as mechanical lyses. Briefly, each filter was aseptically placed in a sample lysis tube (Lysing Matrix B) (MP Biomedicals, Solon, OH, USA) followed by the addition of ice-cold molecular biology grade 1X Phosphate Buffered Saline (PBS) (Gibco-Life Technologies, Grand Island, NY, USA), lysozyme from chicken egg white (10mg/mL, Sigma-Aldrich, St. Louis, MO, USA), lysostaphin from *Staphylococcus staphylolyticus* (5mg/mL Sigma-Aldrich, St. Louis, MO, USA) and mutanolysin from *Streptomyces globisporus* ATCC 21553 (1mg/ml Sigma-Aldrich, St. Louis, MO, USA) and incubation at 37°C for 30 minutes. A second enzymatic lysis step followed, with the addition of Proteinase K (20mg/mL, Invitrogen-Life Technologies, Grand Island, NY, USA) and 10% (w/v) sodium dodecyl sulfate (SDS) (BioRad, Hercules, CA, USA) and incubation at 55 °C for 45 minutes. The samples were then mechanically lysed at 6.0 m/s for 40 seconds using the FastPrep®−24 benchtop homogenizer (MP Biomedicals, Irvine, CA, USA). DNA purification was achieved using the QIAmp DSP DNA mini kit 50, v2 (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol, followed by

additional purification using sodium acetate. DNA quality was assessed using a NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and gel electrophoresis.

2.4. 16S rRNA Gene Amplification and Sequencing

Polymerase chain reaction (PCR) amplification of the V3-V4 hypervariable region of the 16S rRNA gene was achieved, using previously published procedures (Caporaso et al., 2012; Fadrosh et al., 2014; Sellitto et al., 2012) and universal primers, 319F and 806R. Unique 12 base pair (bp) sequence tags were included with the 806R primer to barcode each sample and allow for multiplexing samples in a single Illumina MiSeq (Illumina, San Diego, CA, USA) run (Fadrosh et al., 2014). PCR amplification was performed using Phusion High-Fidelity DNA polymerase and mastermix (Thermo Fisher Scientific, Waltham, MA, USA) along with 20 mg/mL additional bovine serum albumin (BSA) (to overcome PCR inhibition) (Sigma-Aldrich St. Louis, MO, USA) in a DNA Engine Tetrad 2 thermal cycler (Bio-Rad, Hercules, CA, USA). The cycling parameters were as follows: 30 s at 98°C, followed by 30 cycles of 10 s at 98°C, 15 s at 66°C and 15 s at 72°C and a final step of 5 min at 72°C. Negative controls excluding templates were also processed per primer pair. Amplicon presence was confirmed using gel electrophoresis and quantified using a KAPA library quantification kit (KAPA Biosystems, Wilmington, MA, USA). Equimolar (25 ng) PCR amplicons, from each sample, were mixed in a single tube and amplification primers and reaction buffers were removed using the AMPure kit (Agencourt Biosciences, Beverly, MA, USA). Amplicons were pooled and sequenced according to the manufacturer's protocol using the Illumina MiSeq (Illumina, San Diego, CA, USA). Sequence data generated in this study were deposited with GenBank and linked to BioProject number PRJNA415141 in the NCBI BioProject database.

2.5. Analysis Pipeline and Data Normalization

The analysis pipeline used was similar to a previously published method (Pop et al., 2016). The multiplexed 16S rRNA reads were screened for low quality base calls and insufficient raw read lengths. Paired-end sequences were assembled using Paired-End Assembler for DNA sequences (PANDAseq) (Masella et al., 2012) and resulting high-quality consensus sequences were de-multiplexed, trimmed of artificial barcodes and 5' and 3' primer regions followed by *de novo* clustering into operational taxonomic units (OTUs) using DNAclust (Ghodsi et al., 2011) to 99% identity. Taxonomic annotation was performed using the Ribosomal Database Project (RDP) (Cole et al., 2014) (rdp.cme.msu.edu, release 10.4) database. OTUs without a match to the RDP database and with > 97% identity by the Basic Local Alignment Search Tool (BLAST) (Madden, 2003), were assigned an OTU identifier. Chimeras were identified and filtered using Perseus/UCHIME (Edgar et al., 2011). Phylogenetic placement (Nguyen et al., 2014) was used to obtain high confidence taxonomic assignment for *Legionella* and *Mycobacterium* sequences using the Ribosomal Database Project (RDP) 16S rRNA database (Cole et al., 2014).

The number of observed sequences compared to the estimated coverage can be seen in Figure S1. Sufficient sequencing depth was obtained and samples containing fewer than 100

sequences were excluded from downstream analysis (Figure S1). Data were normalized with cumulative sum scaling (CSS) using metagenomeSeq (Paulson et al. 2013).

2.6. Statistical Analysis

Normalized data were used to determine the observed number of OTUs and to estimate the Shannon Index (Shannon and Weaver, 1948) and Simpson's Diversity Index (Simpson, 1949) using R statistical software, version 3.3.0 (R Core Team, 2017) using packages phyloseq, version 1.16.2 (McMurdie and Holmes, 2013) and vegan, version 2.3.5 (Dixon, 2003). The Kruskal-Wallis test was used to evaluate differences in alpha-diversity estimates. Paired t-tests were used to evaluate differences in alpha-diversity estimates across same-day influent-effluent sample pairs. Beta diversity was estimated using Bray-Curtis dissimilarity (Bray and Curtis, 1957) and compared using analysis of similarities (ANOSIM) on the normalized data with 999 permutations. Pairwise differences were calculated using Tukey's test. CSS normalized data were used when estimating differential abundance across samples using metagenomeSeq, version 1.14.2 (Paulson et al., 2013). All visualizations were performed using ggplot2, version 2.1.0 (Wickham, 2009). For the comparison of differential abundance, OTUs present in fewer than half of the samples with counts at least equal to 1 were excluded from the analysis to reduce potential biases in the statistical test due to sparsity (high frequency of unobserved OTUs). In the case of comparison of differential abundance specifically across treatment processes, OTUs present in less than half the samples were excluded from analysis. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Sequencing

After quality control, a total of 6.14×10^6 sequences were obtained from a total of 67 samples, with 107,748 ± 96,514 sequences per sample (mean ± standard deviation). Ten samples were computationally removed from the analysis due to low coverage, all of which had less than 100 sequences after quality control. A total of 1,494 unique assigned-species OTUs were identified and 339 unique unassigned-species OTUs were identified. Figure S1 illustrates the estimated coverage using the Good's coverage metric (Hsieh et al., 2016).

3.2. Differences among Influent Samples from all Wastewater Treatment Plants

No statistically significant differences were detected in the observed number of OTUs, Simpson's index and Shannon index estimates across influent samples from all four wastewater treatment plants (Figure S2). Significantly different abundance (p-value < 0.01) among the top ten genera observed across influent samples from all four wastewater treatment plants can be seen in Figure 1.

The genera *Bifidobacterium*, *Blautia*, *Eubacterium*, *Faecalibacterium*, *Lachnoclostridium*, *Lactococcus* and *Streptococcus* occurred at the highest abundance at Mid-Atlantic WWTP1. The genus *Clostridium* was detected at the highest abundance at Mid-Atlantic WWTP2 and the genus *Paracoccus* was detected at the highest abundance at Midwest WWTP2.

3.3. Differences between Same-day Influent-effluent Pairs from all Wastewater Treatment Plants

Alpha diversity, measured by the Shannon and Simpson indices, as well as observed number of OTUs, was significantly higher in influent samples compared to effluent samples (p-value < 0.01) (Figure 2). Significant differences in abundance of bacterial genera across same-day influent-effluent sample pairs from all four wastewater treatment plants were observed (Figure 3). The bacteria with the most significant differences in abundance in the influent samples compared to the effluent samples belonged to genera predominantly associated with the human microbiome (Human Microbiome Project Consortium, 2012; Vandewalle et al., 2012) and sewer infrastructure, such as *Bifidobacterium*, *Propionibacterium*, *Streptococcus* and *Trichococcus*. Except for one collection date, the abundance of the genus *Mycobacterium* was higher in the effluent samples compared to the influent samples (Figure 3).

3.4. Differences Across Wastewater Treatment Processes

When analyzed collectively, influent samples from all four wastewater treatment plants formed a distinct collective group separate from all other samples collected from downstream treatment processes (Figure 4; ANOSIM statistic R: 0.5632, p-value < 0.01). Figures 5A through 5D illustrate the differentially abundant genera detected in varying treatment process samples from Mid-Atlantic WWTP1 (Figure 5A), Mid-Atlantic WWTP2 (Figure 5B), Midwest WWTP1 (Figure 5C) and Midwest WWTP2 (Figure 5D).

The abundance of *Legionella* spp. was higher in effluent, compared to influent samples, at both Mid-Atlantic WWTP1 (Figure 5A) and Mid-Atlantic WWTP2 (Figure 5B). The abundance of *Mycobacterium* spp. was higher in influent, compared to effluent, samples at MidAtlantic WWTP1 (Figure 5A), and higher in effluent, compared to influent, samples at Midwest WWTP2 (Figure 5D).

With respect to other genera, *Chryseobacterium*, *Clostridium*, *Flavobacterium*, *Janthinobacterium*, *Pedobacter, Pseudomonas* and *Sphingomonas* occurred at the highest abundance in influent samples, while *Massilia* spp. and *Deinococcus* spp. were detected at the highest abundance in the secondary clarifier samples, and *Halomonas* was the only genus detected at a higher abundance in the effluent compared to the influent at Midwest WWTP1 (Figure 5C). *Pseudomonas* was detected at similar abundance between influent and effluent samples at Midwest WWTP2 (Figure 5D).

3.5. Potentially Pathogenic Species of *Legionella* and *Mycobacterium*

Potentially pathogenic species belonging to the genus *Legionella* and genus *Mycobacterium,* with species classifications at > 96% confidence, *Legionella pneumophila* (subsp. *pneumophila* (strain Philadelphia 1) (*L. pneumophila*), *Legionella feeleii* (*L. feeleii*) and *Legionella tusconensis* (*L. tusconensis*), *Mycobacterium brisbanens* (*M. brisbanens*), *Mycobacterium phocaicum* (*M. phocaicum*), *Mycobacterium ilatzerense* (*M. ilatzerense*), *Mycobacterium gordonae* (*M. gordonae*) and *Mycobacterium terrae* (*M. terrae*), were detected in samples collected from three of the four WWTPs.

At Mid-Atlantic WWTP1, *M. brisbanens* was detected in three influent samples and *M. phocaicum* and *M. terrae* in one influent sample. *L. pneumophila*, *L. feeleii* and *M. brisbanens* were detected in one activated sludge sample. *L. pneumophila*, *L. tusconensis, M. brisbanens* and *M. phocaicum* were detected in one secondary clarifier sample and *L. feeleii* in two secondary clarifier samples. *L. pneumophila* and *L. feeleii* were detected in all effluent samples, while *L. tusconensis, M. brisbanens* and *M. phocaicum* were detected in one effluent sample.

At Mid-Atlantic WWTP2, *M. brisbanens*, *M. phocaicum* and *M. terrae* were detected in two influent samples and *M. ilatzerense* was detected in one influent sample. *L. feeleii* was detected in two secondary clarifier samples.

At Midwest WWTP1, *M. brisbanens* and *M. ilatzerense* were detected in one influent sample and *M. phocaicum* and *M. terrae* were detected in two influent samples. *M. brisbanens* and *M. gordonae* were detected in one secondary clarifier sample. *M. brisbanens*, *M. gordonae* and *M. ilatzerense* were detected in two effluent samples, while *M. phocaicum*, *M. terrae* and *L. feeleii* were detected in one effluent sample.

At Midwest WWTP2, no potentially pathogenic species belonging to either genus *Legionella* or genus *Mycobacterium* were detected from any of the samples collected.

3.6. Differential Abundance Across Reuse Site Stages

Figure 6 shows the differences in observed number of OTUs and the Shannon index and Simpson's index estimates within samples across stages from wastewater treatment plant influent from Mid-Atlantic WWTP1 through to the inlet to the pumphouse at the tested spray irrigation site, Mid-Atlantic SI1. Alpha-diversity decreased after wastewater treatment and after UV treatment and increased after open-air storage. Statistically significant (p-value < 0.01) differences were observed for Shannon index and observed OTU number estimates across all samples analyzed at the spray irrigation site. Furthermore, significant differences (p-value < 0.01) between observed OTU number estimates were found between influent and "after UV" and influent and "pond" samples.

Figure 7 illustrates that samples taken from the inlet to the pumphouse clustered apart from all other samples collected at the spray irrigation site (ANOSIM statistic R: 0.5802 p-value < 0.01). Reclaimed water reaches the inlet to the pumphouse after undergoing on-site UV treatment and being stored in an open-air pond.

Figure 8 illustrates the differentially abundant genera across influent and effluent samples (from Mid-Atlantic WWTP1) and spray irrigation site samples (from Mid-Atlantic SI1) before and after on-site UV treatment and storage. The abundance of *Clostridium* spp., *Legionella* spp. *and Streptococcus* spp. was lower, and that of *Mycobacterium* spp. was higher, in the "before UV" samples compared to the effluent samples. The "before UV" samples were the first samples collected at the spray irrigation site after treated effluent reaches it. The reclaimed water then underwent UV treatment and the abundance of *Clostridium* spp., *Legionella* spp., *Mycobacterium* spp. and *Streptococcus* spp. was higher in the "after UV" samples compared to the "before UV" samples. After UV treatment, the

reclaimed water was stored in an open-air storage pond before being pumped to the sprinkler system via a pumphouse. The abundance of *Clostridium* spp., *Legionella* spp. *and Mycobacterium* spp. was higher, and that of *Streptococcus* spp. was lower, in the samples collected after UV treatment compared to those collected in the pond. The abundance of *Legionella* spp., *Mycobacterium* spp. and *Streptococcus* spp. was higher, and that of *Clostridium* spp. was lower, in samples collected in the pond compared to samples collected at the inlet to the pumphouse. *Clostridium* spp., *Legionella* spp., *Mycobacterium* spp. and *Streptococcus* spp. were more abundant in samples collected after UV treatment compared to those collected at the inlet to the pumphouse. The abundance of *Legionella* spp. was higher, and that of *Clostridium* spp., *Mycobacterium* spp. and *Streptococcus* spp. lower, in samples collected at the inlet to the pumphouse compared to samples collected from the influent from the wastewater treatment plant. However, when comparing samples collected from the wastewater treatment plant effluent to those collected from the inlet to the pumphouse, the abundance of *Legionella* spp., *Mycobacterium* spp. and *Streptococcus* spp. was higher while that of *Clostridium* spp. was lower.

Potentially pathogenic species belonging to the genera *Legionella* and *Mycobacterium*, with species classifications at > 96% confidence, *Legionella pneumophila* (subsp. *pneumophila* (strain Philadelphia 1) (*L. pneumophila*), *Legionella cherrii* (*L. cherrii*), *Legionella feeleii* (*L. feeleii*) and *Legionella tusconensis* (*L. tusconensis*), *Mycobacterium brisbanens* (*M. brisbanens*), *Mycobacterium phocaicum* (*M. phocaicum*) and *Mycobacterium gordonae* (*M. gordonae*), were detected in different sample types collected at the spray irrigation site. Specifically, *L. pneumophila*, *L. tusconensis*, *M. gordonae* and *M. phocaicum* were detected in one sample collected before UV treatment, and *L. feeleii* was detected in two samples collected before UV treatment. *L. pneumophila* was detected in one sample collected after UV treatment, and *L. feeleii* and *M. gordonae* in two samples collected after UV treatment. *L. pneumophila* and *L. feeleii* were detected in one sample collected from the pond. *L. cherrii*, *M. brisbanens* and *M. gordonae* were detected in one sample collected from the inlet to the pumphouse and *L. feeleii* from four samples collected from the inlet to the pumphouse.

4. Discussion

In this study, we completed total bacterial community analyses on conventionally treated municipal wastewater and reclaimed water. Influents from all wastewater treatment plants clustered together (Figure 4) indicating structural similarities in the bacterial communities of raw sewage entering wastewater treatment plants despite differences in catchment area characteristics. Influent bacterial community structure has been shown to be influenced by the human microbiome and sewer infrastructure (Human Microbiome Project Consortium, 2012; Vandewalle et al., 2012). However, in the case of all wastewater treatment plants included in the study, the effluent samples showed structural similarity with the treatment processes used at the various wastewater treatment plants, rather than the influent samples (Figure 4), demonstrating that as the wastewater treatment process progressed, changes in bacterial community structure were most likely influenced by the microorganisms used for secondary, biological treatment of wastewater (e.g. activated sludge) and operational parameters of the wastewater treatment plants.

Our data also showed that the bacterial community structure of reclaimed water was also influenced by downstream reuse site practices. At the tested spray irrigation site, reclaimed water samples from the inlet to the pumphouse supplying the sprinkler system clustered together (Figure 7), away from the samples collected after on-site UV treatment, and those collected from the location where UV-treated water entered the open-air storage pond. These results indicate that storage in the open-air pond contributed to significant changes in bacterial community composition of reclaimed water that ultimately comes in contact with individuals working or recreating at this landscape irrigation site. At this site, potentially pathogenic species of the genus *Legionella* and the genus *Mycobacterium* were detected in samples collected after UV treatment, in the pond where UV treated reclaimed water was stored to supply the pumphouse as well as at the inlet to the pumphouse supplying the sprinkler system. Some of these species have been implicated in infections caused by the inhalation of aerosols, namely *L. pneumophila* (Allegra et al., 2016), *L. feeleii* (Centers for Disease Control and Prevention (CDC), 2017; Herwaldt et al., 1984) and *M. gordonae* (Thomson et al., 2013).

4.1. Mid-Atlantic WWTP1

At Mid-Atlantic WWTP1, the genera that were significantly differentially abundant across treatment processes followed a trend that is to be expected in a wastewater treatment plant. Bacteria belonging to genera that are more closely associated with the human microbiome such as *Bifidobacterium*, *Blautia*, and *Streptococcus* (Adamberg et al., 2015) were more abundant in the influent samples, with their abundance decreasing after biological treatment and clarification. Genera that are known to degrade decaying organic material (*Clostridium*) (Gerardi, 2006), those that are known to inhabit aquatic habitats (*Legionella* and *Mycobacterium*) (Kumar, 2003; Steinert et al., 2002) and those that are known to thrive in saline environments (*Halomonas*) (Gerardi, 2006) had higher abundance in secondary biological treatment samples. Since salinity is usually high at all stages within a wastewater treatment plant, it is not surprising that the abundance of *Halomonas* spp. continued to increase along the treatment train with higher abundance in effluent samples compared to influent samples. The abundance of *Legionella* spp. increased along with treatment process steps indicating some influence of conventional wastewater treatment on the growth of *Legionella* spp. in a wastewater treatment plant. Furthermore, potentially pathogenic species of genus *Legionella* were not detected in influent samples but were detected in activated sludge, secondary clarifier and effluent samples. *Legionella* spp. are known to grow in activated sludge systems, within the protozoans *Acanthamoeba*, *Hartmannella* and *Naegleria* that are present in activated sludge, as well as in aerated ponds in the presence of oxygen (Caicedo et al., 2016).

4.2. Mid-Atlantic WWTP2

The differential abundance trends observed for Mid-Atlantic WWTP2 may have been influenced by the fact that only one sampling event was carried out at this treatment plant and sampling occurred in the fall (NOAA., 2013). The genera that were detected at the highest abundance in influent samples from Mid-Atlantic WWTP2 are common inhabitants of raw sewage and sewer infrastructure, namely, *Acinetobacter* (Vandewalle et al., 2012), *Streptococcus* (Gerardi, 2006), *Pseudomonas* (McLellan et al., 2010). Among these, the

genera *Acinetobacter*, *Pseudomonas* and *Streptococcus* contain potentially pathogenic species. The genus *Legionella* was detected at a higher abundance in effluent samples compared to influent samples. *Legionella* spp. are ubiquitous in aquatic environments (Steinert et al., 2002), and as noted above, *Legionella* spp. are known to grow in protozoans present in activated sludge systems and in aerated ponds (Caicedo et al., 2016).

4.3. Midwest WWTP1

The genera with a high abundance in the influent samples from Midwest WWTP1 (*Chryseobacterium*, *Clostridium*, *Janthinobacterium* and *Pseudomonas*) are often isolated from human (Grice et al., 2008) samples. Many more potentially pathogenic species of the genus *Mycobacterium* were isolated from samples collected from this WWTP compared to potentially pathogenic species of the genus *Legionella*.

4.4. Midwest WWTP2

At Midwest WWTP2, the influence of secondary biological (sequencing batch reactor) and tertiary (serial lagooning) treatment was evident in differences in abundance of various genera in the effluent samples compared to the influent samples. At this WWTP, the abundance of the genus *Mycobacterium* increased as biological treatment progressed with influent samples having the lowest abundance and effluent samples the highest. Bacteria belonging to the genus *Mycobacterium* are commonly detected in wastewater treatment plant effluent, especially in wastewater treatment plants using biological treatment (Cai et al., 2014; Cai and Zhang, 2013; Kaevska et al., 2016). *Sporosarcina*, which was detected at the highest abundance in lagoon cell B samples, has been shown to be highly abundant in environmental samples containing urine (Garrity, 2009). The abundance of *Leifsonia* was higher in the effluent compared to the influent, which may be significant considering that this genus contains species that are pathogenic to plants, including Bermuda grass (*Cynodon dactylon*), a popular variety of lawn grass (Monteiro-Vitorello et al., 2013). This WWTP did perform biological treatment but did not conduct any subsequent filtration or chlorination, and yet, no potentially pathogenic species of the genus *Legionella* or the genus *Mycobacterium* were detected in effluent samples collected at this WWTP. The combination of a sequencing batch reactor, followed by serial lagooning, seemed to be very effective in removing these pathogen-containing genera throughout the treatment train; however, the specific mechanisms of action contributing to these removals deserve further study.

4.5. Mid-Atlantic SI1

Conventional wastewater treatment at Mid-Atlantic WWTP1 resulted in a decrease in the abundance of *Mycobacterium* spp. and an increase in that of *Legionella* spp. Transport of treated effluent from Mid-Atlantic WWTP1 to the landscape spray irrigation site, Mid-Atlantic SI1, resulted in an increase in the abundance of *Mycobacterium* spp. and a decrease in that of *Legionella* spp. On-site UV treatment at the spray irrigation site resulted in an increase in the abundance of both *Mycobacterium* spp. and *Legionella* spp., and storage in an open-air pond resulted in decreases of both genera. However, the abundance of *Legionella* spp. detected at the inlet to the pumphouse where, after open-air storage, the reclaimed water is pumped to the sprinkler system, was higher than that detected in influent samples from the supplying wastewater treatment plant (Mid-Atlantic WWTP1). Jjemba et

al. (2010) demonstrated the regrowth of *Mycobacterium* spp. and *Legionella* spp. in effluent reservoirs and reclaimed water distribution systems due to reductions in chlorine residual (Jjemba et al., 2010). *Legionella* spp. are also known to survive in biofilms (Jjemba et al., 2010), which were not sampled in this study, but are often present in reclaimed water distribution systems (Narasimhan et al., 2005). Both *Legionella* spp. and *Mycobacterium* spp. have been known to resist UV treatment at the wavelength (254nm) used by Mid-Atlantic SI1 (Bohrerova and Linden, 2006; Linden and Sobsey, 2005; Liu et al., 1995). Furthermore, potentially pathogenic species of the genus *Legionella* and the genus *Mycobacterium* that are important with respect to aerosolization, and subsequent inhalation exposure (*L. pneumophila*, *L. feeleii* and *M. gordonae*) (Allegra et al., 2016; Centers for Disease Control and Prevention (CDC), 2017; Thomson et al., 2013), were detected in the samples collected from this spray irrigation site even after on-site UV treatment. *L. feeleii*, in particular, was detected in samples collected from all stages at the spray irrigation site including the inlet to the pumphouse supplying the sprinkler system. Exposure to aerosolized water droplets containing *L. feeleii* has resulted in the development of Pontiac fever and pneumonia (Lee et al., 2009). These findings have potential human health implications related to both occupational and recreational exposures that may occur at this type of spray irrigation site.

4.6. Assessment of Conventional Wastewater Treatment and Reclaimed Water in the U.S.

In the U.S., quality of treated effluent, including water destined for reuse, is assessed for the presence of pathogens using surrogate measures. Assessment is performed, most often, by testing for indicator bacteria, such as total or fecal coliforms, in grab samples during and after treatment (Harwood et al., 2005). These indicator bacteria have been shown to be inadequate surrogates for the presence of pathogens (Harwood et al., 2005). For example, despite reductions observed among indicator bacteria after several conventional wastewater treatment configurations, opportunistic pathogens like *Mycobacterium* spp. and *Legionella* spp. have been observed to regrow within reclaimed water distribution systems to concentrations higher than those of indicator bacteria (enterococci, coliforms, and *E. coli*) (Jjemba et al., 2010). At the inlet to the pumphouse supplying the sprinklers at the spray irrigation site analyzed in this study, the abundance of *Legionella* spp. was higher than that in influent from the conventional wastewater treatment plant supplying the spray irrigation site. Furthermore, within this conventional wastewater treatment plant the abundance of *Legionella* spp. was seen to increase as the treatment progressed with higher abundance detected in effluent compared to influent. UV treatment used at the spray irrigation site did not result in the reduction of either *Legionella* spp. or *Mycobacterium* spp. *L. pneumophila* was detected in conventionally treated effluent samples and in samples collected after UV treatment at the spray irrigation site. *L. feeleii* persisted from treatment plant effluent stage through to the sprinkler system pumphouse at the spray irrigation site. Both organisms have been implicated in respiratory illnesses associated with inhalation of aerosolized water containing these organisms (Centers for Disease Control and Prevention (CDC), 2017; Correia et al., 2016; Herwaldt et al., 1984). Our findings provide evidence that conventional wastewater treatment processes and the reuse site practices studied here may not effectively prevent all members of the microbial community, including opportunistic bacterial pathogens, from potentially re-growing in reclaimed water or from forming biofilms. The

study results can thus be used to inform the development of new wastewater treatment strategies and water reuse guidelines that consider the microbial community as a whole.

4.7. Limitations

Our study provides a comprehensive examination of the total bacterial diversity of wastewater and reclaimed water samples collected across multiple wastewater treatment plants in two regions. However, there were some limitations mostly related to unequal sampling from the four wastewater treatment plants and from the various treatment processes within the plants. The grab sampling method and unbalanced sampling pattern across wastewater treatment plants was due to limited access to the tested plants. Since we were only able to sample Mid-Atlantic WWTP2 during one sampling event, the findings from that plant may not be representative of the total bacterial diversity of the plant over time, limiting comparisons with other plants. Furthermore, effluent samples had fewer sequencing reads compared to influent samples, due to lower DNA extraction yields, which may have biased our estimates. In addition, the observed differences in the same-day pairs may have been heavily influenced by the Mid-Atlantic WWTP1 and Midwest WWTP2 samples, the wastewater treatment plants with the most complete pairs of same-day samples available for analysis. Finally, because we used a DNA-based sequencing approach to assess total bacterial diversity across the tested water samples, we were not able to delineate which proportion of detected bacteria represents active, viable bacterial communities. Recently, Carini et al. (2016) used a photoreactive DNA intercalating dye, propidium monoazide, to estimate levels of “relic DNA” in soil samples, showing that up to 40% of detected DNA was “relic DNA”, representing inactive bacteria (Carini et al., 2016). Further studies in our group are ongoing that combine sequencing methods with labeling approaches, teasing out the active/viable proportions of the bacterial microbiota of reclaimed water samples.

5. Conclusions

In summary, potentially pathogenic species of the genus *Legionella* and the genus *Mycobacterium*, some of which are significant with respect to occupational exposures via inhalation and ingestion routes, were detected in effluent samples from three of the four wastewater treatment plants included in the study, as well as in reclaimed water samples recovered at the spray irrigation site. At the spray irrigation site, UV treatment seemed to reduce the abundance of potential pathogens overall, but potentially pathogenic species were detected in reclaimed water collected from the inlet to the pumphouse supplying the sprinkler system after having undergone UV treatment and open-air storage. These findings could inform the development of future reclaimed water treatment technologies and reuse guidelines that address a broader assemblage of the bacterial community of reclaimed water (in addition to indicator bacteria), resulting in reuse practices that may be more protective of public health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

rRNA	ribosomal Ribonucleic Acid
WWTP	Wastewater Treatment Plant
UV	Ultraviolet
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis

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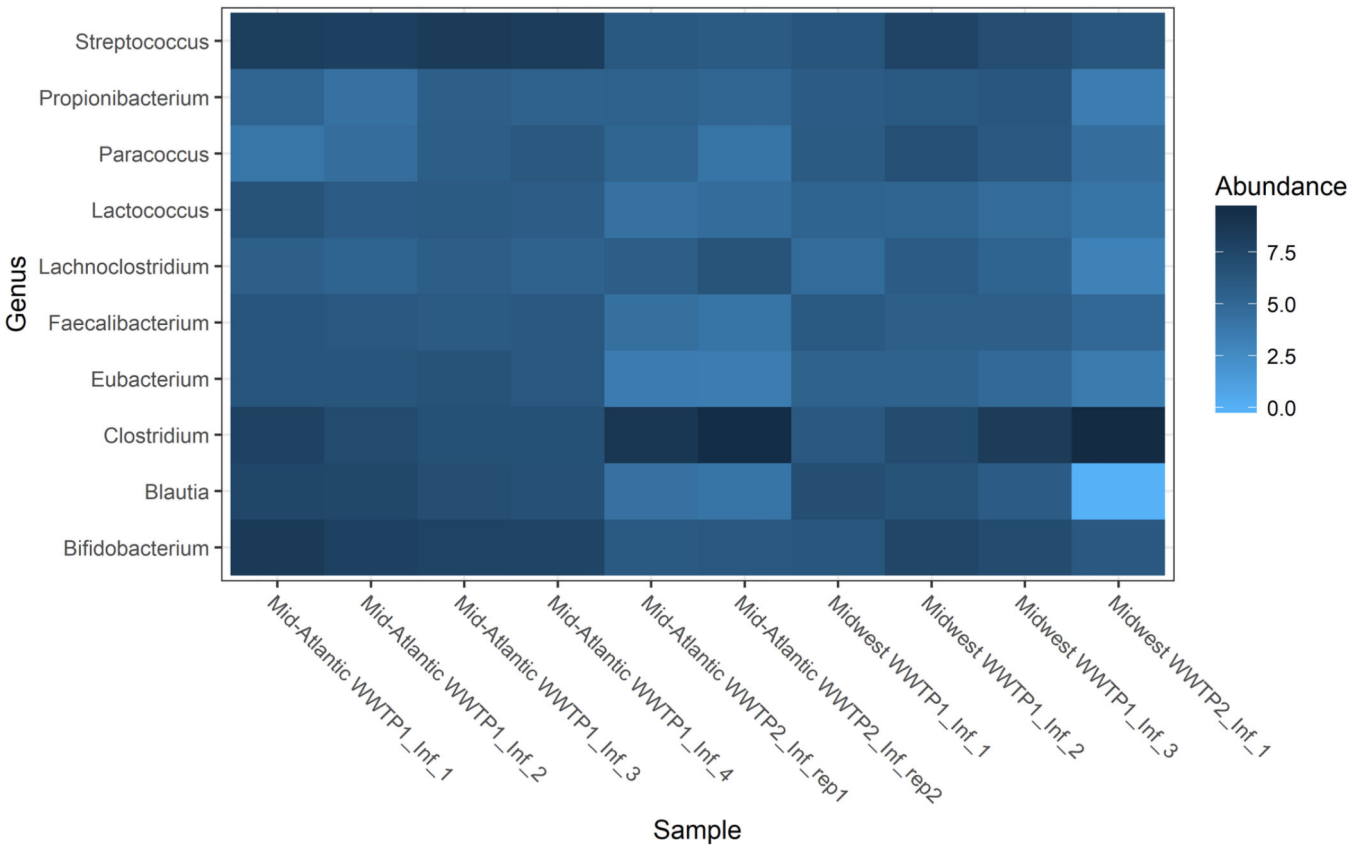

Figure 1.

Heatmap showing genera with significant ($p < 0.01$) differences in abundance between influent samples collected from each of the four wastewater treatment plants. Genera that were highly abundant have been depicted in darker shades of blue while those that were less abundant are depicted in lighter shades of blue.

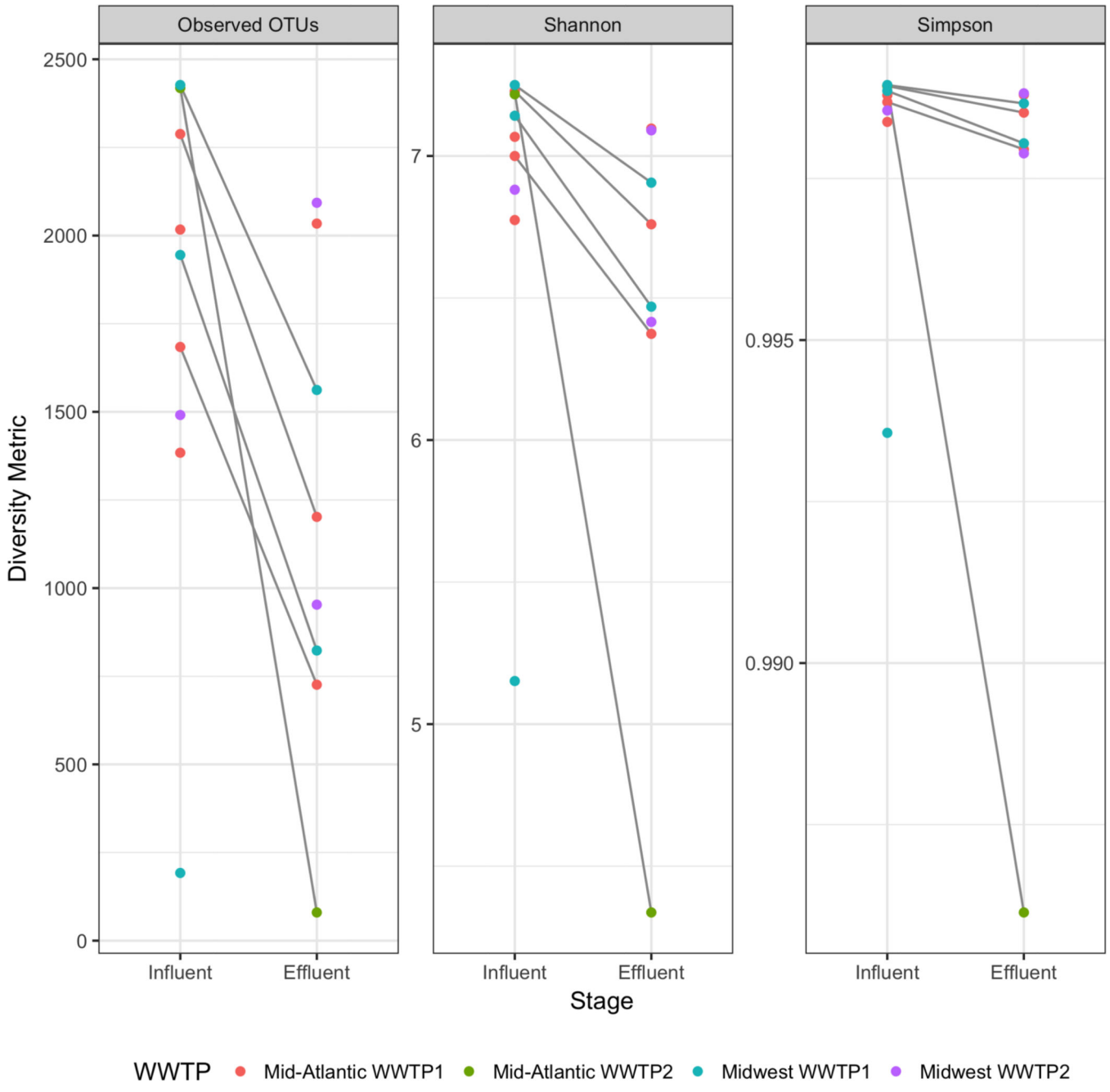

Figure 2.

Alpha-diversity estimates and observed number of operational taxonomic units (OTUs) in same-day influent-effluent pairs from all four wastewater treatment plants. Observed number of OTUs was significantly ($p < 0.01$) higher in influent samples compared to effluent samples.

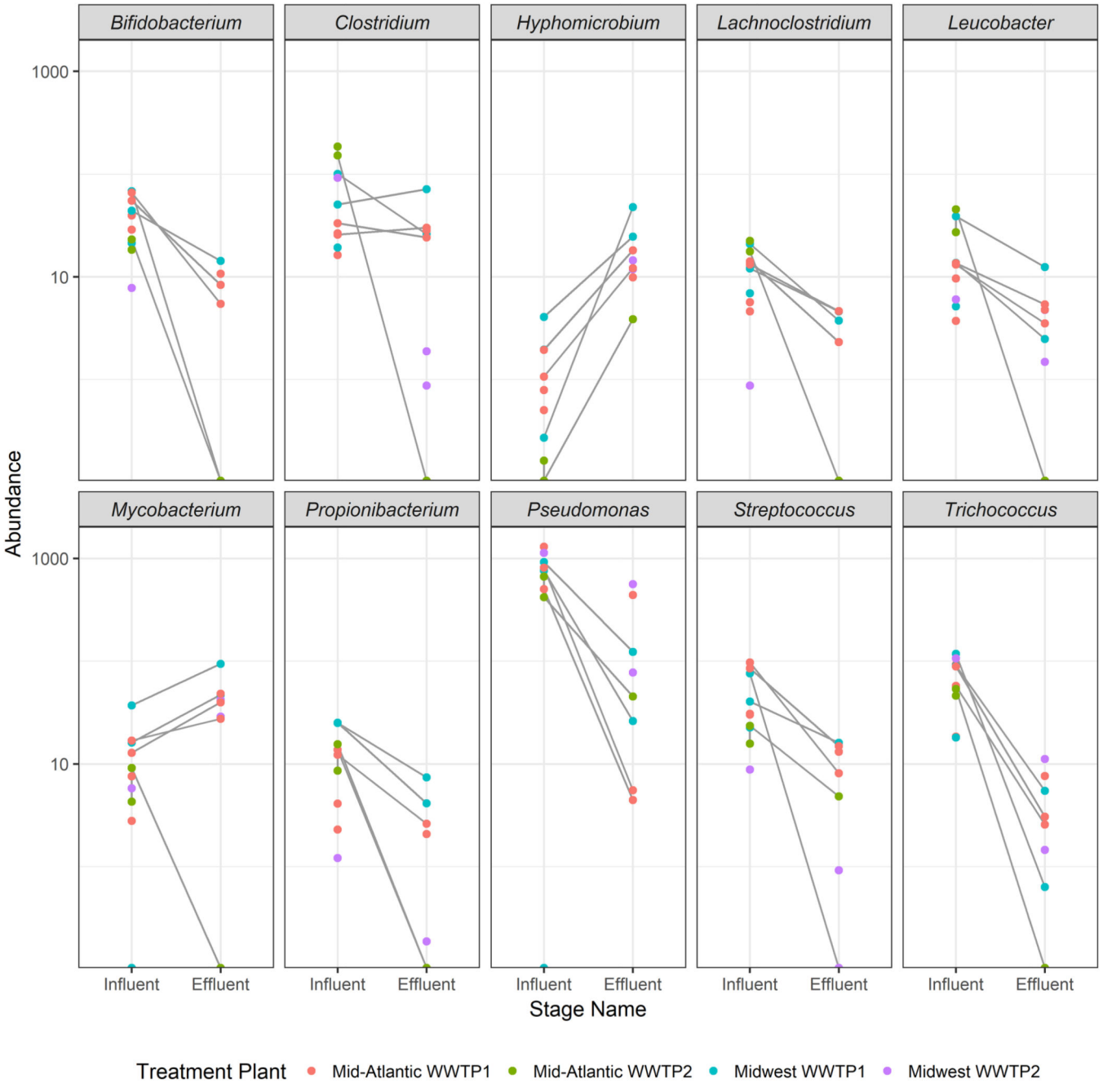

Figure 3.

Significant differentially abundant ($p < 0.01$) bacterial genera across same-day influent-effluent pairs from all four wastewater treatment plants. The cumulative sum scaling (CSS) normalized counts are depicted on the *y* axis (Abundance) and the corresponding sample type on the *x* axis. Grey lines link influent-effluent samples collected on the same day. Except for one collection date, the relative abundance of *Mycobacterium* was higher in effluent samples compared to influent samples.

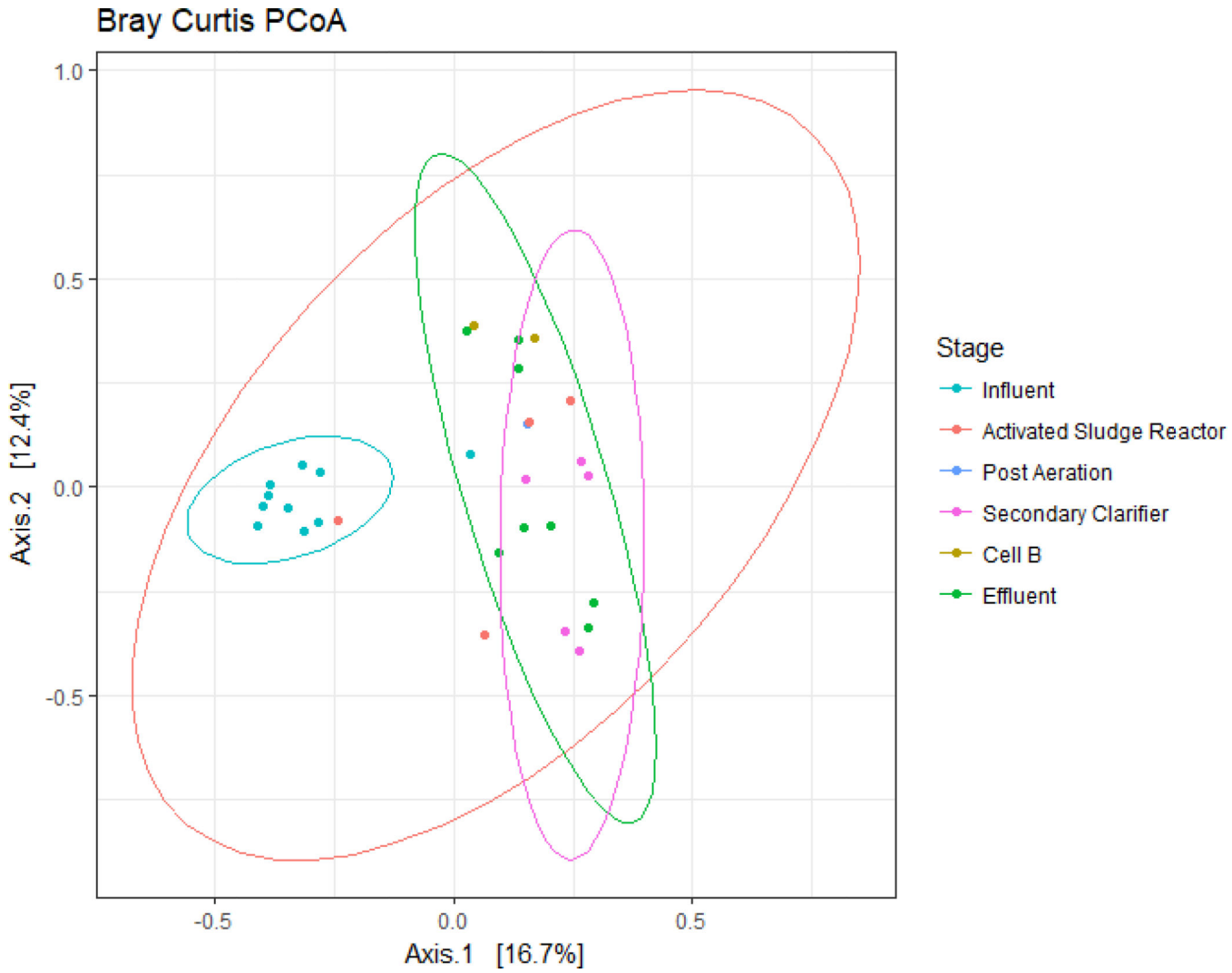

Figure 4.

First two coordinates of the Principal Coordinates Analysis (PCoA) using Bray-Curtis dissimilarity, showing influent samples clustering apart (ANOSIM statistic R: 0.5632, $p < 0.01$) from samples collected from downstream wastewater treatment processes. Axis labels include the percent of total variance in the data explained by the coordinate.

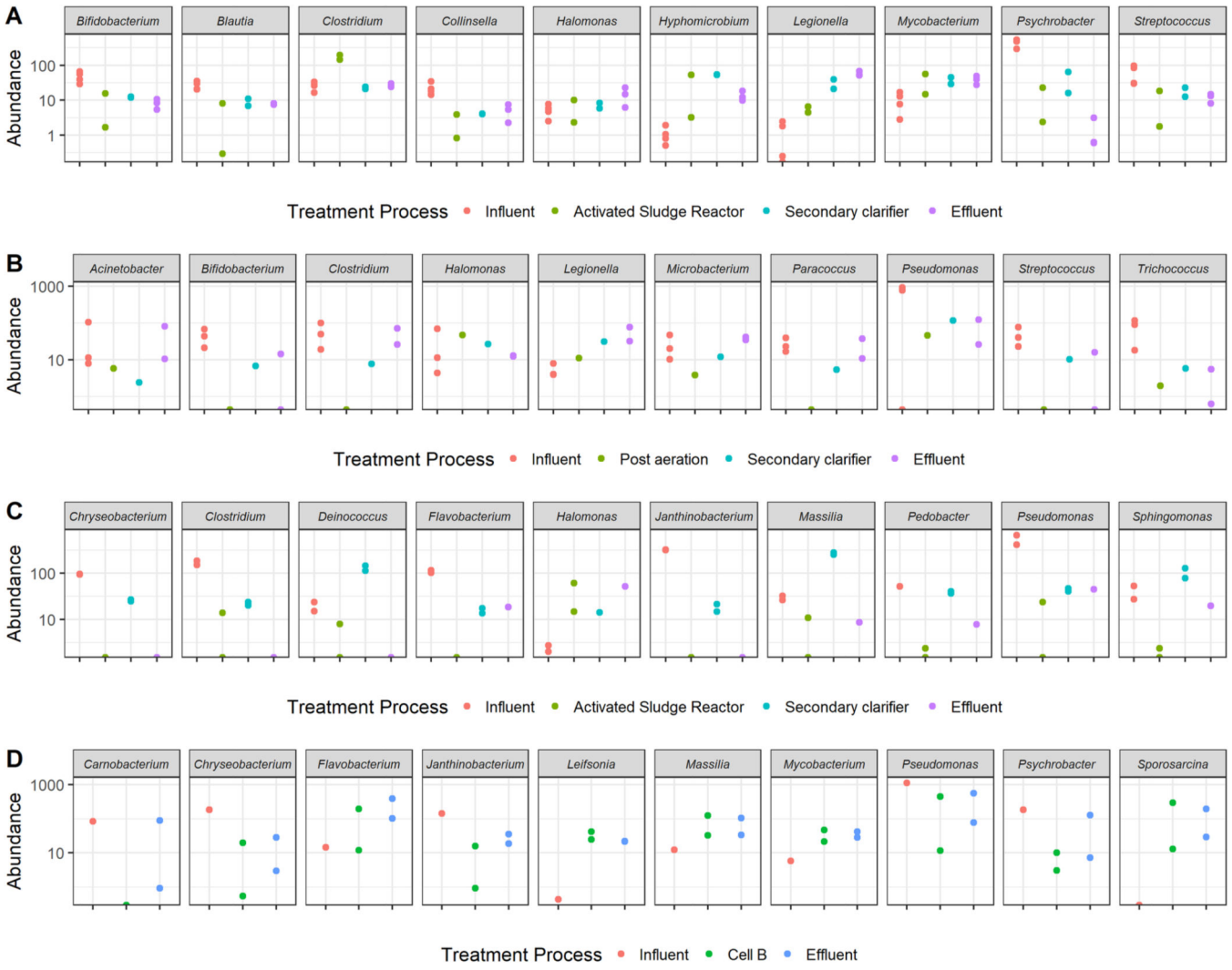

Figure 5.

A – D Significant differentially abundant ($p < 0.01$) bacterial genera (top 10) across the various treatment processes performed at Mid-Atlantic WWTP1 (5A), Mid-Atlantic WWTP2 (5B), Midwest WWTP1 (5C) and Midwest WWTP2 (5D). For each WWTP, the cumulative sum scaling (CSS) normalized counts are depicted on the *y* axis (Abundance) and the corresponding treatment process on the *x* axis.

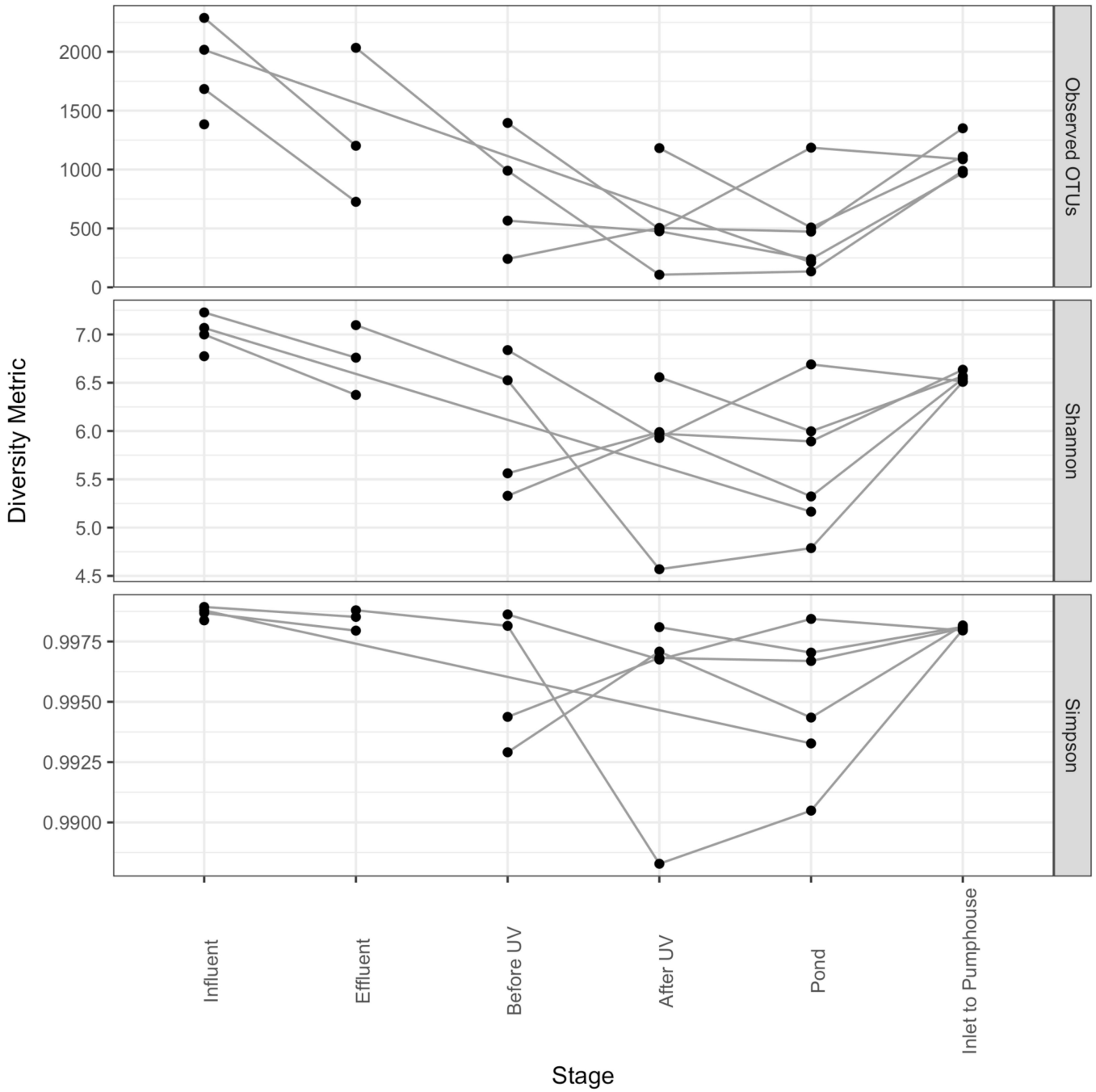

Figure 6.

Alpha-diversity estimates and observed number of OTUs in samples from the influent stage at Mid-Atlantic WWTP1 to the inlet to the pumphouse stage at Mid-Atlantic SI1. Significant differences in alpha-diversity estimates were found for Shannon index ($p < 0.01$) and observed number of OTUs ($p < 0.01$) with alpha-diversity decreasing with wastewater plant treatment and ultraviolet treatment at the spray irrigation site, but increasing after storage in an open-air pond at the spray irrigation site.

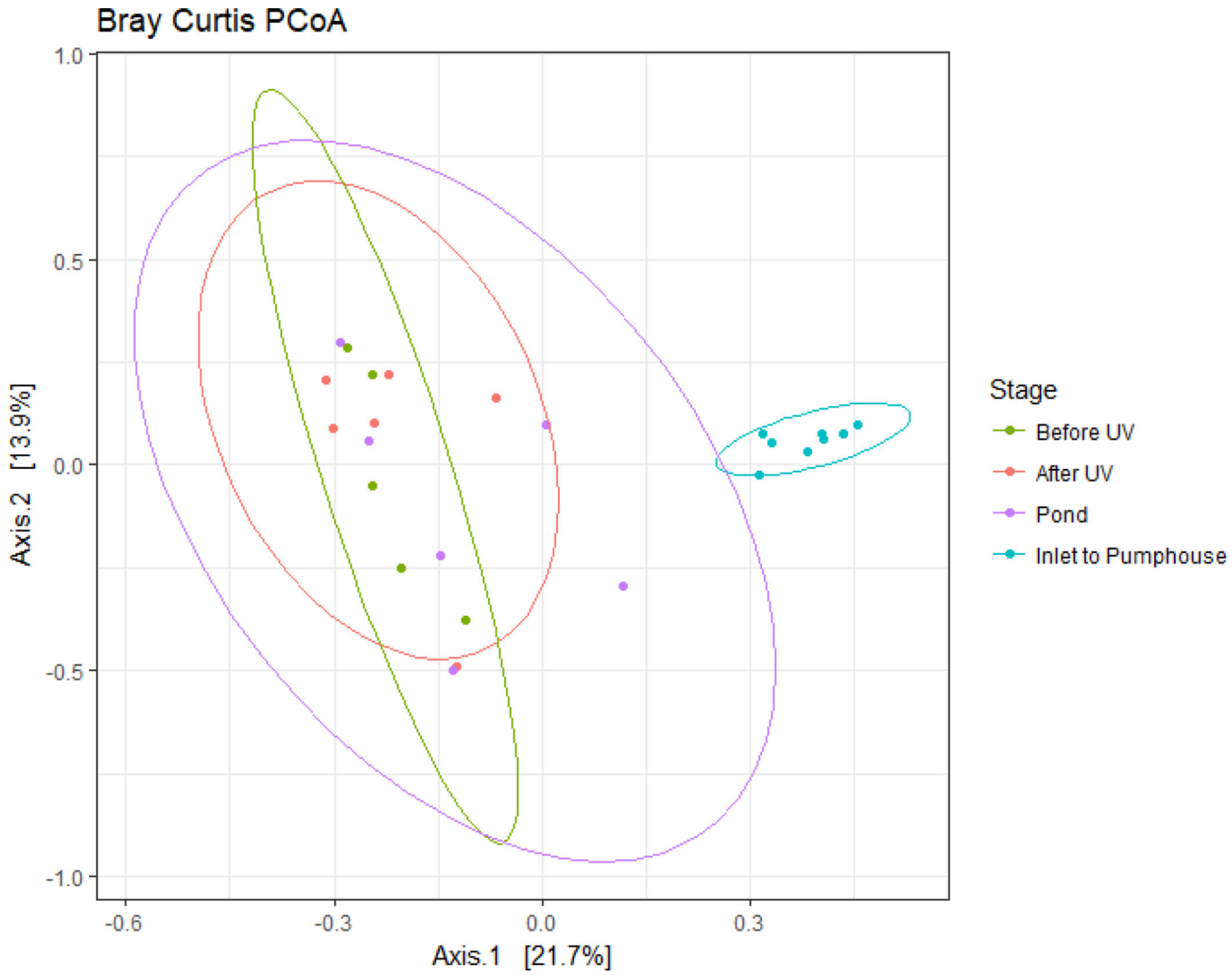

Figure 7.

First two coordinates of the Principal Coordinate Analysis (PCoA) using Bray-Curtis dissimilarity showing samples from the inlet to the pumphouse clustering apart (ANOSIM statistic R: 0.5802 $p < 0.01$) from samples after on-site ultraviolet treatment ("before UV" and "after UV") and on-site open-air storage ("pond"). Axis labels include the percent of the total variance in the data explained by the coordinate.

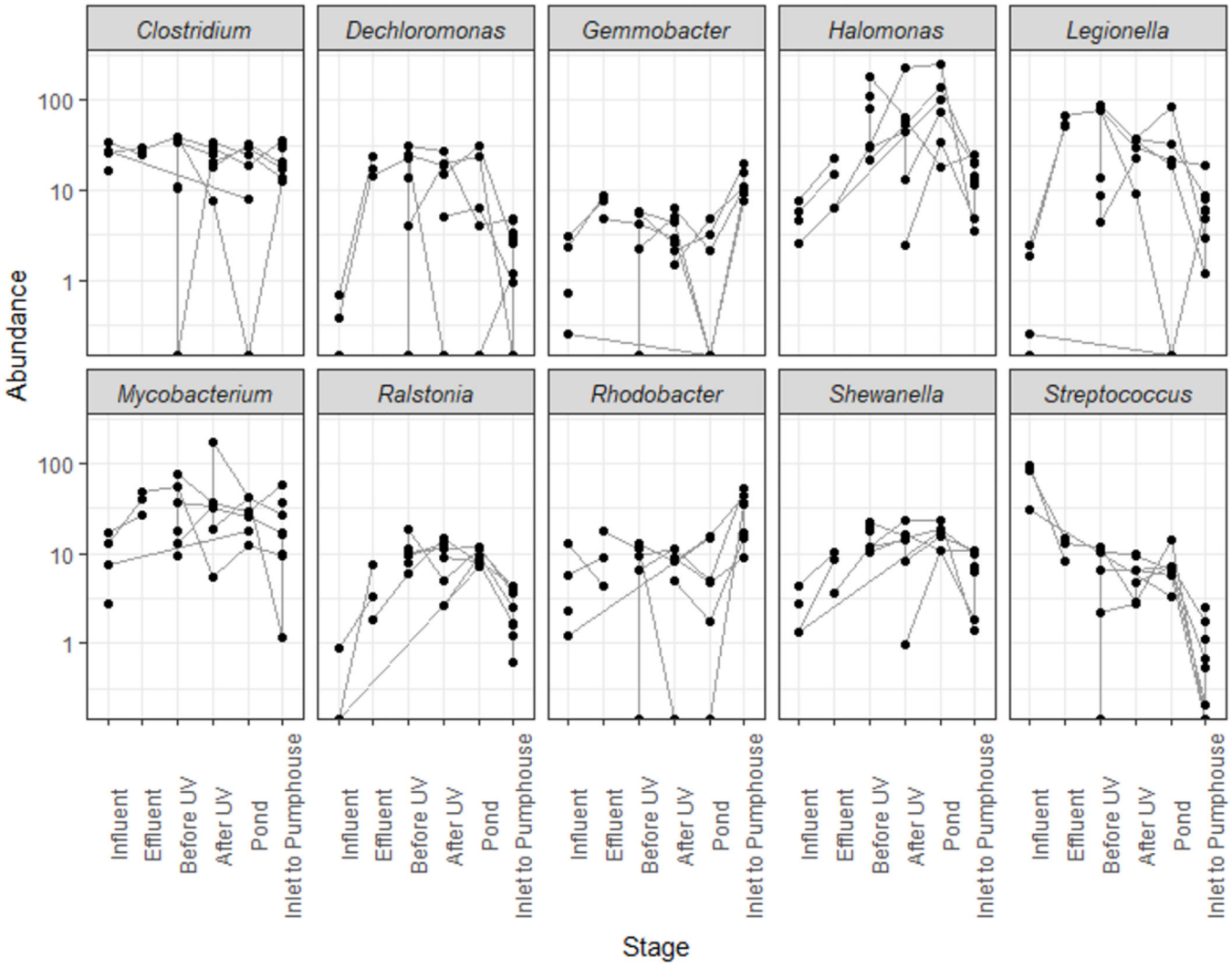

Figure 8.

Significant differentially abundant ($p < 0.01$) bacterial genera (top 10) from treatment at Mid-Atlantic WWTP1 through transport to, treatment and storage at Mid-Atlantic SI1. The cumulative sum scaling (CSS) normalized counts are depicted on the *y* axis (Abundance) and the corresponding stage on the *x* axis.

Table 1

A brief description of the treatment steps at each sampling site

Site (WWTP)[a]	Preliminary Treatment	Primary Treatment	Secondary Treatment	Tertiary Treatment	Discharge
Mid-Atlantic WWTP1	Screens	Primary clarifier	Activated sludge reactor, Secondary clarifier	Rapid sand filters, Chlorination	De-chlorination and surface water discharge with a portion of the effluent diverted to Mid-Atlantic SI1 for use in landscape irrigation
Mid-Atlantic WWTP2	Screens	Primary clarifier	Primary aeration tank, Secondary aeration tank, Secondary clarifier	Multimedia filter, Chlorination	De-chlorination and surface water discharge with a portion of the effluent diverted to a landscape irrigation site
Midwest WWTP1	Screens	NA[b]	Activated sludge lagoons, Clarifiers	Seasonal chlorination (June, July, August)	Seasonal de-chlorination and surface water discharge with a portion of the effluent diverted to a landscape irrigation site
Midwest WWTP2	Screens	NA	Sequencing batch reactor	Lagoon cell A, Lagoon cell B, Lagoon cell C, Lagoon cell D, Lagoon cell E	Effluent transported to an agricultural site for the irrigation of fodder crops
Site (Spray Irrigation)	**After receiving treated effluent**	**On-site Treatment**	**Posttreatment on-site storage**	**Transport to sprinkler system**	
Mid-Atlantic SI1[c]	Screens	Ultraviolet disinfection	Storage in an open-air pond	Stored reclaimed water pumped to sprinkler system through a pumphouse	

[a]WWTP: Wastewater treatment plant;

[b]NA : Not Applicable;

[c]SI: Spray Irrigation