Microbiome Assessment of Water Including Sewage Plant Influent/Effluent

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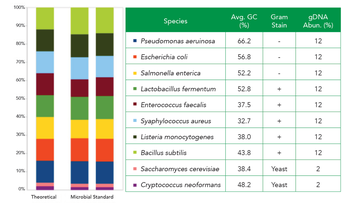
Abstract

Methods used to monitor the microbial content of potable and recreational waters, as well as treated wastewater discharges are limited to dated culture-based methods that utilize indicator organisms as surrogates for pathogenic species. Studies utilizing Next-Generation sequencing (NGS) to profile the microbial composition of water samples are now becoming more routine. However, it is well known that results are prone to bias and errors at every step of the workflow, including sample collection, DNA/RNA extraction, library preparation, sequencing and bioinformatics analysis. Therefore, care must be taken at each step in the workflow to minimize the effects of bias to obtain an accurate microbial profile for each sample. In addition, many studies utilizing 16S rRNA targeted sequencing are phylum- or genus-level resolution, thus limiting the ability to distinguish between innocuous and pathogenic species.

The purpose of the present study was to profile microbiomes of various environmental and commercial water sources using an unbiased, standardized workflow for accurate analysis. In order to determine the precise differences among various samples, water was collected from a variety of salt, fresh, municipal, sewage, and commercial sources. With the inclusion of validated mock microbial communities to ensure unbiased library preparation and sequencing, the microbial profiles of each sample were determined via 16S rRNA targeted sequencing and bioinformatics analysis. Surprisingly, in addition to the expected microbial differences between dissimilar water sources, strikingly different profiles were found among similar sample types collected from different locations. Treated wastewater samples were also subjected to Antibiotic Resistance Gene (ARG) detection, which proved to be widely prevalent. The findings reinforce the need for expanded sampling and the potential of NGS-based microbiomic methods for water monitoring in the future.

Methods: Unbiased Microbial Profiling Workflow



Species	Avg. GC (%)	Gram Stain	gDNA Abun. (%)
Pseudomonas aeruginosa	66.2	-	12
Escherichia coli	56.8	-	12
Salmonella enterica	52.2	-	12
Lactobacillus fermentum	52.8	+	12
Enterococcus faecalis	37.5	+	12
Syaphylococcus aureus	32.7	+	12
Listeria monocytogenes	38.0	+	12
Bacillus subtilis	43.8	+	12
Saccharomyces cerevisiae	38.4	Yeast	2
Cryptococcus neoformans	48.2	Yeast	2

Figure 1. ZymoBIOMICS® Microbial Community Standard. The standard is comprised of microbes with varying sizes and cell wall recalcitrance (i.e., 8 bacteria and 2 yeasts).

POTW	Final Effluent Treatment	Agency
Joint Water Pollution Control Plant (JWPCP)	Advanced Secondary Treatment (Disinfection)	Los Angeles County Sanitation District
Water Reclamation Plant (WRP)	Tertiary Treatment	Los Angeles County Sanitation District
Plant No. 1 (PLANT1)	Advanced Secondary Treatment (No Disinfection)	Orange County Sanitation District
Plant No. 2 (PLANT2)	Advanced Secondary Treatment (No Disinfection)	Orange County Sanitation District
Hyperion Water Reclamation Plant (HTP)	Secondary Treatment	City of Los Angeles
Terminal Island Water Reclamation Plant (TIWRP)	Tertiary Treatment	City of Los Angeles
North City Water Reclamation Plant (NCWRP)	Tertiary Treatment	City of San Diego
South Bay Water Reclamation Plant (SBWWTP)	Tertiary Treatment	City of San Diego
South Bay International Wastewater Treatment Plant (IWTP)	Secondary Treatment	City of San Diego/IBWC
Pt Loma Wastewater Treatment Plant (PLWTP)	Advanced Primary	City of San Diego

Figure 2. Sample collection summary. Influent and effluent samples were collected from ten wastewater treatment plants comprising various levels of treatment techniques.

Quality control studies of microbiomic research suggest that this vast new frontier is littered with potential sources for error and bias. From collection to sequencing, the potential for variation at each step in the microbiomic workflow is enormous[1]. Fortunately, researchers have recognized the potential for bias and are calling for solutions for each step of the workflow including mock microbial controls[2] and even bioinformatics tools[3].

This study utilized methods validated to be unbiased for each step of the process. Microbes were collected on 47 mm 0.2 µm Durapore filters and immediately frozen. When all samples were accumulated and ready for DNA extraction, the frozen samples were thawed in DNA/RNA Shield™ which limits the microbial profile-altering effects of freeze-thaw cycling.

Additionally, the DNA extraction method used has been validated to be unbiased with the use of the ZymoBIOMICS® Microbial Community Standard and 16S sequencing. Lysis of microbes on the filters took place in ZR BashingBead™ Lysis Tubes, containing 0.1 & 0.5 mm bashing beads. Lysis tubes were placed in a Vortex Genie 2 Horizontal Adaptor and process for 40 minutes at maximum speed. DNA extraction was completed using the ZymoBIOMICS® DNA Miniprep Kit. Additionally, to ensure efficient lysis of all microbes across several microbial genera, the ZymoBIOMICS® Microbial Community Standard (Figure 1) was included as a positive control.

During 16S library prep, PCR chimera, formed by primer mismatches and excessive amplification cycles, can spuriously align to microbial species during bioinformatics analysis and artificially inflate their relative abundance. The *Quick*-16S™, NGS Library Prep was utilized in this study because its qPCR-based amplification strategy minimizes the number of PCR cycles required for complete library generation minimizing PCR chimera.

Finally, variations between sequencing runs and across different sequencers can limit data reproducibility. Additionally, trimming parameters are often set by the user based on visual estimations. In order to minimize these variations, an automated trimming program was developed that maximizes usable reads and minimizes error. FIGARO models the error rate for each sequencing run in each direction to find optimal trimming sites that will maximize read retention after filtering while removing some lowest-quality percentile of reads.

The FIGARO application is freely available as source code at:

https://github.com/Zymo-Research/figaro.

16S Sequencing & Pathogen Detection: Environmental and Tap Waters

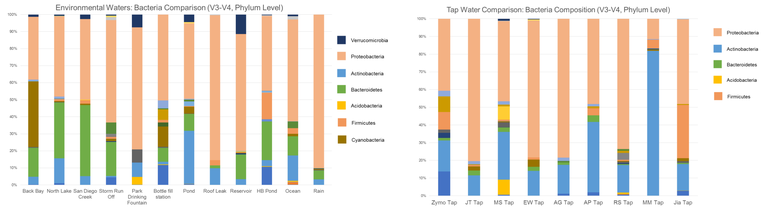


Figure 3. Water microbial profiling. Because various water sources will contain widely different microbial loads, PCR inhibitors, chemicals, and minerals, a pilot study was performed to confirm the ability of this workflow to assess water microbial profiles. 50 ml samples were collected from different environmental sources representing fresh, brackish, and salt waters. Additionally, tap water samples were collected representing different water districts. Phylum level comparisons are shown for simplicity. Additionally, 70 species of known human pathogens were detected in the drinking water samples. Most species make up less than 1% relative abundance and 16S sequencing does not determine between active microbes and 16S gene fragments. But, several notable pathogens were detected: *Escherichia-Shigella coli*, *Legionella pneumophila*, and *Staphylococcus aureus* (data not shown).

Microbial Profile of Sewage Treatment Plants Influent & Effluent

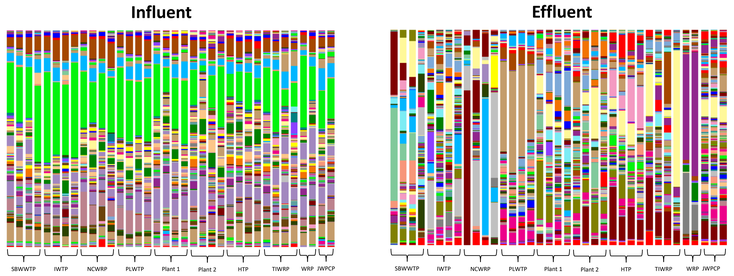


Figure 4. Genus-level relative abundances of 10 wastewater treatment plants' influent and effluent. Untreated wastewater displays similar microbial profiles regardless of geography. But, different levels and techniques of treatment at different plants (Fig. 2) produce strikingly different microbial profiles in effluent samples.

Antibiotic Resistance Gene (ARG) Detection

Plant	MLS ARGs	Sulfonamide ARGs	Aminoglycoside ARGs	Fluoroquinolone ARGs	Tetracycline ARGs	Chloramphenocol ARGs
JWPCP	✓	✓	✓	✓	✓	X
WRP	X	X	X	X	X	X
Plant 1	✓	✓	✓	✓	✓	X
Plant 2	✓	✓	✓	✓	✓	✓
HTP	✓	✓	✓	✓	✓	X
TIWRP	✓	✓	✓	✓	✓	✓
NCWRP	X	X	X	X	X	X
SBWWTP	✓	✓	✓	X	✓	✓
IWTP	✓	✓	✓	✓	✓	X
PLWTP	✓	✓	✓	✓	✓	X

Figure 5. ARGs detected in treated wastewater effluent. A proprietary ARG detection method was used to analyze the wastewater effluent samples. Surprisingly, ARGs were detected in samples from eight of the ten treatment plants. The two plants from which no ARGs were detected, employ Tertiary Treatment methods. Additionally, it was found that the amount of ARGs detected correlates to the treatment level of the plant, with the most detected in Primary and decreasing with Secondary and Tertiary Treatments (data not shown).

Absolute Quantification of Influent vs Effluent

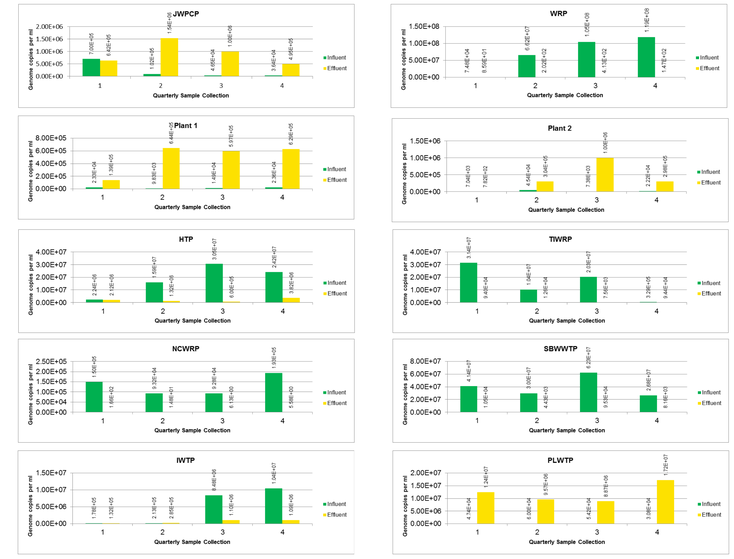


Figure 6. Absolute quantification via qPCR targeting V3-V4 region. Six of the ten plants (Fig. 2) displayed dramatic decreases in bacteria after treatment. Surprisingly, four plants had higher bacterial loads in effluent samples. Downstream treatment of water from these plants is further sanitized which includes incubation with a bacterial "brine". This brine is introduced at the effluent of the sample sites and is most likely the cause of the increase in bacterial load.

Conclusion

Government regulations often require treated wastewater to be tested for Fecal Indicator Bacteria (FIB) to ensure it is safe to release back into the environment. The required tests involve the culture-based methods specifically targeting FIBs which greatly limits the scope of detectable pathogens. Additionally, culture-based methods can be time consuming and phenotypic-based identification of bacteria is becoming a dying art. But, the past two decades a have seen tremendous advancements in complex microbial community identification via Next-Gen Sequencing (NGS), which enables the detection of non-culturable microbes.

16S sequencing was able to detect hundreds of microbial species in treated wastewater effluent but, it should be noted that 16S sequencing does not differentiate between active microbes and 16S gene fragments. This is why we also performed an absolute quantification analysis to assess the reduction of bacterial load. Surprisingly, although most treatment plants displayed lower bacterial loads in effluent samples, four showed an increase. It was later revealed that byproducts from downstream processing may be being reintroduced into the wastewater effluents.

Perhaps more important than the presence of 16S gene bodies in effluent samples, is the presence of antibiotic-resistance genes (ARGs). When bacteria contact these genes, they are able to uptake and express antibiotic resistance, exacerbating the problem of ARG prevalence in environmental and recreational waters[4]. Effluent samples were subjected to a proprietary ARG detection method and shockingly, 32 of the 40 samples contained ARGs. Additionally, it was found that the amount of ARGs detected, decreases with the addition of more advanced treatment methods. For example, two of the Tertiary Treatment plants did not have any detectable ARGs.

Societal pressures to conserve water have municipalities and water districts considering the treatment wastewater for reuse as potable water. But, the outdated culture-based detection methods may not be sufficient to identify unculturable pathogens and ARG spread. NGS-based microbial identification and ARG detection methods have the potential to modernize water safety monitoring by supplying more valuable information to regulators.

References

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