How DNA Sequencing Can Aid Integrated Microbiome Management in Water Systems

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Background

Existing culture-based methods that are used to evaluate water system microbiology miss more than 99% of the microbes present. This is because less than 1% of microbe types can be grown in a lab using culture methods much of the industry relies on (1). Thanks to molecular methods, we have a better than ever understanding of the microbial world around us. By applying a combination of DNA-based methods, water systems can be characterized in a way that has previously been unavailable to industry in terms of how many and what types of microbes are present. 16S and 18S ribosomal ribonucleic acid (rRNA) qPCR, and amplicon sequencing can characterize nearly all the microbes in a system and approximate their quantity.

These methods have response time and accuracy advantages over current culture-based microbiological monitoring methods. In addition, the cost and performance of DNA sequencing continues to improve and open expansive new opportunities for understanding the microbial world. We propose a framework where scientists, engineers, and operators within a facility or industry work together to develop and refine Key Performance Indices (KPIs) and operational decision-making tools based on knowledge gained through operational and microbiome data. If this Integrated Microbiome Management framework is knowledgeably applied across the industry, these tools have the potential to improve operations, reduce downtimes, decrease health risks, and save costs.

Introduction

Problems in Water System Microbiology

Microbes, or microscopic organisms, are ubiquitous in both natural and built environments. While most microbes are harmless and sometimes even beneficial, uncontrolled microbial growth in many built water environments can lead to negative health and operational outcomes. The combined microbial community present within a specific location or system is called the "microbiome." This article outlines limitations and opportunities for microbiome control in clean water systems, including both potable water building plumbing systems as well as cooling water systems.

Biofilms develop in water systems where nutrients are available for growth. Key factors that support biofilm growth include nutrient and carbon availability, moderate-to-high temperatures, and stagnant flow conditions. While the specific issues presented by biofilms differ depending on the type of water system, improved understanding of the responsible microbiome can provide benefits across systems.

In cooling water systems, significant air intake and water evaporation increase concentrations of carbon (i.e., microbe food) and nutrients (i.e., microbe vitamins and minerals) available for biofilm growth. Primary issues associated with cooling towers include legionellosis risk, biological fouling, loss of heat transfer efficiency, and microbial influenced corrosion. Microbially induced corrosion (MIC) can be a byproduct of biofilms, posing a risk to equipment and plant uptime. Legionellosis is a health risk posed by human pathogen *Legionella pneumophila*, which can impart disease if it makes it into the air and then into people's lungs. The presence of biofilms and higher life forms like amoeba and protozoa exacerbate *Legionella pneumophila's* ability to grow and persist in water systems.

Potable water systems can be sourced from a large utility or privately from wells. Either way, water needs to remain safe to drink throughout the distribution system, including premise plumbing within specific properties. The widespread use of free chlorine or chloramine in U.S. potable water systems means that microbes growing in those systems tend to be chlorine resistant. These microbes include *Legionella* spp. and NTMs. Premise plumbing in particular presents microbiological complexities due to more intermittent use, low/no-flow areas, and lower or no disinfectant residual. As a result, the drinking water microbiome and its source biofilms often differ between private and premise plumbing controlled by a property owner and distribution systems controlled by a utility. Key issues associated with drinking water systems include microbial contamination, corrosion, and pathogen regrowth.

Microbial contamination can occur after potable water has left the water utility but before it arrives at the user tap. It may include fecal contamination from sanitary lines, birds, wild animals, and/or agriculture and biological contamination from surface water intrusion. Corrosion is affected by many complex factors, including pipe material, water quality, nitrification, and historical as well as current operation. Pathogen regrowth, especially for opportunistic pathogens like *Legionella* spp. and non-tuberculous mycobacteria (NTM), is supported by general biofilm growth as well as stagnant-flow conditions that remove disinfectant residuals.

Current Methods to Measure and Address Microbial Problems

Current methods to measure and quantify microbiological problems in cooling water systems rely on culture-based methods that take days to weeks to grow and count targeted microbes in the lab. Heterotrophic plate count (HPC) is a culture method to measure general bacterial abundance but does not provide any information about what types of organisms are present. Specific culture analysis of *Legionella* spp. typically takes 14 days, and this delay can limit an operator's ability to understand and control associated risks (2–4).

More recently, some facilities have started using quantitative polymerase chain reaction (qPCR), which is a rapid test that specifically copies *Legionella* spp. deoxyribonucleic acid (DNA) containing the 16S rRNA (ribonucleic acid) gene and back-cal-

culates original concentrations from the amplified signal (2, 5, 6). This method yields more accurate results faster and can also measure both dead cells and viable but not culturable (VBNC) cells. VBNC cells are cells that can grow and multiply but do not grow well in the specific conditions specified by a culture method. Operators can use oxidizing and nonoxidizing biocides, either on a continuous or intermittent basis, to control microbial growth and biofilms (3, 7, 8).

Potable water microbiome sampling typically focuses on culture of pathogens or pathogen indicators (e.g., fecal coliforms) or bulk microbiology measurements (e.g., HPC and BacT). Some commercial laboratory services provide culture tests for a wider range of bacterial pathogens that are of concern in premise plumbing water systems.

Molecular Methods

The cost of DNA sequencing and associated molecular biology methods has decreased astronomically over the past two decades. The cost of sequencing human genomes in 2001 was estimated at about $100 million dollars. In 2020, the estimating sequencing cost was about $1,000, or 100,000 times lower (9). This change has largely been due to advances in sequencing technology, which have spurred innovative microbial ecology techniques. The study of microbiomes in built environments like indoor air, water systems, and wastewater treatment has benefited from these changes. Less than 1% of microbe types can be grown in a lab using these culture methods on which much of the industry relies (1). Thanks to molecular methods, we have a better than ever understanding of the microbial world around us.

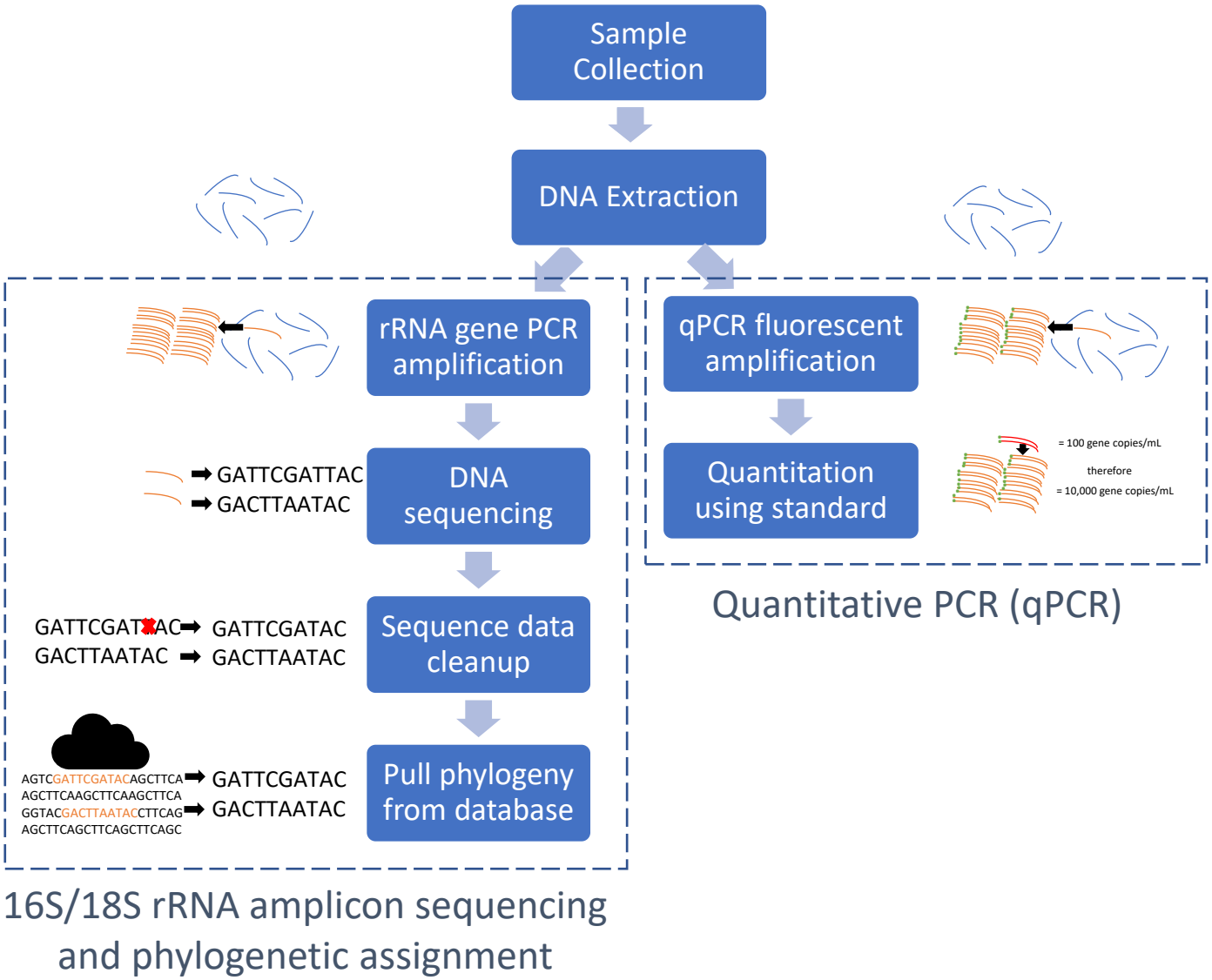
Bacteria and protozoa have significantly smaller genomes, which means you can garner lots of useful information about them from relatively fewer sequences than for human genomes. Several different categories of molecular methods have evolved to help elucidate the structure and function of microbiomes (10). The most appropriate method or combination of methods for a given application depends on the specific questions that require answers. Table A outlines these methods, what they are used for, and key limitations.

Table A: Summary of Molecular and DNA-Based Methods for Microbial Ecology

Analysis Name	*What It Measures*	*Method Description*	*What Questions It Answers*	*Limitations*
Quantitative polymerase chain reaction (qPCR)	Targeted genes and targeted microbes	PCR amplification of DNA	Who is there and how much? What are they doing?	Needs to be targeted to one specific gene or microbe type. Need to know what you are looking for.
Phylogenetic microbiome profiling (16S/18S rRNA amplicon sequencing)	All 16S rDNA genes (targeted gene for all microbes)	DNA Sequencing	Who is there?	Doesn't tell you what they are doing, limited to genus specificity. Targets bacteria/archaea or eukarya, not both. Doesn't capture virus DNA.
Metagenomics	All DNA (untargeted)	DNA Sequencing	Who is there and what can they do?	More prone to errors, limited to family specificity.
Metatranscriptomics	All RNA (untargeted)	Reverse-transcription to DNA and DNA Sequencing	What are they doing?	RNA is short-lived and unstable, so it's hard to get enough of it. Can be lots of noise from common but unimportant transcripts.
Metaproteomics	Proteins (targeted or untargeted)	Mass spectrometry	What are they doing?	Less developed, can't read all proteins.
Metabolomics	Targeted chemical metabolites	Analytical chemistry methods	What are they doing?	Difficult to link compounds to microbes.

The two most common methods used in engineered systems are also the most affordable and simplest. Specific steps for both methods are diagrammed in Figure 1.

Figure 1: Pictorial summary of 16S/18S rRNA amplicon sequencing and qPCR



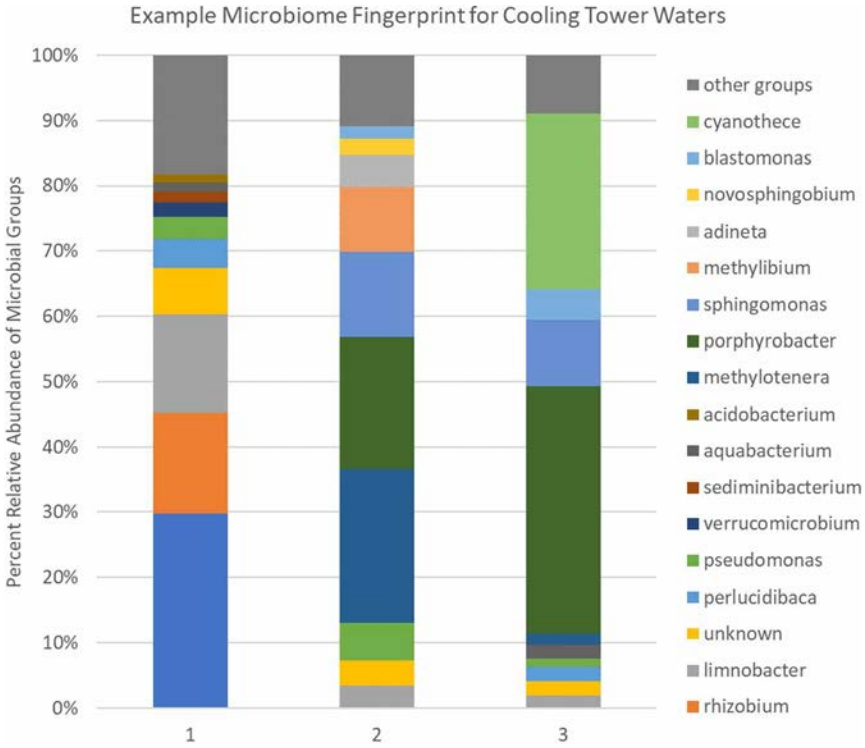
qPCR is in wide use for measuring *Legionella* spp., *Legionella pneumophila*, and more recently, SARS COV-2, in built environments. You need to have a specific gene target in mind to conduct qPCR. In the case of *Legionella* analyses, you target a variant of the 16S rRNA gene that is very specific to the genus (*Legionella* spp.) or species (*Legionella pneumophila*) that you are looking for. You can also target specific genes. For example, you can target an iron-oxidizing gene to quantify microbes that may be involved in MIC. qPCR is highly effective at estimating quantities of targeted microbial types or genes and can now be done on site with fairly simple and affordable equipment.

Phylogenetics describes the study of DNA to better understand how different types of life (which are mostly microbial!) are related to each other. This knowledge has supported development of methods that delineate specific microbial groups within a given sample. 16S/18S rRNA amplicon sequencing is a microbiome fingerprinting method that can identify nearly all microbes in a system. Well-established methods typically read down to a genus level, but some newer sequencing platforms can sequence longer reads that enable species-level identification (11). For example, genus-level data means that you can identify how much of the community is *Legionella* spp., while species level data means you can identify how much is the *Legionella pneumophila*. However, it provides information on nearly all microbes present, not just ones that you specifically target. This makes the method unbiased to any previous theories about the microbiome and provides a clear picture of what types of microbes are present and who dominates.

The 16S rRNA analysis provides data on bacteria and archaea (like *Legionella* spp., methanogens, and most pathogens), while

the 18S rRNA analysis provides data on eukarya (higher life forms like protozoa and fungi). This method does not provide quantitative information but can be paired with qPCR targeting the 16S and 18S rRNA gene to translate the microbial fingerprint into semiquantitative results that can be compared between samples and locations. In addition, microbial fingerprinting data provides insight into community diversity, which is an ecological indicator of community stability and complexity. An example outcome of microbiome analysis of cooling waters using 16S rRNA amplicon sequencing is shown in Figure 2.

Figure 2: Example of microbiome results for cooling tower waters.



DNA sequencing for phylogenetics or metagenomics can be accomplished on a variety of different platforms. Current methods primarily use Illumina MiSeq and HiSeq technology, which require large sequencing machines in a laboratory setting and can only sequence DNA segments up to 300 to 600 base pairs (DNA letters) at a time. DNA sequencing and other powerful, modern molecular test methods are now becoming available in the form of smart handheld devices that can interface with a personal computer. These handheld DNA-sequencing devices are easily transportable and capable of basic DNA sequencing on site at the customer location, with results within one hour (1, 12).

The Oxford Nanopore MinION portable sequencing platform can sequence longer reads than most established sequencing platforms, including the full 1,600 base pair 16S rRNA gene. This means that 16S rRNA amplicon sequencing with this platform could identify microbiome members down to the species level instead of just the genus level (11–13). This change has big implications for pathogen monitoring, as most pathogens cannot be detected by genus-level assays.

Previous use of DNA-based methods to understand water system microbiomes. Previous phylogenetic studies have found cooling tower systems dominated by biofilm formers like *Sphingomonadaceae* and *Pseudomonadaceae* (14–16). These systems also commonly contain opportunistic pathogens like non-tuberculous mycobacteria (NTM), which can cause respiratory disease in immunocompromised individuals (16); however, these studies have not been well integrated with plant engineering and operations to link the microbiome to operational outcomes.

The microbiome of potable water distribution systems and premise plumbing is highly impacted by pipe material and other biofilm conditions, residual chlorine disinfectant, and water quality (17–19). NTMs and *Legionella* spp. are widespread, especially in areas that lay stagnant with minimal flow for extended periods of time (17, 18, 20).

As with previous cooling water studies, these studies have used limited integration of engineering and operations knowledge and data. Collaborative DNA-based studies between scientists, design engineers, and water system operators have the potential to redefine our understanding of the microbiome and how we

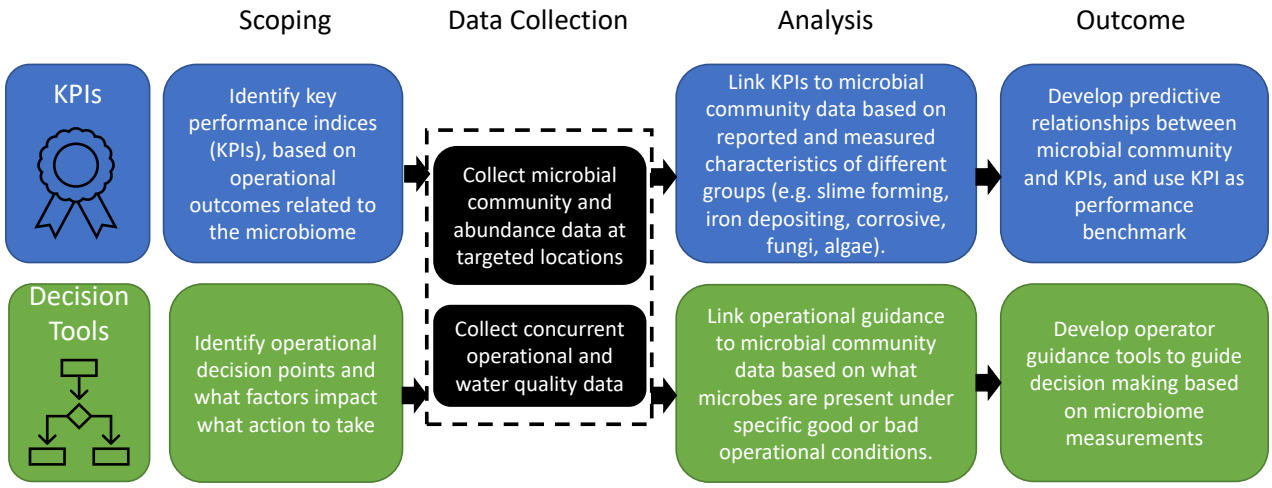
use that knowledge to make decisions and improve operations. One Midwestern city sampled eight potable distribution system locations on consecutive days and found significant amounts of human-associated bacteria in some locations. This finding spurred the city to inspect nearby facilities and find a lack of backflow preventers that was later addressed. That work demonstrated the ability of phylogenetic DNA technology to identify potential water system problems (21).

A recent study conducted by a service company[A] used microbiome analysis to help troubleshoot MIC in a stainless-steel potable water system. Following disinfection of the system, total quantities of microbes decreased, but biodiversity remained high, suggesting that biofilms persist in the system through disinfection, and that the threat of MIC will persist unless the biofilms are mitigated. Microbial diversity data (i.e., how many types of microbes are present) provide insight into the community complexity, with biofilms harboring more types of microbes than are typically free-floating (planktonic) in water systems.

Proposed Approach and Opportunity

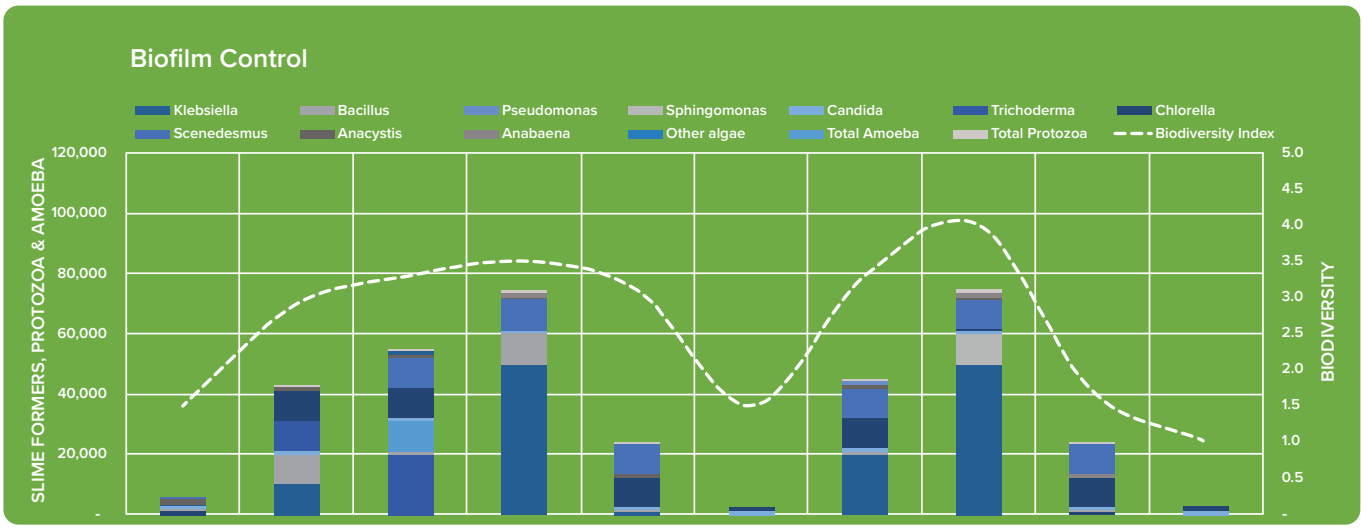
Existing culture-based methods used to evaluate water system microbiology miss more than 99% of the microbes present. Water system engineers could leverage the abundance and community data provided by the combination of 16S/18S rRNA qPCR and amplicon sequencing to improve system understanding and, subsequently, response times and accuracy of response measures. ***Note:*** *Forging a direct connection between microbiome data and operational decisions could reduce the costs associated with health risks, loss of heat transfer efficiency, equipment damage, and unplanned downtime.* Figure 3 outlines an example of a study designed to manifest these improvements by developing a.) KPIs and b.) decision-making tools.

Figure 3: Example framework for Integrated Microbiome Management: Developing meaningful operational outcomes from microbial community data.



In this article, the authors have outlined some potential KPIs and decision-making tools that could be developed and refined for cooling water systems using microbiome data. This would include linking microbiome data to culture-based measurements for HPC and *Legionella* spp. Figure 4 shows an example dashboard for a biofilm control KPI.

Figure 4: Example of KPI dashboard and biofilm control.



Sample ID	1	2	3	4	5	6	7	8	9	10
Score	Good	Critical	Critical	Critical	Warning	Good	Critical	Critical	Warning	Good
Biofilm Slime Formers	5,100	42,100	54,000	73,510	23,500	2,100	44,000	73,510	23,500	2,100
Potential Legionella Hosts	-	215	595	845	70	-	490	1,085	70	-
Biodiversity	1.5	2.9	3.3	3.5	3.1	1.5	3.3	4.0	1.7	1.0

Potential KPIs:

- **General biofilm KPI:** Biofilm health is related to many specific microbes that form biofilms as well as high microbial diversity, which indicates a complex microbial community most likely to be present in biofilms.
- **Microbially induced corrosion KPI:** Identify the types of MIC organisms present and target biocides to them, including microbes that cycle iron and sulfur.
- ***Legionella* KPI:** If one can knock down or eliminate biofilms, *Legionella* has fewer places to grow. An effective *Legionella* KPI would predict growth opportunities by linking to levels of general biofilms as well as identified *Legionella* hosts.

Potential Decision-Making Tools:

- **Improved biocide guidance:** Biocide selection guides, combined with knowledge of what microbes are present and how many, eliminate a lot of guesswork and allow a water treatment professional to select optimal biocides and biocontrol strategies. For even greater precision and cost effectiveness, data on which microbes survive in the presence of specific biocides can be used to develop biocide resistance charts, which can be used to select a biocide based on direct and comprehensive measurement of results.
- **Biofilm preventative maintenance guidance:** Use the biofilm KPI to detect the presence and location of biofilms. Provide specifications on how to remove the biofilm from the water system and prevent it from returning in the most cost-effective and environmentally responsible manner based on specific types of biofilm organisms present.

The application and dissemination of any innovative technology will be met with technical hurdles. These anticipated roadblocks are outlined in Table B.

Table B: Potential Roadblocks to Integrated Microbiome Management and Recommended Mitigation Strategies

Potential Roadblock	*Issue Summary*	*Recommended Mitigation Strategy*
Lack of industry standards for methods	Multiple valid methodologies exist for phylogenetic analysis. These variations can include primers, DNA sequencing platforms, sequence assembly, data cleanup, and phylogeny assignments.	Develop and publish standard methodology for 16S/18S rRNA analyses in the water and wastewater field.
Sample consistency	Microbial community can vary significantly between sites and times and depending on sample collection procedures.	Samples should be collected at a set location using a standard method each time.
Sample contamination	Because this method measures all microbes present, DNA contamination from the sampler's hands or other surfaces can bias microbial community results.	Use DNA-free sample containers and clean nitrile gloves for each sample. Take care not to touch face or other items before sampling.
Sample hold times	DNA degrades quickly in water, and degradation rates vary by microbe. So, the microbial community you see could change from the actual community during storage.	Conduct qPCR on site if possible. Evaluate and vet DNA preservatives for sampling and shipping.
DNA sequencing depth	For microbial fingerprinting technologies to be effective, you need to sequence enough DNA segments to characterize more than 90–95% of the community.	Monitor number of community members observed (OTU count) and compare to estimated community size (Chao1) to estimate percent coverage.

Summary and Outlook

Normalization of environmental genomics data to one industry standard is critical for molecular methods to advance toward mainstream adoption in the water and wastewater industry.

One of the barriers to commercial adoption of more real-time DNA sequencing devices and other rapid molecular test methods is the current lack of industry standards for environmental genomics data. If using a smart handheld device to support DNA sequencing needs in the field, it will need a pre-established framework for translating results to actionable decisions. This framework could be developed now using existing lab-based methods and the Integrated Microbiome Management process described in the article, which can then be applied in the future once more real-time sequencing methods are available.

The authors believe the rapid rate of change in cost performance will continue to drive a rapid evolution toward smart handheld genomic devices and online DNA sensors. Different specialty service companies[B] and other firms are likely to commercialize these types of mobile, onsite, near real-time DNA analysis solutions over the next few years.

These developments represent a unique opportunity for water professionals to improve our collective understanding and control of water system microbiomes such as distributed and premise plumbing potable water systems and open recirculating cooling water systems. This could start with an industrywide survey of microbiome and operational data, followed by a collaborative study to develop and refine KPIs and decision-making tools.

By combining microbiome data with operational knowledge and understanding, we have the opportunity to improve the protection of health and the environment in our work while cutting costs and improving service. In the process, water treatment businesses may also increase their appeal and attract future employees who could become the next generation of water treatment innovators and leaders.

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Endnotes

[A] Water Detectives is a service company based in Downers Grove, Illinois, that is a part of WaterTrust. One aspect of the company's business is to offer test methods for detecting the DNA of microorganisms.

[B] In the text, the authors are referring to Water Detectives and other companies that offer products and test methods to identify the DNA of target microbials.

Alison Ling, Ph.D., P.E., is an environmental engineer with Barr Engineering Co. in Minneapolis, Minnesota, and scientific a advisor at Microbe Detectives. At Barr, Dr. Ling is engaged in front-end design and troubleshooting for water and wastewater treatment projects. Her work includes process modeling, bench testing, and technical advising for clients in the power, mining, food and beverage, and municipal sectors. Dr. Ling holds a Ph.D. in civil engineering from the University of Colorado at Boulder, where she split her time between environmental engineering and microbial ecology lab groups.

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