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# the ANALYST

THE VOICE OF THE WATER TREATMENT INDUSTRY

# AMPLIFY AWT

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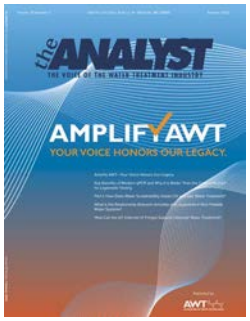


Cover

*Amplify AWT—Your Voice Honors Our Legacy*

*Cover graphics: Kerry Bankert*

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# Features



# Departments

# Key Benefits of Modern qPCR and Why It Is Better Than the Culture Method for *Legionella* Testing

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*A picture of the Stafford Hotel in Philadelphia, where the 1976 outbreak of Legionella occurred. Source: www.alchetron.com.*

Since the discovery of *Legionella* bacteria during the 1976 outbreak that occurred in Philadelphia at the Bellevue Stratford Hotel (pictured on left), the culture method has been used to identify the presence of *Legionella* in water.

The culture method was not intended to effectively quantify the bacteria and has many deficiencies and subjectivity in the method itself. However, the culture test is the predominant method now in use by laboratories. Polymerase chain reaction (PCR) methods have become more established and have supplemented or replaced culture methods in many industries and for many types of bacteria. The Centers for Disease Control and Prevention (CDC) and other organizations recognize PCR as an approved method for *Legionella* analysis, but most people and organizations remain unaware of newer quantitative PCR (qPCR) methods and their ability to identify *Legionella* type and with much greater precision. Today, the modern qPCR method can have better usefulness than culture methods for routine monitoring and remediation work.

This article will discuss monitoring for the presence of *Legionella* and how the qPCR technology can improve biocontrol in cooling water and building water systems.

## Background

Several test methods for *Legionella* are currently available on the market. Some are designed for field work, and others are typical for laboratory testing. Two lab methods are culture and PCR or qPCR.

The culture method commonly used by CDC elite labs follows the procedure established by the International Organization for Standardization labeled ISO 11730 (1). There is some subjectivity in the procedure to remove interfering bacteria, with the goal of trying to get *Legionella* bacteria cells or groups of cells to grow on an agar plate over the course of days, creating colonies big

enough to recognize and count as *Legionella* colonies. Each is recorded as colony-forming units (CFUs).

PCR is a molecular method that amplifies target genetic material with special enzymes, primers, and reagents and an instrument called a thermocycler to achieve high enough levels so that they can be detected. Newer, modern techniques can quantify the amount of the targeted genomic sequence and quantify them as number of genomic units (GU) or *Legionella* cells present in the water sample (qPCR), and even classify them as *Legionella* species, *Legionella pneumophila*, Sero Group 1, or Sero Group 2-15.

A GU is a specific section of the gene that is recognized with laboratory techniques such as PCR and can be quantified to associate with the amount of genetic material coming from, in this case, a *Legionella* cell. A CFU is different in that it is a colony grown on an agar, containing perhaps millions of freshly grown bacteria cells.

Since the two are very different, there is no good way to compare and contrast the two. Figure 1 shows data from PCR amplification of genetic material created from each cycle of amplification.

**Figure 1: Charts from PCR amplification of genetic material.**

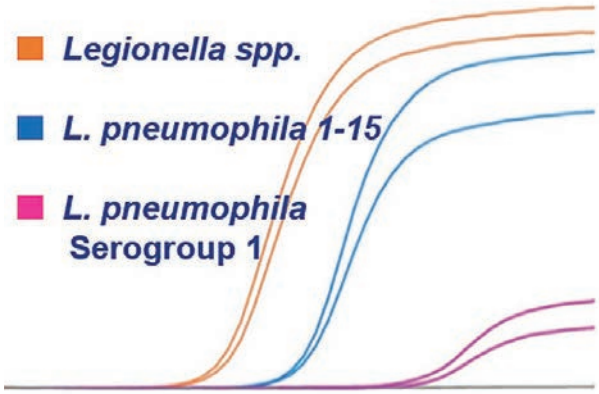



Chart courtesy of BIOTECON Diagnostics Co.

## Moving Forward With qPCR

Today, the question should be, "Why and how to proceed with qPCR?"

If we had used qPCR methods that are available now, going back to 1976, we would not even discuss changing to the culture method for regular and remediation work.

To help with getting qPCR accepted and used more, we need GU alert and action levels for building/potable waters and for cooling tower waters that are as safe as CFU guidelines but not set too low. Various studies suggest a one log increase or a little more compared to CFU as alert and action levels. To offer some higher safety, it may make sense to look at half that. This is open for discussion, and there is probably enough published data available to establish a guideline (2, 3).

Table A provides suggested guidelines for *Legionella* alert and action levels.

**Table A: Suggested Guidelines**

**Building Potable Water Supplies:**
- Alert: 5 to 10 GU per milliliter (GU/mL)
- Action: 50 to 100 GU/mL

**Cooling Towers:**
- Alert: 50 to 100 GU/mL
- Action: 500 to 1,000 GU/mL

As data builds, we will more likely be able to establish better correlation data to *Legionella* GU level and the risk of Legionnaires' disease and create better alert and action guidelines.

Because CFU levels are colonies on a plate and not necessarily actual cell numbers in the water, they may not provide very good alert or action control guidelines. Use of qPCR allows for a much better risk assessment and with more reliable data. The sooner we start using GU, the faster we will improve control and reduce risk.

## Modern (Elite) qPCR Versus Culture Method

Many people familiar with PCR recognize it as a rapid results screening tool to see if *Legionella* are present. If so, they may then move onto further testing using the culture method. But modern qPCR, which we can even further categorize as elite qPCR methods, can do so much more. Test kits are available with the reagents basically prepared and freeze dried to increase ease and accuracy in the testing. Figure 2 shows PCR tube strips containing extracted DNA material along with necessary primers and components to duplicate and amplify targeted DNA sequences. These tubes are placed into the rack of a thermocycler machine.

**Figure 2: Tube strip for qPCR analysis.**

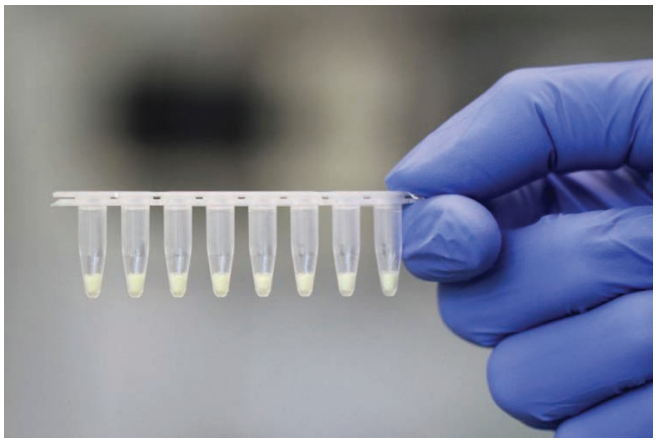


Photo courtesy of BIOTECON Diagnostics Co.

The efficiency or consistency of qPCR is very high, with some studies showing >90%, while the culture method is so low that arguments have been made that a difference in tested levels during a remediation or other actionable effort cannot statistically detect changes.

The lab time required for qPCR can compare favorably to the culture method. It depends on several factors, such as how many *Legionella* tests are being performed on a given day, the complexity of the water matrix, and options available to the culture method that may influence how a particular lab runs the culture method. Figure 3 is a workflow chart taken from BIOTECON Diagnostics' qPCR instructions manual.

Because GU and CFU are different units, there is no direct and consistent conversion from one to the other. There are statistical methods based upon comparative testing results on large sample sizes that can yield some algorithm to make conversions, but it is best to understand what each method and unit are providing. The GU count is generally expected to be higher than CFU count for several reasons. Modern qPCR can be calculated to report results as GU, which is equivalent to one *Legionella* cell, while a CFU can develop from one or more cells. As an example, if the average isolate originates from groups of three cells, then 1 CFU would equal 3 GU. If each CFU originates from single *Legionella* cell, then 1 CFU = 1 GU. Also, qPCR detects most all *Legionella* in the sample (Limit of Detection [LoD] of 5), while the culture method can miss some or even most of *Legionella* present.

**Figure 3: Example test procedure flow and time required for each stage.**


Image courtesy of BIOTECON Diagnostics Co.

Table B highlights some of the major differences in the test methods and value differences between qPCR and culture.

**Table B: Comparison of Test Methods**

| Process | qPCR | Culture |
|---|---|---|
| Time for results | 4 hours | 7–14 days |
| Recovery of *Legionella* present | High | Low |
| Can distinguish live from dead cells | Yes | Yes |
| Can be used to speciate types of *Legionella* | Yes | Yes |
| Repeatability of results | Consistent | Inconsistent |
| Total laboratory time | Similar to culture | Similar to qPCR |
| How are results reported | Genomic Units (GUs) | CFUs |
| Comparison of reporting units | Higher than CFU | Lower than GU |
| Actionable control response benefits | High value | Low or no value |
| Laboratory cost | Similar to culture | Similar to qPCR |

## Establishing qPCR as the New Gold Standard

Since the water treatment community (including water treatment companies and water management plan writers), relevant associations, healthcare facilities, government regulators, laboratories, and various other organizations may not be aware of modern qPCR capabilities, we need to better publicize this needed change of best practice (5).

For the water treater and end-user, they will want guidelines based on GUs to be used in conjunction with CFU guidelines until CFU guidelines become outdated. As with any newer method, it usually requires key early adopters.

With understanding of modern qPCR capabilities, a next step includes getting GUs written into guidelines and procedures.

## Culture Method

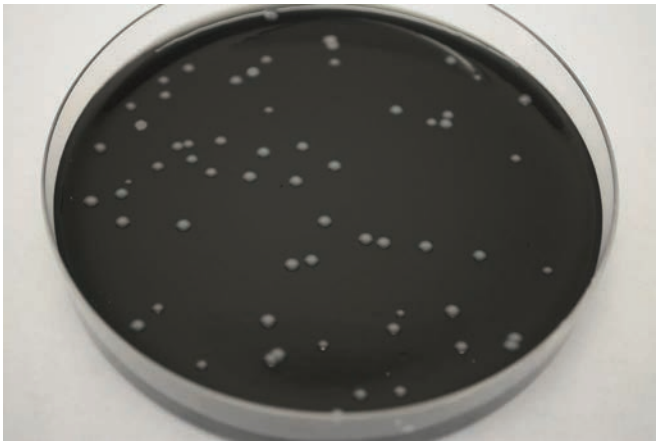
Because the culture method has been in use for so long, it has been accepted, and many who rely on it have not critically examined it or know the details of its procedure and its many inherent inaccuracies and problems. There are many liberties that can be taken by the laboratory technician. Waters can be plated directly or concentrated with filtration. The volume of water can be discretionary. It suggests one liter, but smaller volumes are acceptable.

Acid treatment to help remove non-*Legionella* bacteria before culturing may be necessary, but again, this is up to the lab to decide. Even the agar material, incubation process, and time varies from lab to lab. Actual identification of *Legionella*-cultured isolates can be quite difficult. Some labs confirm *Legionella* by replating suspected isolates grown on the first plate, while other labs do not.

So, even though the ISO 11731 standard method and the "CDC Laboratory Guidance for Processing Environmental Samples" (4) may be used, it is still a highly subjective procedure. Results take days to attain, (three days for tentative indications and up to 14 days for confirmations). Results are erratic and unreliable (e.g., poor sensitivity/recovery, poor efficiency).

The colonies are difficult to isolate, grow, and count. The culture method relies on cells to be viable and culturable. The organisms are typically alive but in a stressed state. As a result, only a portion, and likely a small portion of the organisms present, are actually cultured, even under the best of conditions. Figure 4 shows an example of cultures grown on the black Buffered Charcoal Yeast Extract (BCYE) plates.

**Figure 4: Example of cultures grown on a BCYE.**



Culture plate courtesy of Q Laboratories, Cincinnati, Ohio.

Attempts to culture them may be suppressed by anywhere from 5 to 99% due to the selective nature of the medium required and isolation techniques used. Many cells can be in a state of metabolic dormancy, so they are Viable But Non-Culturable (VBNC), but they could perhaps resuscitate within a water system and pose a risk to human health.

Where waters are more complex and a series of screenings are required and the plating of isolates are performed, the test can be labor intensive.

PCR is a molecular method that amplifies target genetic material with special enzymes, primers, and reagents and an instrument called a thermocycler to achieve high enough levels so that they can be detected.

Results are reported in CFUs, which can have very arbitrary value since the CFU is a million or more cells on the growth media, but it does not consider the genesis of the colony. Each colony could have grown from quite a different number of initiators of the colony. It could have grown from one single *Legionella* cell, a group of agglomerated cells, a large group of cells clinging to a suspended solid, one or many cells within an amoeba, or a portion of a detached biofilm containing perhaps thousands of cells.

Figure 5 demonstrates how CFUs can vary due to the source of the *Legionella* cells while the quantification of genetic material is not influenced by the condition of the cells.

Almost universally, the culture method is recognized as a very poor and problematic procedure and is still ironically called the "gold standard" by some, but it is also recognized that the time delay for results adversely affects human health.

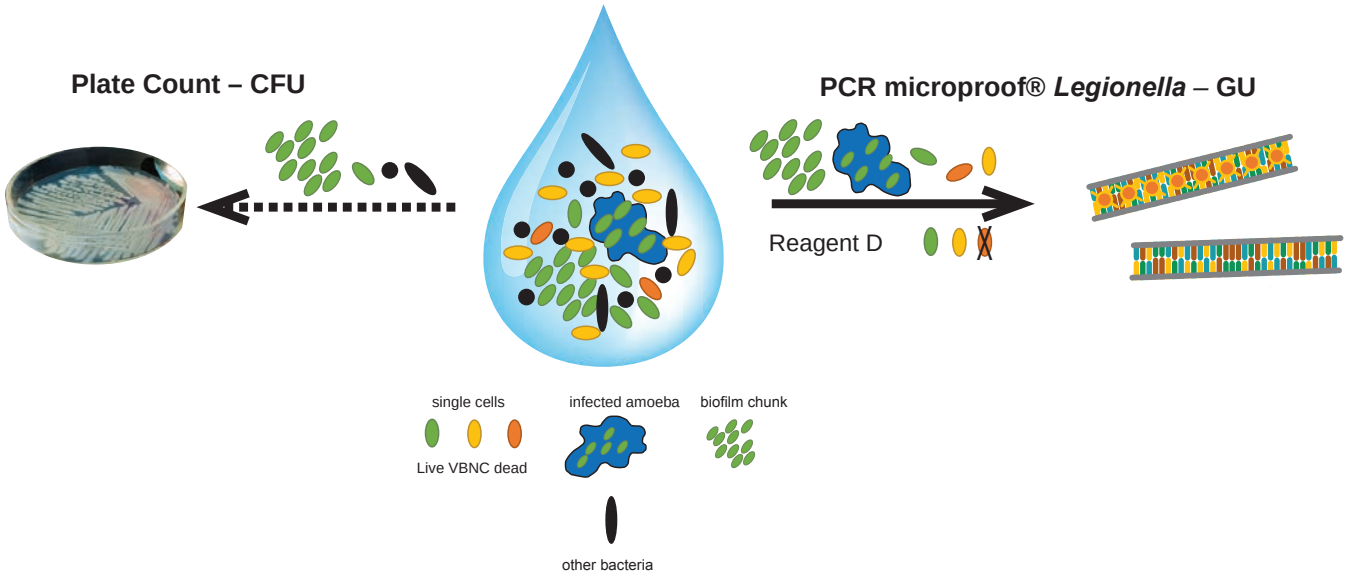
Reasons that the culture method is still used:

- The CFU results are an established convention, and response actions have been provided within written procedures.
- It takes time and effort to make changes to established procedures and guidelines.
- The capabilities of the modern qPCR method are not yet well known.
- Culture method is useful to trace origin of an outbreak by genetic analysis.

## Other Considerations for *Legionella* Testing and Control of Legionnaires' Disease

*Legionella* are associated with biofilm and mostly located within biofilm. Sloughing of biofilm is the major source of *Legionella* going into the water for most systems. If steady state is reached, the sloughing of biofilm can be thought to be more consistent while a certain biofilm thickness is maintained, but most likely there are events over the minutes, hours, days, and weeks that inconsistently release biofilm. The concentration of *Legionella* that are contained in the water that is aerosolized into

**Figure 5: Genesis of CFU versus GU.**




Drawing courtesy of BIOTECON Diagnostics Co.

5-micron (µm) water droplets would vary considerably based on the specific dynamics, system designs, and retention times.

## Does the system have a steady state condition, or could there be major disruptions?

Table C looks at some of the major contributions to disruptions to steady state and how that can influence bacteria levels.

**Table C: Disruptions to Steady-State Conditions**

| System Parameter | Cause or Consequence to Steady State |
|---|---|
| Flow rate changes | Low flow—biofilm growth; High flow—biofilm releases |
| Water hammer | Pump or valve operation can rattle pipes and release biofilm |
| Water treatment changes | Biocides, surfactants, other chemical concentrations changes |
| Temperature | Affects growth rate of biofilm |
| Intermittent use of systems | Allows stagnation, adding variability to total system |
| Water supply variations | Bacteria concentrations or loading rate changes |
| Nutrient loading | Biofilm growth rate |

How valuable is any testing method if there is infrequent testing, no consistent steady-state condition, a poor water management plan, and no effective critical control points? In non-steady-state systems (which is probably most systems), sampling and testing every hour or 24 hours would likely show high variability with both culture and qPCR methods.

The value of testing in a water management plan is highly dependent on how good the plan is and how well it is implemented. If there is no water management plan for preventing Legionnaires' disease, then there is little value for water testing. Where a good plan exists and is followed, and some level of steady state exists, the testing is more valuable for confirmation and prevention of Legionnaires' disease.

Is the water sample point appropriate to the point where water is aerosolized and related to the risk of *Legionella* bacteria being contained in the water droplet?

## Questions to Consider

### *Example 1*

What if the *Legionella* source is contained in biofilm within the return header to the cooling tower and

sampling is done from the cold well sump or to supply water after filtration and treatment?

How does the procedure of filtering the sample onto 0.45-µm paper and then resuspending the sample into a buffer solution affect the grouping of bacteria and resultant CFU?

### *Example 2*

If the bacteria are grouped in the system water, do they get separated in the testing procedure? Alternatively, could some cells get grouped in the process? How would that affect risk correlation of CFU to risk of acquiring Legionnaires' Disease?

Should there be a correlation between risk of disease from the amount and form of *Legionella* bacteria inhaled? Right now, there does not seem to be a correlation between CFU detected in a water supply and risk of disease. Considerations include:

- Which *Legionella* cells are most likely to be contained in aerosolized 5 µm or less water droplets, and how does that relate to the CFU determined in the bulk water sample that was tested?
- Is there a correlation between the ability to culture a single cell or small group of cells that could be contained in the aerosol and the most problematic form causing disease?
- Is the probability of successfully culturing a single cell less likely than successfully culturing a larger group of cells? Or put another way, which approach could distort the total CFU number?
- Does an aerosol most likely contain one or several *Legionella* cells that are 1 µm in diameter and up to 20 µm long, and are these harder to culture than larger groups or biofilm fragments.
- Does the sample point relate consistently to the point where the water is aerosolized?

How is facility liability and potential litigation affected? As a modern, elite qPCR method becomes more well-known and implemented, it is possible or even likely that those using an inferior method such as culture could be at greater liability risk in litigated cases of Legionnaires' disease.

## What if qPCR Was Established and Culture Was the New Method?

To implement change, sometimes it makes sense to poke fun at the existing condition and turn the table upside down. In an attempt to do that, it appears absurd to think that we would implement culture as the best available technology for *Legionella* testing if modern qPCR had been in use since 1977 instead of the culture method. The absurdity may be recognized where someone tries to pitch culture as a new proposed method in an effort to try to get funding or acceptance. To illustrate, here is a sample what such a discussion might be like on the well-known *Shark Tank* TV program.

## Shark Tank Routine Pitching Culture

**Pitchman:** I have a new test method that I think would be useful for testing for *Legionella* bacteria and should be part of a plan to reduce the risk of Legionnaires' disease.

**Committee:** How does it work?

**Pitchman:** Well, you take a sample of water, filter it to concentrate the bacteria, resuspend what was filtered, plate it on a culture media and grow the bacteria, and then count the colony forming units.

**Committee:** So, one *Legionella* bacteria in the water will create one colony-forming unit—right?

**Pitchman:** Well not exactly. A colony-forming unit can be created by one cell, or two, or three, or 10, or 100, or even 1,000. A colony-forming unit could be created by a fragment of biofilm that may have even thousands of *Legionella* bacteria cells contained in it.

**Committee:** Oh, I see. But at least it does detect all the *Legionella* in the water sample?

**Pitchman:** Well, no, not really. *Legionella* are very hard to grow, and there can be so many other types of bacteria there, so we need to do pretreatment steps that probably kill a lot of the *Legionella* or stress them so much that they will not grow on the agar. They are viable but not culturable.

**Committee:** Are the viable but not culturable bacteria dangerous, and could they cause Legionnaires' disease?

**Pitchman:** Well, I guess so. Yes, probably. That is a concern.

**Committee:** So, the method grows all the *Legionella* in the water except those that we kill or inhibit from growing?

**Pitchman:** Well, not quite. Often, bacteria that are contained within amoeba do not culture either, but maybe they do if they make it out of the amoeba, and in that case could grow.

**Committee:** So, let me get this straight, cells that can be exposed to the culture material and still are viable to grow, will show up as a colony-forming unit on the plate and can be recognized and counted as a *Legionella* bacteria?

**Pitchman:** Sort of, but not exactly. In the end, I must admit that the recovery rate can be quite low, and also inconsistent. It takes a certain skill to recognize a *Legionella* colony and to distinguish it from non-*Legionella* colonies, so we take an isolate sample from a representative amount of the colonies that grew and replate on two different types of other plates. One plate type can grow the *Legionella* and the other typically will not support growth. That way we can confirm that it really was *Legionella*.

**Committee:** Hmm, this sounds like you might have to spend quite a bit of time to finally get an answer?

**Pitchman:** Yes, but it is really dependent on the water sample and the matrix of that water. We often might have preliminary indication after three to seven days, but some samples will take 14 days or longer.

**Committee:** Are you kidding? I'd have to wait up to 14 days compared to 4 hours? You can probably recognize the different species of bacteria that are growing as colonies though, right?

**Pitchman:** Oh no. There are other steps to try to find the specific species.

**Committee:** So, let me understand this so far:

- The test can find *Legionella* in the water but may miss many or even most.

- It takes seven to 14 days to get an answer.
- The colony-forming unit is a colony that results from the genesis of many possible combinations of *Legionella* groupings, so it doesn't represent any consistent amount of *Legionella*.
- The *Legionella* are extremely difficult to separate from background flora and are hard to culture and grow and then identify, so it can be quite dependent on a laboratory's skill and experience.
- And it is not really useful in quantifying or giving consistent results.
- qPCR on the other hand is:
  - Precise
  - Consistent
  - Provides results in 4 hours of lab work
  - Can identify *Legionella* species, *Legionella pneumophila*, SG-1,and SG-15 all in one step
  - Does not require much lab training
  - May be more useful in linking bacteria concentration to risk of Legionnaires' disease
  - Is cost effective

**Pitchman:** That about sums it up, but we think calling culture the *Gold Standard* could help get it better accepted.

## Conclusion

Since the discovery of *Legionella* bacteria, the culture method has been used. Even with improvements, it remains a very difficult culture test for capturing, growing, and identifying *Legionella* bacteria in a water sample. A rather high level of experience is required to make some of the subjective decisions described in standard procedures, it is imprecise, does not accurately quantify, and takes days for results.

Increased awareness of modern qPCR capabilities should lead to it becoming the preferred laboratory test method for *Legionella* bacteria. For most regular monitoring of water systems, *Legionella* action and alert efforts, or remediation processes, modern qPCR testing should improve water safety.

An important step for acceptance and greater awareness would be for the AOAC International (Association of Official Analytic Chemists) to approve the qPCR method through their development process. On its website, it states that "approved methods undergo rigorous, systematic, scientific scrutiny to ensure they are highly credible and defensible—and can be used with confidence by industry, regulatory agencies, research organizations, testing laboratories, and academic institutions." This would allow agencies, organizations, and end-users to have confidence in the method and results.

Statements made in publications such as AWT's 2019 *Legionella: A Position Statement and Guidance Document,* and the 2020 publication *Management of Legionella in Water Systems,* from the National Academies of Sciences, Engineering, and Medicine, all need to be updated to recognize the full capabilities of today's qPCR method and that qPCR could replace culture for most monitoring and mediation work.

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