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Review

From Culture to Omics: Laboratory Methods for Environmental Detection of *Legionella* in Water Systems

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Abstract

Legionella remains a persistent public health concern in engineered water systems, where complex ecological interactions, biofilm dynamics, and disinfection pressures complicate reliable detection and interpretation. Traditional culture-based methods (ISO, AFNOR, CDC) continue to serve as the gold standard and are indispensable for compliance, yet their long turnaround times, labor-intensive workflows, and inability to detect viable but non-culturable (VBNC) cells limit their usefulness in proactive or high-frequency surveillance. These constraints have stimulated the development of a broad spectrum of alternative approaches, ranging from more or less rapid culture assays (Legiolert®, Scan-VIT®, IMC, MICA *Legionella*, IMC) and hybrid methods (PVT VIABLE®, LeVoScan) to molecular techniques (qPCR, vPCR, dPCR, LAMP) and rapid/emerging platforms (FCM, IMS and biosensors). Collectively, these methods promise faster results, improved sensitivity, and deeper insight into microbial ecology. This review evaluates the used currently and emerging techniques, detailing their analytical mechanisms, performance characteristics, and operational constraints. The comparative evaluation revealed that single-method strategies inevitably redistribute rather than resolve the fundamental trade-offs between processing speed, taxonomic specificity and biological relevance. To address these limitations, we propose a tiered, fit-for-purpose surveillance framework integrating complementary tools across four functional levels: rapid screening for early warning, quantitative assays for regulatory compliance, high-resolution methods for identification and epidemiological characterization, and viability-oriented approaches useful in a research setting. This multi-modal strategy shifts Legionella monitoring from a method-centric paradigm toward a decision-oriented process, aligning analytical performance to viability assessment and enabling more timely interventions.

Keywords: system; surveillance; culture

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Academic Editor: Alexander Machado Cardoso

Received: 2 September 2026
Revised: 6 November 2026
Accepted: 11 November 2026
Published: 18 November 2026

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1. Introduction

Waterborne pathogens remain a persistent challenge for public health, particularly in complex engineered water systems where microbial ecology, infrastructure design, and operational conditions interact to favor the persistence of opportunistic microorganisms [1,2]. Among these, *Legionella spp.* represent a major concern due to their environmental ubiquity, resistance to disinfection, and association with severe respiratory infections, including Pontiac fever—a milder, flu-like disease—and Legionnaires' disease (LD), especially in elderly and immunocompromised populations, which, if untreated, usually worsens, becoming fatal [1,3]. The incidence of legionellosis has been increasing due to urbanization

(inhalation most often occurs in the built environment and the risk of inhalation is greatly increased also during construction activities) [4], climate change [5] and also increased surveillance.

The genus *Legionella* comprises more than 60 species distributed across natural and man-made aquatic environments, with *Legionella pneumophila*—particularly serogroup 1—accounting for the majority of reported cases [6]. However, an increasing number of non-*pneumophila* species have been implicated in human disease, highlighting the ecological and clinical diversity of this genus [3,7,8].

Legionellae are ubiquitously present in both natural and anthropogenic freshwater environments, where they can withstand temperatures of 5.0 °C–63 °C (replication begins at 20 °C. They are especially localized in engineered water systems, including building plumbing, cooling towers, and healthcare facilities. Environmental stressors such as temperature fluctuations, stagnation, and disinfectant exposure promote bacterial persistence and adaptation, often within biofilms and protozoan hosts [9–11].

An adaptive strategy of *Legionella* is the transition into the viable but non-culturable (VBNC) state, in which cells remain metabolically active yet fail to grow on standard culture media [12]. Various environmental stresses—such as sub-lethal temperatures, nutrient deprivation, and chemical exposure—can also induce the viable but non-culturable state (VBNC) [13–15]. However, successfully recovering viable *Legionella* depends on several critical factors. These include proper sample collection and transport and storage (transport and storage below 6 °C might induce a non-culturable state and reducer recovery of *Legionella* by culture, above 20 °C is likely to result in growth of non-target species that might interfere with detection by culture) [16], appropriate sample pre-treatments to enrich viable cells, and the selection of suitable culture media [17,18]. VBNC cells can be resuscitated naturally under specific environmental conditions, but they can also be resuscitated in the laboratory by co-culture with natural protozoan hosts [19]. This adaptative status poses a critical challenge for environmental monitoring: by decoupling microbial presence from cultivability, it complicates the interpretation of laboratory results, underscoring the need for both a clearer definition of "viability" and rapid methods to measure it. A key, yet frequently underestimated, determinant of surveillance performance is sampling strategy. Most analytical approaches focus on planktonic cells, despite substantial evidence that biofilms represent the primary ecological reservoir for *Legionella* persistence, protection from disinfectants, and amplification within engineered water systems [10,13]. This discrepancy introduces a structural bias in environmental monitoring, whereby negative water samples may not indicate true absence of contamination but rather spatial compartmentalization within biofilm-associated niches.

Legionella testing can be done as part of an "investigation into reported Legionnaires' disease cases", as "routine testing" in situations with no reported cases of Legionnaires' disease. Routine testing for *Legionella* is one way to check that a program to maintain water quality is working. Testing can also provide a baseline measurement, so that *Legionella* growth, if found, can be checked over time [20].

For decades, environmental monitoring of *Legionella* has relied on culture-based methods standardized by the ISO (11731:1998; ISO 11731:2017) [21,22], Association Française de Normalisation (AFNOR) [23], and Centers for Disease Control and Prevention (CDC) [24] that provide colony-forming unit (CFU)-based quantification and enable isolate recovery for epidemiological investigations. While these methods remain the regulatory cornerstone, their limitations—particularly long turnaround times and inability to detect VBNC populations—have become increasingly evident.

In response, a diverse array of alternative and complementary laboratory approaches has emerged, including rapid culture-based assays and molecular techniques [25]. These

methods offer improved analytical speed and sensitivity, but they introduce a critical interpretative challenge: the dissociation between detection and biological relevance [26,27], herein referred to as the "viability dilemma".

Despite substantial technological advances, current surveillance strategies remain largely method-centric, with individual techniques applied in isolation rather than as components of an integrated decision-making process. This fragmentation limits the ability to translate analytical outputs into actionable risk management strategies, particularly in complex systems where microbial dynamics are spatially and temporally heterogeneous. Taken together, these limitations are not independent but systemic, reflecting a fragmentation of surveillance across disconnected analytical layers.

The purpose of this narrative review is to provide a comprehensive overview of the published literature on laboratory methods for the environmental detection of *Legionella spp*. in water systems, with a particular focus on analytical objectives, performance characteristics, and operational constraints. The advantages, limitations, and applicability of current laboratory methods are discussed, alongside their potential to accurately identify sources of infection and contribute to the prevention and mitigation of the risk of legionellosis and in order to provide a current snapshot of ongoing scientific research and the extent to which these findings are transferable and applicable to routine testing laboratories.

Beyond method comparison, we propose a conceptual shift toward a tiered, decision-oriented surveillance framework, in which complementary analytical tools are integrated across functional levels, ranging from rapid screening to strains characterization for epidemiological investigation and research purposes.

2. Methods

A broad literature search was performed to identify significant contributions addressing laboratory methods for environmental detection of *Legionella spp*. in water systems. The search included the following electronic databases, PubMed, Medline, Scopus, Web of Science (WoS) and Google Scholar, using the combinations of keywords "*Legionella*" OR "*Legionella pneumophila*") AND ("water" OR "drinking water" OR "cooling tower" OR "environmental samples") AND ("detection methods" OR "novel methods" OR "rapid detection"). To further refine the search, "MeSH Terms" (on PubMed and Medline) were also used, extracted from the first relevant articles found (e.g., Sensitivity and Specificity, Limit of Detection, Time-to-Result).

Access to articles retrieved from selected databases was provided through the UNITO University Library System (SBA) https://www.unito.it/ateneo/organizzazione-strutture-e-sedi/strutture/biblioteche (accessed on 1 April 2026).

A manual search was also conducted by screening guidelines and the reference lists of included studies (backward citation searching) and identifying subsequent studies that cited the included studies (forward citation searching) to capture additional relevant evidence not identified through the electronic database searches.

The scientific evidence is presented by summarizing the results in order to provide an overview of the topic through a descriptive analysis. Methodological quality appraisal was not undertaken in this review. The search was limited to English-language publications published between January 2000 and September 2026. Reference time was selected to capture contemporary and evolving evidence on *Legionella* laboratory methods. Studies published before 2000 were excluded.

The review focused on research, including qualitative, quantitative, and mixed-methods studies.

All retrieved records were imported into Zotero software for reference management and organization.

Findings were then synthesized to identify advantages (Section 3) and disadvantages (Section 4).

3. Results

3.1. Traditional and Rapid Culture Methods

Culture-based methods are the most commonly used method for environmental surveillance of *Legionella spp*., representing the standard for regulatory monitoring and compliance. Their main strength lies in the ability to recover viable organisms and obtain isolates suitable for downstream phenotypic and genotypic characterization, which is essential for outbreak investigations and source attribution [17]. However, despite their central role in surveillance frameworks, culture methods are intrinsically limited by long incubation times [21,23,24], susceptibility to environmental inhibitors, presence of disinfectants, inability to detect VBNC populations and reduced sensitivity in complex microbial communities [28].

In recent years, a series of optimized and alternative culture-based approaches has emerged, aiming to improve turnaround time, reduce operator-dependent variability, and enhance analytical performance while maintaining compatibility with CFU-based regulatory reporting [29–32].

3.1.1. ISO 11731:2017 Water Quality—Enumeration of *Legionella*

ISO 11731:2017 [21] is the internationally recognized reference method for the detection and enumeration of *Legionella* in water samples.Figure 1 illustrates a typical workflow for this method.

The choice of the method (fourteen different procedures are described) used for the enumeration of *Legionella* species depends on the origin/characteristics of the sample and the reason for sampling or investigation. Once the desired lower limit of detection (LOD) level for a sample is known, this will play an important role in the selection of the most appropriate method. The decision matrix (Annex J, Figure J.1 of ISO 11731) summarizes all possible types of water (samples), methods, treatments, culture media and the procedures. Depending on the type of sample, it is possible to plate the sample on buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYE), as well as on media with selective agents glycine–vancomycin–polymyxin–cycloheximide agar (GVPC) [33], modified Wadowsky and Yee agar (MWY) and buffered charcoal yeast extract agar supplemented with selective supplement (BCYE+AB) [34]. Selective agents are essential to suppress competing environmental microbiota, but they also introduce selective pressure that can reduce recovery of stressed *Legionella* cells.

After incubation at 36 °C for 7 to 10 days, presumptive colonies are recognized based on morphology. Regarding the morphological recognition of colonies, *Legionella* colonies typically present a smooth texture, intact edges, a white-grey color, and a characteristic ground-glass appearance. To confirm the presence of *Legionella*, presumptive colonies should be inoculated onto cysteine-free BCYE agar (BCYE-cys). Isolates that grow on BCYE but fail to grow on BCYE-cys are confirmed as *Legionella*, supported by biochemical or serological testing (Annex G of ISO 11731). The results are expressed as CFU/L.

For special situations like outbreak investigations, at least five presumptive colonies if only one morphology is present, or two presumptive colonies for each type of morphology, shall be confirmed according to the method listed in Annex G (G.1) of ISO 11731.

Several trials were undertaken to determine the performance characteristics of the ISO procedures. Results from an intralaboratory trial and three interlaboratory studies are summarized in Annex H (Table H.1 and Table H.2) of ISO 11731. The LODs for this method are reported in Table J.1 of ISO 11731.

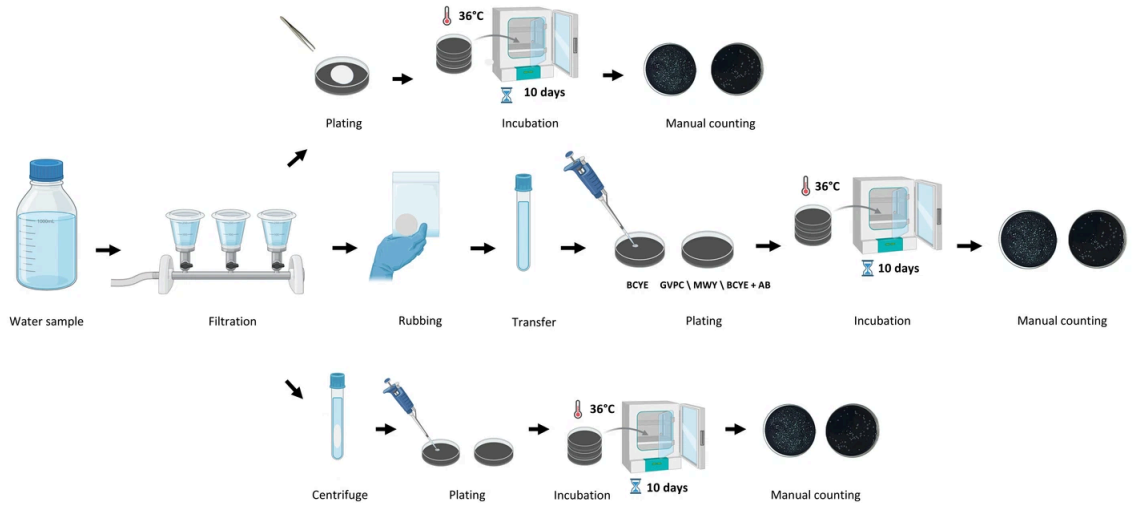


Figure 1. ISO 11731:2017: brief schematic procedure.

3.1.2. Legiolert™

Legiolert™ (IDEXX Laboratories, Inc., Westbrook, ME, USA) is a liquid culture-based method utilizing the Most Probable Number (MPN) principle for the detection and enumeration of *L. pneumophila* across various water matrices (such as potable water, non-potable water, cooling towers, and public bath water). Some validation studies have reported no significant difference from the ISO 11731 plate culture method in potable samples [29,35].

The workflow begins with evaluating sample water hardness—excessive hardness can interfere with test performance. The water sample is then mixed with the specialized Legiolert® liquid reagent medium, which contains a substrate specifically metabolized by *L. pneumophila*. This mixture is dispensed into a multi-well Quanti-Tray®/Legiolert® vessel, sealed, and incubated for up to 7 days. Metabolism of the substrate produces brown pigmentation or turbidity in positive wells, which are then counted to calculate the *L. pneumophila* concentration using MPN tables [29,36]. Figure 2 illustrates a typical workflow for this method.



Figure 2. Legiolert™: brief schematic procedure.

This approach offers several operational advantages, including a simplified test procedure and an easy-to-read positive signal. A major advantage of the method is that VBNC *L. pneumophila* can reproduce in Legiolert® liquid medium [36,37].

Legiolert® allows researchers to sample the positive culture broth, thereby allowing the *L. pneumophila* strain to be isolated and serotyped. Enumeration by the MPN technique Legiolert has a detection limit of 1 MPN in 100 mL of sample (10 MPN/L) for drinking water and 100 MPN in 100 mL of sample (1000 MPN/L) for non-drinking water. Quanti-Tray/Legiolert provides Most Probable Number (MPN) results up to 2272 per 100 mL sample.

The IDEXX Legiolert™ test has AFNOR Certification (No. IDX 33/06-06/19). Furthermore, the IDEXX Legiolert™ method is standardized and published as an official

international method by the ASTM International standards organization (ASTM D8429-21 standard) [35], and it is now specified as a recommended method in the Standing Committee of Analysts publication [16] and in the Laboratory Handbook (D 5.1) by the EURL-PH-LEGI [38].

3.1.3. Scan-VIT-*Legionella*^{TM}

The Scan-VIT-*Legionella*^{TM} test by Vermicon (Vermicon AG, Hallbergmoos, Germany) is a rapid, fluorescence-based molecular method used to detect and quantify living *Legionella* species and *L. pneumophila* in water matrices (such as drinking water and cooling water). The Scan-VIT-*Legionella*^{TM} system utilizes fluorescence in situ hybridization (FISH) to identify *Legionella* colonies after a shortened incubation period. This method targets ribosomal RNA (rRNA) sequences, which are abundant in metabolically active cells. Following a 3-day incubation period on MWY or GVPC agar, membranes are exposed to fluorescently labeled gene probes specific for both the *Legionella* genus and the *L. pneumophila* species [30,39]. The probes penetrate the bacterial cell wall and hybridize with their target sequences, allowing for identification under a fluorescence microscope [40]. After fluorescence excitation, a dual-color signal identifies *L. pneumophila*—concurrent green and red fluorescence—while other *Legionella* non-*pneumophila* species exhibit only green fluorescence. Figure 3 illustrates a typical workflow for this method.

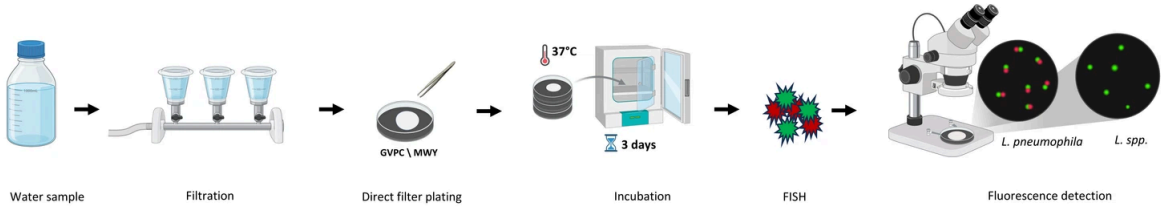


Figure 3. Scan-VIT-*Legionella*^{TM}: brief schematic procedure.

Scan-VIT-*Legionella*^{TM} allows the use of small samples (50/100 mL vs. 1 L), permitting wide-scale sampling with smaller sample volumes that are easier to handle in sample collection, transport and storage. Furthermore, this method reduces total analysis time to 72–75 h and simplifies colony recognition, with the possibility to identify *Legionella* spp. and *L. pneumophila* simultaneously and, given the simplicity of colony identification by fluorescence, the ScanVIT test can be used in laboratories where staff is not experienced in identifying typical colonies of *Legionella*, increasing throughput in routine laboratories. The method expresses the results as CFU/L and does not require any further equivalence with the CFU to establish an environmental intervention on the contaminated source [39]. The Scan-VIT-*Legionella*^{TM} method is supported by peer-reviewed scientific studies and an interlaboratory validation dossier published by research institutes and the manufacturer itself [40,41]. However, it lacks any formal certification of validation. The LOD is 1 CFU/100 mL of sample.

3.1.4. MICA *Legionella*—Microcolony Identification Assay for *Legionella*

The MICA *Legionella* method (Diamidex, Marseille, France) represents a modern evolution of rapid culture, utilizing "click-chemistry" to label *L. pneumophila* microcolonies. After sample filtration, membranes are incubated on GVPC agar for 48 h. Growing cells internalize a modified sugar (pLeg-N_3) and incorporate it into their lipopolysaccharide cell wall structure. A fluorescent bioprobe then binds specifically to this sugar via a highly selective chemical reaction. This labeling occurs at the microcolony stage, long before colonies are visible to the naked eye. After incubation, the membranes are scanned by an automated

AI-driven counter, which identifies and enumerates the fluorescent microcolonies [32,42]. Figure 4 illustrates a typical workflow for this method.

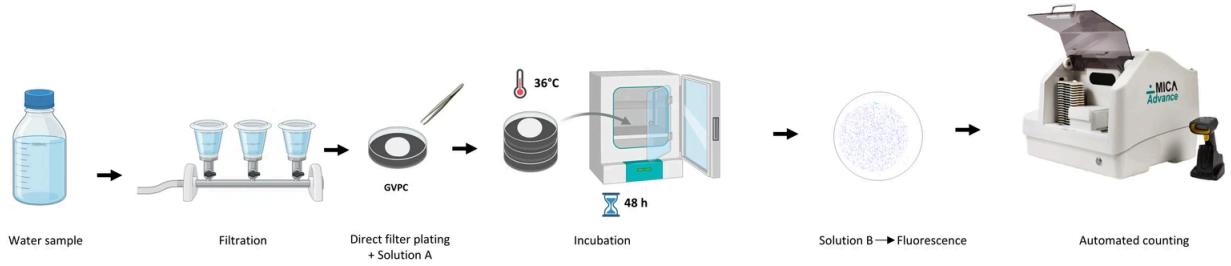


Figure 4. MICA *Legionella*: brief schematic procedure.

MICA is described as a useful diagnostic tool, easily usable even in laboratories with little experience because it reduces the time to obtain confirmed results. It features very simple and rapid sample preparation and avoids the need for large-volume filtration, colony isolation, and additional confirmation or identification. Moreover, the MICA test uses AI and automated imaging to detect and count microcolonies, reducing manual counting errors and it shows a strong correlation with CFU counts. On complex matrices such as cooling tower waters, MICA's timeframe prevents interfering background flora from overgrowing the plates, yielding more reliable results than traditional culture [32,42,43].

The performance data were calculated by comparing the results obtained from analyzing water samples using the MICA method and the ISO 11731:2017 method [32] and therefore officially validated and certified for the detection of *L. pneumophila* (all serogroups) in hot sanitary water and cooling towers within 48 h (Association of Official Analytical Chemists Research Institute Performance Tested Method, certificate number 032201). The LOD for the MICA *Legionella* system depends on the type of water sample being analyzed: 1 CFU/L for domestic/sanitary water and 100 CFU/L cooling tower water.

3.1.5. IMC—Isothermal Microcalorimetry

Isothermal microcalorimetry (IMC) measures the heat flow generated by microbial metabolism under constant temperature conditions [31,44]. As bacteria cells grow in sealed ampoules containing selective broth, thermoelectric sensors detect the minute amounts of heat produced by their catabolic processes. This heat flow signal is proportional to the metabolic activity and number of growing cells, generating a characteristic growth curve. Figure 5 illustrates a typical workflow for this method.

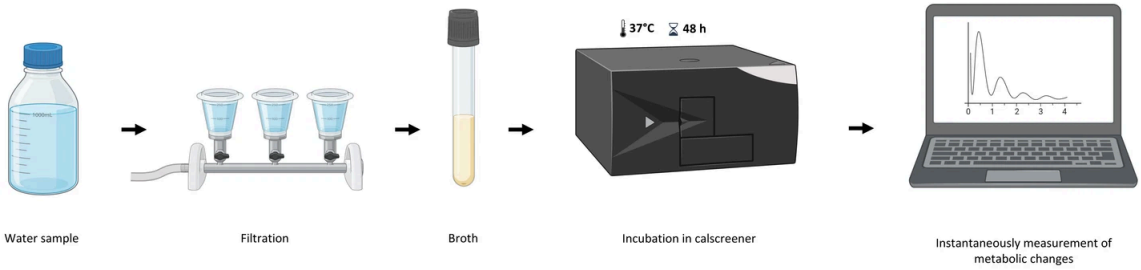


Figure 5. Isothermal microcalorimetry: brief schematic procedure.

IMC has many strengths: it produces metabolic heat in real time, and heat output is treated as an indicator of metabolically active cells [31]. IMC recognizes only active and cultivable bacteria; furthermore, IMC is very easy to implement and data interpretation does not require specific expertise. This method can also be applied for detecting *Legionella*

in environmental samples, according to Fricke et al. [45]. Even though it is a non-specific method, IMC becomes highly selective through the application of chemical (acid treatment) and thermal (50 °C for 30 min) pre-treatments and the use of selective media according to ISO 11731:2017. IMC can detect the presence of Legionella within 24–48 h; critical concentrations (10^2 CFU/100 mL) can be detected after two days and highly contaminated samples (>10^6 CFU/100 mL) can be detected within a few hours to one day. This method allows for the monitoring of microbial activity directly in the samples without prior preparation and offers a very low detection limit. For this reason, IMC can be an early warning system for Legionella contamination. Moreover, it can highlight the metabolic activity of *Legionella* embedded in biofilms and evaluate the efficacy of biocides.

3.1.6. Amoebal Co-Culture

Free-living amoebae play an important role in the environmental persistence, multiplication, and transmission of *Legionella spp*. In particular, *L. pneumophila* can replicate intracellularly within amoebae, which may release vesicles containing large numbers of viable bacteria and potentially facilitate their transmission to humans [44].

Amoebal co-culture exploits the natural intracellular lifecycle of *Legionella* within free-living protozoa such as *Acanthamoeba spp*. and *Vermamoeba vermiformis* [19,46]. Amoebal co-culture serves as a powerful diagnostic tool by resuscitating viable but non-culturable (VBNC) states of *Legionella*., that escape detection by standard culture [47]. Once the bacteria have multiplied and eventually lysed the amoeba host, the sample is inoculated on *Legionella* selective media and incubated, and presumptive colonies are identified. Figure 6 illustrates a typical workflow for this method.

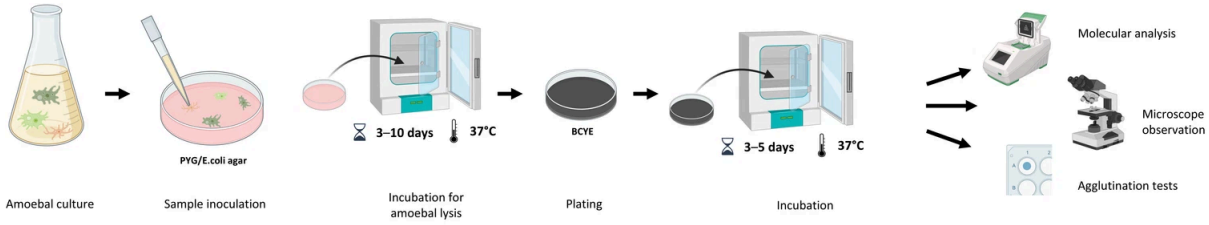


Figure 6. Amoebal co-culture: brief schematic procedure.

3.2. Molecular Methods

While culture-based methods remain essential for regulatory compliance and isolate recovery, their inherent delays and sensitivity limitations have driven the development of faster, culture-independent approaches. Molecular techniques have thus emerged as powerful complementary tools, enabling rapid detection and improved analytical sensitivity across diverse water matrices [12,25,48,49].

When monitoring *Legionella*, there is a well-documented discrepancy between traditional culture and molecular method results. Traditional methods measure colony forming units (CFUs), focusing solely on bacteria capable of replication, while molecular techniques measure genomic units (GUs), which account for the total DNA present. Consequently, GU values are typically much higher because they include DNA from dead or VBNC cells that traditional cultures fail to detect [50].

3.2.1. Quantitative PCR (qPCR) and Viability PCR (v-qPCR)

Quantitative polymerase chain reaction (qPCR) has been developed for real-time monitoring of *Legionella* in water systems and for the follow-up of disinfection treatments. The method is both rapid, specific and sensitive and can target genes such as 16S rRNA or

MIP to estimate the concentration of *Legionella* in the sample by expressing the result as GU/L. Its main advantage is speed, providing results within a few hours, which is particularly valuable in outbreaks [51–54]. Figure 7 illustrates a typical workflow for this method.

The performance of qPCR may be reduced in complex environmental samples with high turbidity or organic load, obtaining a reduction in the qPCR signal from dead cells, [53,55]. However, the interpretation of qPCR results has been largely controversial: several studies comparing culture and real-time PCR methods in different water types showed a higher rate of positive results and higher quantification values with real-time PCR compared to the standard culture method [54,56].

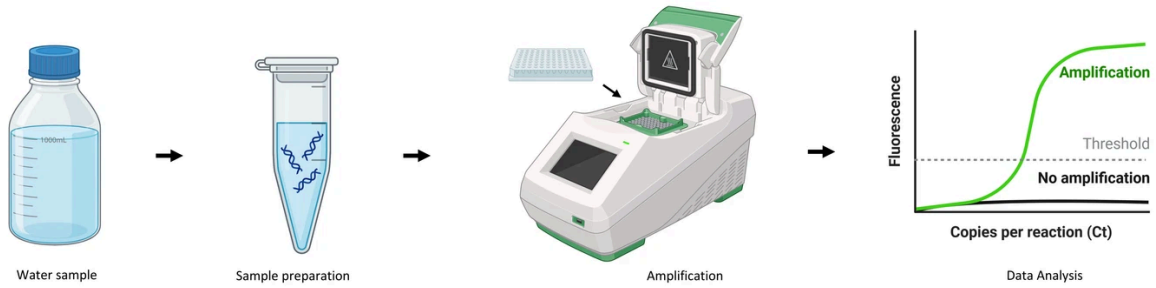


Figure 7. PCR: brief schematic procedure.

To refine this gap, intercalating dyes (such as propidium monoazide—PMA—or ethidium monoazide—EMA) can be used; these dyes selectively penetrate cells with damaged membranes and prevent the amplification of their DNA; therefore, this molecular approach, known as viability PCR (v-qPCR), improves the correlation with viable populations [27,57–59].

There are validated commercial kits for qPCR (NF Validation Certificate No.: BRD 07/15-12/07; BRD 07/16-12/07) [60,61] and v-qPCR (manufacturer-associated validation: AOAC Performance Tested Method^{SM} 082303) [62] designed specifically for the detection and quantification *Legionella* in aqueous and environmental matrices [51,63].

The LOD for *Legionella* using the qPCR method, depending on the specific assay design, ranges from 5 copies/reaction [60] to 10 copies/reaction [62].

Overall, together, qPCR and v-qPCR are mainly used as rapid screening tools for environmental surveillance.

3.2.2. Digital PCR (dPCR and ddPCR)

Digital PCR (dPCR) represents an evolution of conventional PCR [64] by enabling absolute quantification of target DNA without the need for standard curves. The sample is partitioned into thousands of micro-reactions, and target concentration is calculated based on the proportion of positive partitions using Poisson statistics [65,66].

Compared to traditional qPCR, dPCR offers superior quantitative precision, inter-laboratory reproducibility, and a tolerance to enzymatic inhibitors commonly present in complex environmental water samples, such as those collected from industrial cooling towers or heavy industrial water loops [64,66]. Figure 8 illustrates a typical workflow for this method.

Droplet Digital PCR (ddPCR) is a sub-category of digital PCR, with ddPCR offering higher precision, high-throughput capabilities [67,68], and the partitioning of low concentrations of *L. pneumophila* [69]. Unlike RT-qPCR, ddPCR is less affected by the presence of fragmented DNA, making it particularly suitable for ultra-sensitive monitoring near regulatory action thresholds and ensuring robust data acquisition in highly challenging matrices [70]. LODs vary from reaction to reaction (sensitivity increases with the number

of droplets per reaction) [71]. The LOD for ddPCR targeting *Legionella* typically ranges from 1 to 5 copies of target DNA per reaction (or roughly 1 to a few genomic units per well/reaction), depending on the specific assay (such as targets for the mip gene or 5S rRNA gene) [54]. Figure 9 illustrates a typical workflow for this method.

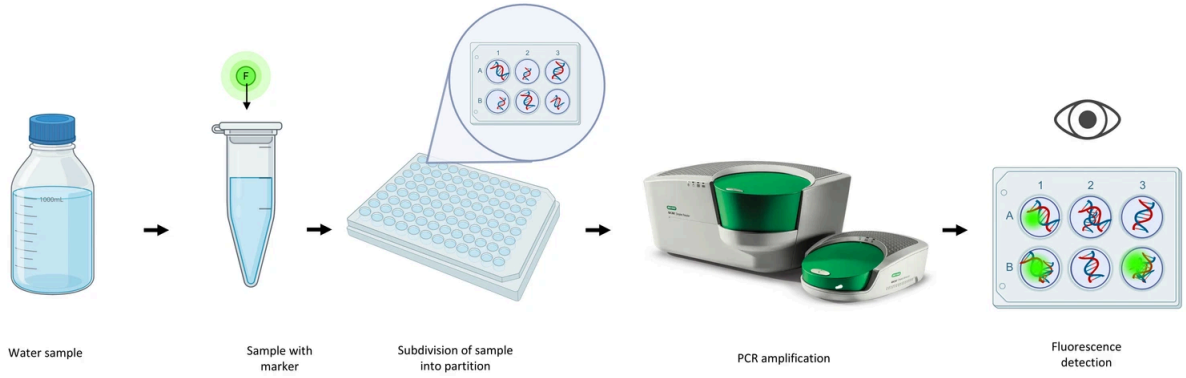


Figure 8. dPCR: brief schematic procedure.

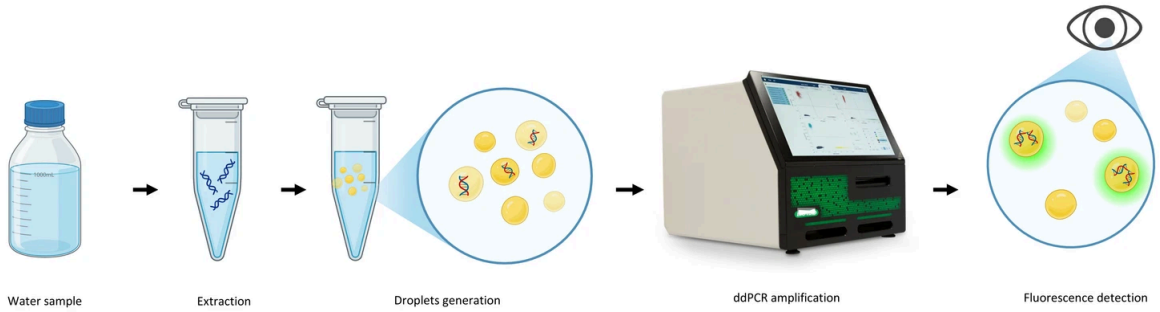


Figure 9. ddPCR: brief schematic procedure.

3.2.3. LAMP—Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is an innovative isothermal nucleic acid amplification technique that uses only one type of enzyme and enables rapid DNA amplification at a constant temperature (60–65 °C), completely eliminating the need for complex thermal cycling instrumentation [72–74]. By utilizing 4 to 6 specialized primers (targeting 16S rRNA gene for *Legionella* species or *L. pneumophila*-specific mip gene) [75] alongside a strand-displacing DNA polymerase, LAMP rapidly forms loop structures that drive exponential target amplification in a single step, with results readable by simple turbidity, fluorescence or color changes. Figure 10 illustrates a typical workflow for this method.

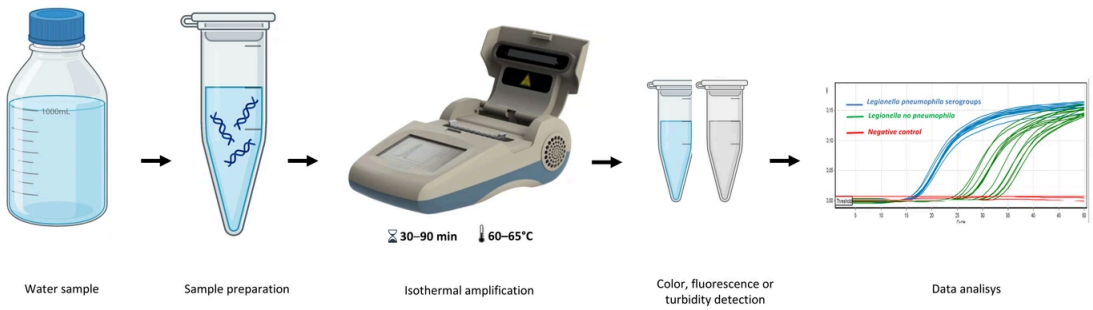


Figure 10. LAMP: brief schematic procedure.

This specific design makes it exceptionally suitable for rapid, decentralized and point-of-care testing for highly specific species-level identification of *L. pneumophila* [76,77].

The matrices suitable for analysis using this test are environmental water (hot springs, cooling towers, showers, domestic hot water). The performance data were calculated by comparing the results obtained from analyzing water samples using the LAMP method and the ISO 11731:2017 method [77]. Overall, the commercial tests based on this technology have been validated by independent laboratory, demonstrating equivalence to the standard culture-based reference method [74,77].

The LOD for *Legionella* using LAMP method varies depending on the specific assay design, targeting mechanism, and detection principle. It generally ranges from 2 genomic copies/reaction [78] to 5.2 to 52 genomic copies/reaction [74] or 28 plasmid copies/µL [77] (validation according ISO/TS 12869:2019) [79].

3.3. Hybrid Viability Enrichment Approach

To bridge the operational gap between slow conventional culture and rapid molecular assays, such as quantitative polymerase chain reaction (qPCR), recent strategies have introduced integrated culture-based molecular platforms.

3.3.1. PVT VIABLE®

To overcome the diagnostic bottleneck of traditional culture methods, the PVT VIABLE® (Viability Identification Assay by *Legionella* Enrichment (Phigenics, Warrenville, IL, USA) method has emerged as an alternative. This protocol employs a brief selective liquid enrichment stage followed by a multiplex molecular readout. It relies on the ability of intercalating DNA dyes to penetrate the cell and render the DNA useless for PCR, quantifying the change in target DNA concentration over a short incubation window. Since only living cells can replicate, a positive delta selectively confirms bacterial viability [80]. Figure 11 illustrates a typical workflow for this method.

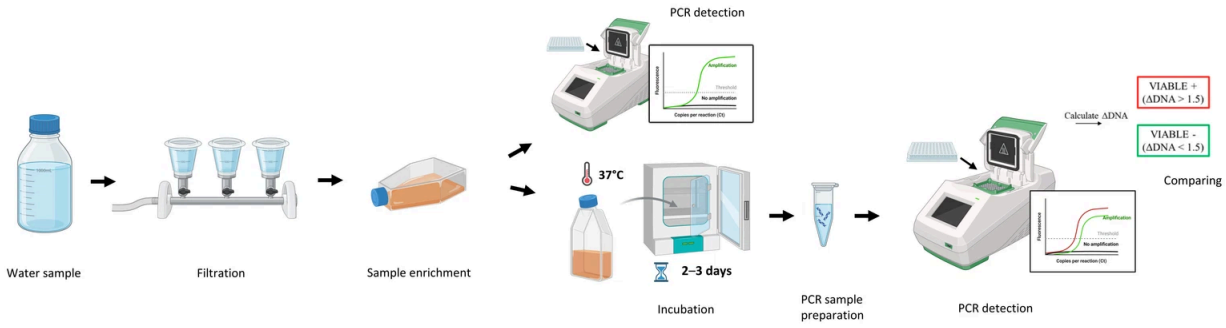


Figure 11. PVT VIABLE®: brief schematic procedure.

By utilizing a liquid enrichment matrix, the turnaround time is to 2–3 days. The liquid culture is intended to facilitate the recovery of sublethal injured or VBNC cells that are missed by standard solid agar media. Furthermore, it intercalates dye function by preventing the amplification of DNA from membrane-damaged cells rather than serving as a direct indicator of metabolic activity or cell viability. The validation study was conducted under the AOAC Research Institute Performance Tested Method℠ in the laboratories of Phigenics on potable water sample and non-potable water samples against the ISO 11731:2017 reference standard. Statistical analysis showed comparable results between PVT VIABLE presumptive and ISO 11731:2017 for the matrixes tested in this study [80].

The PVT VIABLE® assay has a nominal LOD equivalent to 1 CFU per 100 mL sample volume.

3.3.2. LeVoScan

Similar to the PVT method is the LeVoScan method (Vitens/KWR Water Research Institute, Nieuwegein, The Netherlands), which combines a 48 h selective liquid pre-enrichment step at 37 °C with a subsequent diagnostic PCR assay [81]. This hybrid system selectively amplifies only culturable *L. pneumophila* cells capable of multiplying in the liquid medium. It delivers verified culturable results in approximately 50 h—five days faster than standard plate culture. Overall, the sample pre-enrichment strategy makes it possible to reduce the analysis time by several days.

Validation of LeVoScan followed ISO 16140-4 [82] with ISO 11731:2017 as a reference method using naturally and artificially contaminated and uncontaminated drinking water samples. The results of the Relative Limit of Detection (RLOD) show that at least 1 CFU/100 mL of *L. pneumophila* can be detected with LeVoScan.

3.4. Rapid and/or Emerging Detection Techniques

To complement existing analytical frameworks, recent innovations have stimulated the development of rapid, real-time monitoring technologies that prioritize analytical speed, automation, and system-level dynamics. These emerging platforms aim to provide high-throughput performance and immediate operational insights, serving as efficient early-warning systems within engineered water infrastructures.

3.4.1. IMS—Immunomagnetic Separation

Immunomagnetic separation (IMS) for detecting *Legionella* utilizes anti-*Legionella* magnetic beads combined with an enzyme-linked colorimetric assay to rapidly isolate target cells. Following the ISO 11731:2017 protocol, the water sample is filtered, concentrated, and eluted. When immunomodified magnetic beads are introduced, they bind to any target *Legionella* cells present. These bacteria–bead complexes are isolated via a magnetic field, washed, and incubated with a horseradish peroxidase (HRP)-conjugated antibody. The final addition of HRP substrates triggers a colorimetric reaction that visualizes the captured bacteria [83,84]. Figure 12 illustrates a typical workflow for this method.

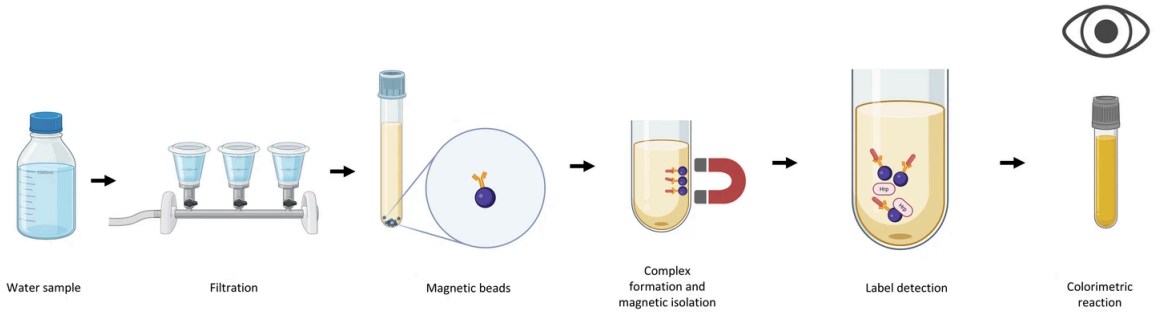


Figure 12. Immunomagnetic separation: brief schematic procedure.

The most remarkable aspect of this approach (the elimination of pre-enrichment steps, DNA extraction and amplification techniques) results in analytical simplification and a significant reduction in the total analysis time, obtaining the results within hours [83]. This IMS technology seems to offer substantial performance benefits over traditional culture methods, reporting high efficiency relative to culture [85].

Linking to this, IMS technology has shown high sensitivity and specificity when compared to culture (ISO 11731: 2017). Another advantage of IMS methods is the speed of the analysis; as the method does not rely on the proliferation of *Legionella*, results can be

obtained within a few hours, even in heavily contaminated water and in the presence of growth inhibitors.

One specific commercial test based on this technology has been validated by a manufacturer-associated laboratory according to the AOAC Performance Tested Method^{SM} (certificate number 052002) [86,87], demonstrating equivalence to the standard culture-based reference method ISO 11731 (LOD at 21 CFU_{eq}/L and the LOQ at 80 CFU_{eq}/L for potable water). Moreover, the method demonstrates superior repeatability and reproducibility, as well as equivalent or even superior to the standard method 11731:2017 across three different types of water matrixes (potable, surface, and industrial process water).

3.4.2. FCM—Flow Cytometry

Recent advancements have sought to address the lack of taxonomic specificity in traditional flow cytometry (FCM) by combining viability dyes with target-specific labeling [88–90]. The FCM technique characterizes and quantifies individual suspended particles by passing them one at a time through a light source, typically a laser beam. Fluorescent particles (autofluorescent or made fluorescent, as in the case of bacteria after staining with fluorescent dyes or fluorescent antibodies) are excited by the light source and emit light at a longer wavelength for the rapid and selective detection and enumeration of *Legionella* cells.Figure 13 illustrates a typical workflow for this method. FCM offers valuable capabilities for detecting VBNC *Legionella* populations that are missed by standard BCYE agar culture. The method is also reproducible, rapid and suitable to analyze all types of water samples (drinking water, process water, environmental water). For the detection and counting of bacteria such as *Legionella* in water samples, it is often used in combination with the IMS method: according to Keserue et al. [87], by using the IMS-FCM method, the recovery is over 85% of spiked *L. pneumophila* sg.1 cells in 1 L of tap water and detection limits are around 50 and 15 cells/L for total and viable *L. pneumophila* sg.1, respectively.

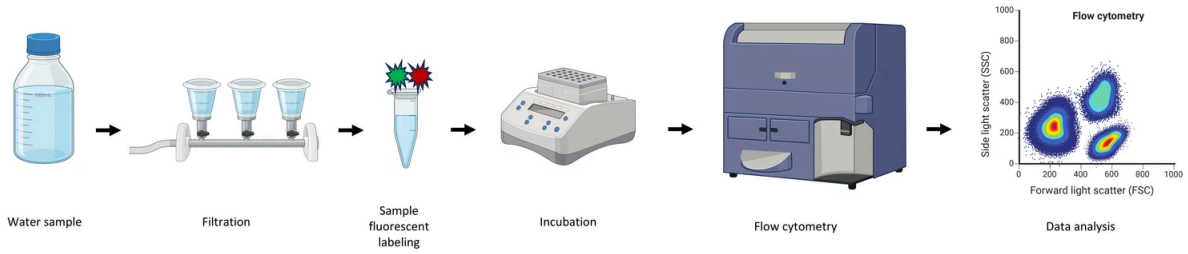


Figure 13. FCM: brief schematic procedure.

The LOD varies depending on the protocol used and ranges from 50 to 86.5 intact *Legionella* cells per 100 mL of sample (ILC/100 mL) [91].

3.4.3. Biosensors and On-Site Detection

Biosensor technologies aim to decentralize environmental testing by bringing the laboratory directly to the field. In 2–3 h, these compact devices integrate a biological recognition element (such as highly specific monoclonal antibodies, engineered aptamers, antimicrobial peptides, or targeted DNA probes) with a high-sensitivity transducer (optical, electrochemical, or piezoelectric) that automatically converts a binding event into an instantaneous, measurable electronic signal [25,92]. Figure 14 illustrates a typical workflow for this method. Biosensors provide real-time and in situ analysis of the targeted analyte, without costly and complicated sample preparation.

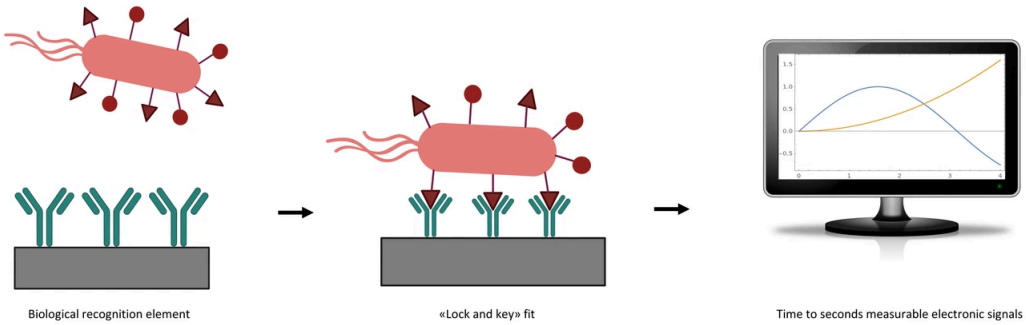


Figure 14. Biosensor technology: brief schematic generic procedure.

Biosensors have wide applications, including biomarker detection for medical diagnostics, as well as pathogen and toxin detection in food and water.

A recent systematic review of biosensing technologies for *Legionella* detection [93,94] catalogued a broad diversity of sensing architectures—including advanced optical resonators, electrochemical immunosensors, magnetic nanoparticle assays, and mass-sensitive platforms—noting their analytical performance, low limits of detection, and rapid response times under optimized conditions.

Most biosensors that are associated with *Legionella* detection are immunosensors [95,96] and genosensors [97–99].

The LOD for *Legionella* biosensor methods varies by sensing modality, target type (whole cells vs. DNA/RNA), and sample preconcentration strategy [92].

3.5. Methods for Strain Characterization and Epidemiological Investigation

When a sporadic case or outbreak of Legionnaires' disease is declared, epidemiological studies must be carried out to enable investigators to precisely identify the exact source of LD and determining risk factors in order to prevent future outbreaks. Certain laboratory techniques may be more effective in investigating specific outbreaks, as they can identify particular phenotypic and genotypic traits that allow for the differentiation of the strains. Therefore, the integration of conventional microbiological methods with other to enhance surveillance accuracy, support outbreak investigations, and strengthen public health responses.

3.5.1. Subtyping by Dresden Panel: Phenotypic Typing

Phenotypic subtyping toolkits like the Dresden Panel—a standardized panel of monoclonal antibodies (Mab)—provide critical epidemiological utility by differentiating *L. pneumophila* serogroup 1 into sub-groups associated with severe clinical outbreaks and reproducible classification data.

It is well-established that the epitope recognized by Mab 3/1 is present in select *L. pneumophila* sg 1 subgroups, including Knoxville, Philadelphia, Benidorm, and France/Allentown. These strains are predominantly implicated in nosocomial, travel-associated, and community-acquired infection outbreaks [6,100,101].

3.5.2. Subtyping by Sequencing

The molecular epidemiological tools currently used in *Legionella* laboratories include sequence-based typing (SBT) [102–104], and recently, Next-Generation Sequencing (NGS) [105] and Whole Genome Sequencing (WGS) [106] have become increasingly popular. These methods have great discriminatory power, but they are designed to be applied after obtaining the microbial strains from samples [107].

SBT was considered the gold standard in outbreak detection for many years. SBT of *L. pneumophila* is usually performed by Sanger sequencing of seven gene fragments, and it provides a specific sequence type that can be matched against an international database [101–103]. However, WGS has revolutionized the typing of *L. pneumophila*, surpassing the limited resolution of SBT [106,107].

Expanding beyond targeted amplification, NGS and specifically WGS represent the highest level of molecular resolution, shifting the paradigm from single-locus detection to comprehensive genomic characterization. WGS offers exceptional strain-level resolution, making it an invaluable tool for epidemiological outbreak investigations and source tracking. Furthermore, in silico analyses can identify candidate virulence factors, mobile genetic elements, and potential antimicrobial resistance determinants. However, genomic sequence identification alone does not establish functional expression or phenotypic pathogenicity; as emphasized in environmental-isolate studies, sequence-based predictions of virulence and resistance remain indicative and require downstream experimental and phenotypic confirmation [108].

3.5.3. Fourier Transform Infrared Spectroscopy

Recent advancements in microbial phenotyping have highlighted the potential of Fourier Transform Infrared (FT-IR Biotyper) spectroscopy as a rapid, cost-effective, and easy-to-use alternative to traditional bacterial typing [109]. Operating downstream of primary isolation, the system exploits the infrared absorption spectra of surface biomolecules—such as lipopolysaccharides—to generate a strain-specific molecular fingerprint from cultured isolates. While further expansion of reference spectral databases is required to refine classification algorithms and minimize misclassifications, FT-IR spectroscopy represents a promising high-throughput tool for post-culture characterization. If integrated into routine surveillance, this approach may support epidemiological cluster investigations [110].

Table 1 summarizes the key characteristics and operational advantages of the primary laboratory approaches for environmental *Legionella* detection.

Table 1. Systematic comparison of discussed methods.

Method	Principle	Target	Matrix	Output Result	Time to Result	LOD LOQ	Cells Target	Validation	Strengths	Limitations	References
ISO 11731:2017	Culture on selective solid media	*Legionella* species	• Potable water • Non-potable water • Water-related matrix	• Quantitative CFU/L	10 days	• Annex J (Table J1)	• Viables culturable	• Vitens Laboratory Annex H	• Gold standard [a] • Isolation • Typing	• High sample volume • Long turnaround time • Inability to detect stressed cells or VBNC • High operational complexity • Inter-lab variability	[21]
Legiolert®	Liquid culture with chromogenic enzymatic substrate	*L. pneumophila*	• Potable water • Non-potable water • Water-related matrix	• Quantitative MPN/L	7 days	• LOD 1 MPN/100 mL (10 MPN/L) Potable water • LOD 100 MPN/100 mL (1000 MPN/L) Non potable water • LOQ up to 2272 MPN/100 mL sample	• Viables culturable	• AFNOR N°: IDX 33/06-06/19	• Small sample volume • Standardization • Isolation • Typing • Easy workflow • Reduced operator bias • Strong correlation with ISO 11731	• Species-specific • Inability to detect VBNC • Matrix interference	[29,36–38,111]
Scan-VIT ®	Fluorescence (in situ hybridization targeting rRNA)	*Legionella* species	• Potable water • Water-related matrix (short pre-enrichment)	• Quantitative CFU/L • Qualitative	72–75 h	• LOD 1 CFU/100 mL • LOQ Technical Counting Limit (100–150 CFU/filter)	• Viables culturable	• No validation • Peer-reviewed scientific studies	• Small sample volume • Early detection • Faster identification • Easier colony recognition	• RNA content dependent • No quantification of high *Legionella* concentrations • No recovered colonies from the filter for typing • Expensive • Inability to detect VBNC	[30,39–41]
MICA	Fluorescent labeling of microcolonies via click-chemistry	*L. pneumophila*	• Potable water • Non-potable water • Water-related matrix	• Quantitative CFU/L	48 h	• LOD 1 CFU/L Potable water • LOD 50–100 CFU/L Non potable water • LOQ 1–2 × 10^6 CFU/L	• Viables culturable	• AOAC Research Institute Certificate N° 032201	• Early detection • Rapid, automated AI counting • High throughput • Small sample volume	• Species-specific • No recovered colonies from the filter for typing • Expensive • Inability to detect VBNC	[32,43]
IMC	Thermoelectric measurement of microbial metabolic heat flow in selective broth	*Legionella* species	• Potable water • Non-potable water • Water-related matrix	• Qualitative µW	24–48 h [a] few hours–24 h [b]	• LOD 10^2 CFU/100 mL • LOQ **	• Viables culturable	• Peer-reviewed scientific studies	• Small sample volume • Early detection • High sensitivity • Real-time kinetics	• No intrinsic taxonomic • Low specificity • Indirect interpretation • Post-analysis confirmation • Expensive • Inability to detect VBNC	[31,45,112,113]

Table 1. *Cont.*

Method	Principle	Target	Matrix	Output Result	Time to Result	LOD LOQ	Cells Target	Validation	Strengths	Limitations	References
Amoebal co-culture	Intracellular replication within free-living protozoa hosts	*Legionella* species	• Potable water • Water-related matrix	• Qualitative	Days–weeks	**	• Viables culturable • VBNCs	**	• Detects VBNCs • High sensitivity	• Long turnaround time • Labor-intensive • Poorly standardized • Data interpretation challenges • Risk of host-specificity bias	[19,46]
qPCR	Fluorogenic nucleic acid amplification	*Legionella* species	• Potable water • Water-related matrix	• Quantitative GU/L	2–4 h	• LOD 5 GU/reaction • LOQ 15 GU/reaction	• Viables culturable • VBNc	• AFNOR N°: BRD 07/15-12/07; • AFNOR N°: BRD 07/16-12/07	• Early detection • High sensitivity • Low risk of false negative results	• No viability distinction • High risk of false positive results (dead whole cells, free DNA) • Matrix interference • Overestimation of *Legionella* count	[50,53,60,61]
v-qPCR	qPCR combined with membrane-impermeable intercalating dyes (e.g., PMA)	*Legionella* species	• Potable water • Water-related matrix	• Quantitative GU/L	3–5 h	• LOD 10 GU/reaction • LOQ 20 GU/reaction	• Viables culturable • VBNCs	• Validation According to ISO/TS 12869:2012 (Manufacturer-associated validation)	• Early detection • Improves qPCR • High sensitivity • Low risk of false negative results	• Matrix interference	[14,27,54,59,62,63,114–117]
dPCR/ddPCR	Partitioned micro-reaction PCR	*Legionella* species	• Potable water • Water-related matrix	• Quantitative GU/L	3–6 h	• LOD 1–5 GU/reaction (depending on the specific assay (such as targets for the mip gene or 5S rRNA gene) • LOQ **	• Viables culturable • VBNCs	**	• Early detection • High precision • Poisson absolute quantification • Low matrix interference • Low risk of false negative results	• No viability distinction • High risk of false positive results (dead whole cells, free DNA) • Long time preparation and processing samples • Overestimation of *Legionella* count • High *Legionella* load cannot be accurately quantified • Expensive	[64,69–71,118,119]
LAMP	Isothermal DNA amplification with visual or electrochemical readout	*Legionella* species	• Potable water • Water-related matrix	• Quantitative GU/L	30–90 min	• LOD 2–52 genomic copies/reaction • 28 plasmid copies/µL • LOQ **	• Viables culturable • VBNCs	• Validation According to ISO/TS 12869:2012 (Manufacturer-associated validation)	• Small sample volume • Fast detection • In-field application • No thermal cycler required • High sensitivity	• No viability distinction • High risk of false positive results (dead whole cells, free DNA) • High risk of false positive results (primer hybridization)	[72–78,120]

Table 1. *Cont.*

Method	Principle	Target	Matrix	Output Result	Time to Result	LOD LOQ	Cells Target	Validation	Strengths	Limitations	References
PVT VIABLE®	Liquid enrichment + qPCR	*Legionella* species	• Potable water • Water-related matrix	• Qualitative GU/L	2–3 days	• LOD 1 CFU/100 mL • LOQ **	• Viables culturable • VBNCs	• AOAC Performance Tested Method℠ 082303 (Manufacturer-associated validation)	• Small sample volume • Early detection	• Risk of false positive results • Expensive • Semi-quantitative/qualitative results	[80]
LeVoScan	Liquid enrichment + qPCR	*Legionella* species	• Potable water	• Qualitative GU/L	50 h	• RLOD 1 CFU/100 mL • LOQ **	• Viables • VBNCs	• Validation According to ISO 16140-4 (Manufacturer-associated validation)	• Small sample volume • Early detection	• Risk of false positive results related to high microbiota concentration	[81,82]
IMS	Immunomagnetic beads isolation paired with antibody-HRP colorimetric assay	*Legionella* species	• Potable water • Water-related matrix	• Quantitative CFU/L	3–5 h	• LOD 21 CFU_{eq}/L • LOQ 80 CFU_{eq}/L • LOQ **	• Viables • VBNCs	• AOAC Performance Tested Method℠ 052002 (Manufacturer-associated validation)	• Early detection • No DNA extraction or amplification needed • High sensitivity and specificity • Colorimetric counting	• Risk of overestimation of *Legionella* count (damaged bacteria, cellular fragment)	[84–87,91]
FCM	Targeted fluorescent antibody labeling paired with membrane-integrity dyes	*L. pneumophila*	• Potable water	• Cell/100 mL	2–3 h	• LOD 50–86.5 ILC/100 mL (depending on the specific assay)	• Viable • VBNCs	**	• Fast detection • Onsite decentralized testing • Automated counting • Detects VBNCs	• Matrix interference • Antibody cross-reactivity • Bacterial aggregates leads quantification error • Pre-analytical steps required • Subjectivity in data analysis	[89,90,121–123]
Biosensors	Integrated biorecognition + transducer signal	*Legionella* species	• Potable water	***	Min.–hours	**	• Cells • DNA • RNA	**	• Fast detection • Simple sample preparation • On-site decentralized testing • High sensitivity	• Risk of false positive results related to biofouling and matrix complexity	[92,93,98,99,124]

ISO 11731 is the most widely used and recognized official standard throughout Europe—it is also widely adopted by American accredited laboratories as a "gold standard" for the detection and counting of *Legionella* in aqueous matrices; ** data not available; *** the output of results differs depending on the type of analysis (qualitative or quantitative) and the transduction system employed; 24–48 h [a] (critical concentration: 10^2 CFU/100 mL); few hours–24 h [b] (high concentration: $> 10^6$ CFU/100 mL).

4. Discussion

The surveillance of *Legionella* in engineered water systems is undergoing a fundamental transition, driven not only by technological innovation but by a growing recognition of the conceptual limitations inherent to single-method approaches.

Historically, culture-based detection methods [21,23,24] were considered the "gold standard" for clinical and environmental monitoring, as they provide quantification based on CFU. The result, expressed in CFU/L, plays an important role in defining the risk levels associated with exposure to sources of infection contaminated with *Legionella* [125–128]. Furthermore, the culture-based method allows for the recovery of isolates and their subsequent characterization, a fundamental tool for linking clinical cases to sources of infection. Unfortunately, the inability to detect VBNC cells [12], which leads to systematic underestimation of environmental contamination, combined with prolonged incubation times and susceptibility to environmental interference, such as the potential proliferation of *Pseudomonas*, results in a delayed and potentially inaccurate assessment of viable populations, particularly in disinfected or stressed systems, that may not reflect real-world risk [14,15,129,130]. Moreover, a persistent bottleneck in the culture methods remain the confirmation step. To address these issues, laboratories have integrated different analytical assays for rapid species identification [110,122,131,132].

The emergence of rapid culture-based and culture-independent rapid commercial methods reflects an effort to optimize this paradigm without fundamentally altering its core logic. Techniques such as Legiolert™, MICA *Legionella*, and Scan-VIT-*Legionella*™ improve workflow efficiency and reduce time to result, yet they largely preserve the reliance on cultivability or present specific analytical constraints [29,32,36,39,40,43,50,111]: while the Scan-VIT-*Legionella*™ method is able to detect *Legionella spp.* and *L. pneumophila* simultaneously, MICA and Legiolert™ are exclusively designed for *L. pneumophila*, thereby lacking the genus-level scope required to detect other clinically relevant *Legionella* non-pneumophila species [48,133,134]. This means that other clinically relevant *Legionella* non-*pneumophila* species in water systems may go undetected, potentially leading to an underestimation of total *Legionella* risk [135].

Furthermore, MICA and Scan-VIT-*Legionella*™ do not provide serogroup information useful for establishing an epidemiological link between human cases and environmental source; Legiolert™ allows one to sample the positive culture broth, thereby allowing the *Legionella* strain to be isolated and serotyped.

Comparative data regarding the MICA method show a sensitivity ranging from 67.4% according to Ditommaso et al. [43] to 94% according to Passot et al. [32].

It should be noted that those results were obtained using different plate-culture procedures (sample volume plated and culture media), analyzing different types of samples (spiked samples, samples with high *Legionella* load) or applying different methods for results calculation/interpretation.

On the other hand, the Scan-VIT-Legionella™ test underestimates bacterial concentration in several samples with high *Legionella* density. This occurs because the upper counting limit is 100–150 CFU/filter; beyond this threshold, overlapping microorganisms prevent an accurate count. Furthermore, since FISH signal intensity is directly dependent on cellular rRNA content, the reduced metabolic activity of stressed cells can diminish fluorescence sensitivity, further contributing to underestimation. Finally, the inability to easily recover viable colonies from the Scan-VIT-*Legionella*™ membranes for downstream typing or biomolecular analysis limits the utility of this system in epidemiological source tracking.

Several scientific studies have shown that IMC is compatible with simple sample preparation procedures and can provide accurate results in short times, but the method is

still subject to variables related to the standardization of culture media and cell age. IMC lacks intrinsic taxonomic specificity and, since all growing microbes generate heat, requires highly selective media or post-analysis confirmation to ensure that the thermal signal originates specifically from *Legionella* [45,112,113]. Furthermore, to generate a detectable thermal signal, a minimum mass or concentration of active cells is required, and the exact determination of the beginning of metabolic heat is often very difficult because at the onset of the calorimetric signal the thermal stabilization and the metabolic heat development overlap. Finally, this method requires a significant financial investment and an extremely stable temperature control system [136].

Amoebal co-culture is one of the most sensitive tools available for recovering environmental bacteria, but it provides only qualitative results due to the intracellular replication. Therefore, such data present interpretation challenges when evaluating the degree of water system contamination [19,44,47]. Its implementation is constrained by significant methodological limitations; specifically, the technique is labor-intensive, technically complex, lacks standardized quantitative metrics (e.g., CFU/L equivalents), and carries a risk of host-specificity bias, where the selected protozoan host may fail to support the replication of certain bacterial strains. Although it is a scientifically well-documented technique for academic and epidemiological research purposes, it is not included in the official reference protocols.

These alternative culture methods redistribute rather than resolve the core trade-off between speed, taxonomic breadth, and biological relevance.

To overcome the problem of false-negative results with the culture method caused by VBNCs [12], hybrid methods such as PVT Viable® [80] and LeVoScan [81] can be used.

The PVT Viable® platform presents distinct limitations: it tends to give false negative results due to high concentrations of competing microbiota. Moreover, it relies on a proprietary, semi-closed commercial system that increases operational costs, and it provides a semi-quantitative or qualitative result rather than absolute colony enumeration (CFU/mL) mandated by strict regulatory frameworks like ISO 11731.

LeVoScan is a rapid pre-enrichment PCR method designed to specifically detect culturable *L. pneumophila* species in water samples that contain low levels of interfering background flora, such as drinking water. When applied to samples with low background flora, LeVoScan performs at least equivalent to the current standardized culture-based ISO 11731 reference method.

To overcome cultivation methods' limitations, molecular techniques have been innovated.

qPCR has become a central tool for environmental screening, to reduce turnaround time by several days [51,53,114]. However, qPCR cannot distinguish between DNA from viable and non-viable cells, thereby often leading to an overestimation of the total genomic signal relative to culturable cells [63], though not necessarily correlating with actual infection risk. This discrepancy is not merely technical but conceptual, reflecting the "viability dilemma" that defines modern *Legionella* surveillance [54].

Attempting to address this limitation, v-qPCR improves specificity toward membrane-intact cells but remains susceptible to environmental interference and does not fully resolve the relationship between detection and risk of infection [14,117]. The claim that links PMA treatment to the inhibition of DNA amplification in PCR must be considered with some caution. A difference in the ability of PMA to suppress the PCR signal was observed in samples containing different amounts of bacteria, causing the detection of viable *Legionella* cells, VBNCs and traces of DNA from non-viable cells in the analyzed samples [27]. However, for both the EMA-PCR and PMA-PCR viability assays, complete dead cell signal suppression was observed when long-amplicon qPCR was amplified [115,116,137].

Moreover, when applied to sessile reservoirs, v-qPCR efficiency can be severely compromised by the biofilm matrix itself, where extracellular polymeric substances and humic acids act as potent enzymatic inhibitors, potentially leading to false-negative results if extraction protocols are not rigorously optimized [53,55]. However, v-qPCR remains a tool for water system management and potential risk determination, and the results can be used for risk management in water systems.

Digital PCR further enhances quantitative reliability through absolute quantification and improved tolerance to inhibitors, particularly in complex environmental matrices such as cooling towers and industrial water systems [64]. Nevertheless, some studies reported higher levels of inhibition in dPCR measurements compared to qPCR when analyzing wastewater samples [138]. Moreover, dPCR has relatively longer preparation compared to qPCR with higher risk for contamination (more complex workflow that requires more hands-on time with multiple steps, which increases the time to result and the possibility of contamination) [118]. The study of Cao et al. [71] revealed three main limitations of ddPCR: the need to dilute samples, especially those from highly polluted water, to achieve exact quantification; analytical sensitivity variability, where ddPCR sensitivity depends on the number of droplets per reaction, resulting in different detection limits from reaction to reaction; and the potential for some scenarios to affect the partitioning of molecular targets into the water-in-oil droplets during ddPCR. Still, improved quantitative precision does not resolve the lack of viability information. Like other molecular methods, dPCR and ddPCR do not provide information on cell viability. Furthermore, their higher cost and limited availability currently restrict their routine use in surveillance; nevertheless, their high analytical robustness makes them valuable tools for quantitative environmental monitoring.

Isothermal amplification methods such as LAMP extend molecular detection into rapid and deployable formats, reinforcing their role as high-sensitivity screening tools, particularly in decentralized or field-based applications [70,72–74,77]. Standard LAMP does not differentiate viable from non-viable cells; however, integrating viability dyes—such as in PMA-LAMP assays developed for *Legionella* field testing [120]—allows for the selective targeting of membrane-intact bacteria.

Importantly, while PMA pre-treatment offers a surrogate proxy for cell viability, membrane integrity alone does not directly equate to infectivity or human exposure risk. Additionally, the high amplification efficiency of LAMP retains a significant risk of carryover contamination [87], meaning that even viability-coupled isothermal assays should be interpreted as enhanced screening tools rather than direct measures of infection risk [76].

Furthermore, given the high amplification efficiency of LAMP, even minor contamination can lead to false-positive results. The use of multiple primers (4–6 different primers) increases the risk that the primers will amplify non-target sequences (primer hybridizations that give rise to false-positive results) [139]. Primers must be carefully designed to ensure high specificity, as primer design is complex and time-consuming. Overall, LAMP represents a rapid and sensitive screening tool but not a standalone method for viability assessment in *Legionella* surveillance.

IMS appears to be particularly useful in the early identification of potential risk sources in a Legionnaire's disease outbreak, allowing for the rapid implementation of interventions [84,85,87,91]. One limitation is that antibodies target cell-wall antigens, meaning the magnetic beads can also bind damaged bacteria or cellular fragments. This can lead to an overestimation of risk compared to traditional cultures [86,87].

Parallel to organism-specific detection, system-level monitoring tools such as flow cytometry and biosensors shift the analytical focus toward microbial dynamics.

Flow cytometry enables rapid quantification of total and intact cells, functioning as an early warning indicator of system instability and microbial regrowth potential [25,89].

This rapid resolution is particularly critical given that artificial water systems frequently harbor VBNC *Legionella* populations that escape traditional culture-based detection [12]. To elevate FCM to a pathogen-specific screening level, antibody-based adaptations are increasingly used [58,59]; however, this targeted format still faces limitations regarding potential antibody cross-reactivity and background interference from complex environmental matrices [88]. Targeted FCM applications—incorporating specific fluorescent labeling or cell sorting combined with qPCR—are necessary for robust environmental surveillance [123]. Furthermore, while it confirms cellular integrity and taxonomic presence, it does not fully elucidate the viability of all detected cells [90]. Consequently, this targeted FCM method alone should be viewed as a rapid screening tool for high-throughput surveillance, best implemented alongside standard molecular or culture-based frameworks to ensure rigorous risk management. Additionally, the limitations of this method arise from the subjectivity of data analysis (variable instrument settings and the need for manual gating to separate bacterial signals from background noise) and from issues relating to the handling of bacterial aggregates, leading to quantification errors. Also, characterizing the biofilm reservoir via FCM requires aggressive physical disruption, such as sonication or chemical detaching; these pre-analytical steps present a distinct methodological challenge, as the mechanical shear stress can artificially compromise cell membranes, thereby distorting the true ratio of intact to damaged cells [90].

Hence, Streich et al. [91] formulated cartridge-based IMS coupled with FCM, demonstrating it on evaporative-cooling-system samples as a rapid monitoring method. It is an innovative antibody-based method for general analytical procedures for the culture-independent quantification of *L. pneumophila*, using a panel of antibodies against serogroup 1–15. The IMS-FCM method resulted in being used for different purposes, such as outbreak management, risk assessment or routine monitoring.

To address these diagnostic gaps, biosensor platforms—ranging from electrochemical to optical systems—have also been developed, demonstrating promising sensitivity and rapid response times for pathogen screening under controlled conditions [92,93,95–98]. A critical practical barrier in environmental applications is the low concentration of *Legionella* relative to the large sample volumes typical of water monitoring. Modern biosensor designs are increasingly integrating microfluidics and pre-concentration strategies [124]. Electrochemical biosensors based on DNA probes have demonstrated detection limits in the picomolar range, while formats coupling LAMP amplification with electrochemical readout have pushed sensitivity to the level of single genome copies [92]. However, despite their potential for timely risk assessment, significant challenges related to matrix interference, biofouling, and long-term stability remain major barriers to the widespread, routine implementation of these sensor technologies [140]. This is especially evident in biofilm-rich environments, where the rapid accumulation of organic matter and non-target microflora on sensor surfaces leads to severe biofouling, drastically reducing signal transduction accuracy and operational lifespan.

Considering the information provided thus far, laboratories involved in environmental surveillance for *Legionella*—in addition to keeping abreast of developments in available diagnostic techniques and understanding their advantages and disadvantages—must be able to offer the method best suited to the following objectives: i) routine purposes (perform an environmental assessment to identify areas with increased risk of *Legionella* growth and spread, establish a baseline measurement for performance indicators, and evaluate potential growth and transmission sources, validating a water safety plan); ii) non-routine purposes (support environmental assessment during public health investigations, confirm success or failure of remedial treatment, and investigate potential sources of environmental exposure for people with disease).

All risk assessment, epidemiological investigation and management procedures are based on the results of microbiological monitoring. The laboratories that perform analyses to detect *Legionella* in environmental samples must ensure that results are produced using validated procedures, qualified instrumentation, and personnel with verified expertise because the test report issued by a laboratory is an important document for prevention, epidemiological investigation, and legal purposes. Personnel with specific expertise in *Legionella* risk management defines the frequency, the scope of the sampling plan and the selection of critical points. However, the laboratory must provide support in interpreting the results in order to integrate analytical data into the technical decision-making process, taking into account the specific characteristics of the system and the facility. In addition, the laboratory must assist clients by (i) ensuring that samples are collected in accordance with correct operating procedures (sample volume, collection with or without flushing, sample storage/transport) and (ii) providing support during the interpretation of results (including distinguishing between qualitative and quantitative results, units of measurement used to express the results, and comparison between different methods).

In order to overcome the limitations of each method when used individually, this review proposes a shift from method-centric environmental monitoring to an approach structured within a multilevel framework in which various analytical tools play functional roles (Table 2) and/or sequential steps (Figure 15). The integration of multiple methods provides more accurate and reliable information regarding the actual *Legionella* contamination present in the samples analyzed and, consequently, facilitates hazard assessment and the establishment of *Legionella* control measures in the monitored system or at the point of use.

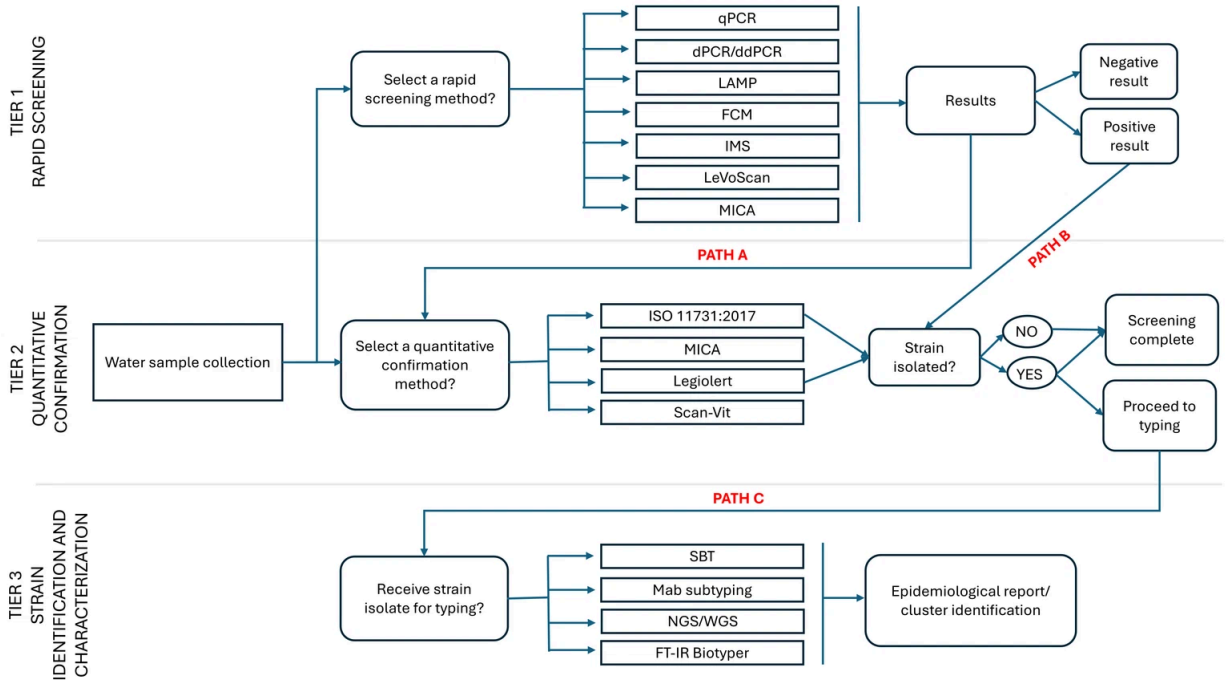


Figure 15. Schematic illustration of a sequential architecture integrating complementary analytical approaches across three functional tiers for environmental surveillance of *Legionella* in water systems.

In this architecture, Tier 1 (rapid screening and early warning) encompasses fast diagnostic tools designed for rapid early warning, system stability screening, and quick pathogen exclusion.

Table 2. Conceptual functional tiered framework for environmental surveillance of *Legionella* in water systems.

Tier	Functional Role	Methods	Output	Time to Resullt	Primary Application
1	Rapid screening and early warning	qPCR	GU/L	2–4 h	• Screening • On-site test • Early disinfection efficacy evaluation
		qPCR	GU/L	3–5 h	
		dPCR/ddPCR	GU/L	3–6 h	
		LAMP	GU/L	30–90 min	
		FCM	Intact cells/100 mL	2–3 h	
		IMC	qualitative	few hours–2 day	
		IMS	CFU/L	3–5 h	
		Biosensors	Qualitative/Quantitative	min-hours	
		PVT VIABLE	GU/L	48–72 h	
		LeVoScan	GU/L	50 h	
		MICA	CFU/L	48 h	
		Scan-Vit	CFU/L	73–75 h	
2	Quantitative confirmation	ISO 11731:2017	CFU/L	10 days	• Worldwide regulatory compliance • Routine official monitoring for *Legionella spp.* detection • Strain isolation
		Legiolert	MPN/L	7 days	• No worldwide regulatory compliance • Routine official monitoring for *L. pneumophila* detection • Strain isolation
		MICA	CFU/L	48 h	• Screening • Routine monitoring for *L. pneumophila* detection • No strain isolation
		Scan-Vit	CFU/L	73–75 h	• Screening • Routine monitoring for *Legionella spp.* detection • No strain isolation

Table 2. *Cont.*

Tier	Functional Role	Methods	Output	Time to Resullt	Primary Application
3	Strains characterization and epidemiological investigation	Mab subtyping	Groups strains into distinct antigenic variants (e.g., Pontiac, Philadelphia, Benidorm, Knoxville, Bellingham), providing reproducible classification data and helping distinguish high-risk clinical strains.	hours days	• Outbreak investigation • Cluster identification • Source tracking and attribution • Investigating pathogenicity and antibiotic resistance
		SBT	Provides a specific sequence type (seven-gene fragment) that can be matched with an international database. Relies on a standardized, universally accepted numbering scheme.		
		WGS	Reads (sequence fragments of) the entire Legionella genome, processed into standardized genetic profiles, clustering data, and epidemiological metrics.		
		NGS	Lists all specific SNP positions and nucleotide mutations discovered in the isolate, allowing users to obtain the phylogenetic structure used to visualize outbreak clusters in third-party software.		
		FT-IR Biotyper	Allows laboratories to process many isolation strains simultaneously, generating "fingerprints" on the same day, and match patients' clinical isolates with environmental isolates from suspicious water systems.		
4	Isolation of low amount of viable and VBNC cells	Amoeba co-culture	Strain isolation	Weeks	• Academic and epidemiological research purposes

Tier 2 (quantitative confirmation) comprises CFU-compatible and culture-based methods that satisfy regulatory compliance and enable colony recovery for downstream characterization.

Tier 3 (strain characterization and epidemiological characterization) integrates high-resolution techniques to support outbreak investigations, cluster identification, and precise source tracking.

Tier 4 (isolation of low amount of viable and VBNC cells) is intended for use in research settings.

5. Conclusions

Within this framework, the value of individual methods lies not in their standalone performance but in their integration within a dynamic and context-dependent workflow.

Faster methods act as triggers for targeted confirmatory analyses, while more complex approaches refine interpretation and epidemiological attribution.

The future of *Legionella* surveillance lies not in the identification of a single superior method but in the strategic integration of complementary analytical tools. Bridging the gap between detection and decision-making requires not only technological innovation but conceptual alignment between analytical outputs and risk management needs.

This review represents an update to previously published literature. It serves as a valuable resource not only for laboratory experts but also as a guidance tool for stakeholders who request analytical services, such as hospital health management, Water Safety Plan (WSP) teams, and Hospital-Acquired Infection (HAI) committees. In our daily practice, we frequently encounter laboratory test reports that are difficult for clients to interpret and offer limited utility for their intended purposes, whether for routine monitoring or epidemiological investigations. Furthermore, the analysis proposed by a laboratory is not always aligned with the specific objective; in many instances, laboratories tend to offer the simplest test to perform (e.g., qPCR using commercial kits) that does not require a high level of specialized training or expertise, such as the morphological recognition of typical colonies.

In conclusion, laboratories carrying out the isolation and enumeration of *Legionella* in water samples could adopt our proposed framework to define their own workflows, thereby overcoming the issue of false-positive and false-negative results while accounting for the specific requirements of clients such as healthcare facilities, building managers and water system operators.

Author Contributions: Conceptualization, S.D. and C.S.; methodology, S.D. and C.S.; software, C.S.; validation, S.D. and M.G.; formal analysis, C.S.; investigation, C.S. and S.D.; resources, C.S., S.D., M.G. and F.B.; data curation, M.G.; writing—original draft preparation, C.S and S.D.; writing—review and editing, C.S., S.D., M.G, and F.B.; visualization, C.S., S.D. and M.G.; supervision, F.B.; project administration, F.B.; funding acquisition, F.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by EU FUNDING within the MUR PNRR Extended Partnership Initiative on Emerging Infectious Disease Project no. PE00000007, INF-ACT.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflicts of interest.

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