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The Design of a PNA Probe to Improve *Legionella pneumophila* Monitoring

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Abstract: *Legionella* is a significant public health threat in engineered water systems, requiring rapid and accurate environmental monitoring to prevent outbreaks. Traditional detection methods, such as culture-based assays and PCR, are limited by long processing times and the potential for false negatives due to viable but non-culturable (VBNC) cells. This study addresses these limitations by developing a novel Peptide Nucleic Acid-Fluorescence in situ Hybridization (PNA-FISH) method for the specific and sensitive detection of *Legionella pneumophila*. In silico analysis predicted high theoretical specificity (100.0%) and sensitivity (99.8%), results that were confirmed by experimental validation against 17 *L. pneumophila* strains and 37 non-target strains (including *Pseudomonas*, *Acinetobacter*, and other *Legionella* species), demonstrating strong fluorescence signals with no cross-reactivity. Furthermore, the method was successfully applied to artificially contaminated tap water, achieving a limit of detection of 10^3 CFU mL^{-1} directly on the filter membrane. This work highlights the potential of PNA-based probes to improve bacterial monitoring, offering fast, reliable, and field-adaptable detection of *L. pneumophila*. The findings support the integration of this probe into routine water system monitoring workflows, facilitating timely assessment of contamination and outbreak prevention.

Keywords: *Legionella pneumophila*; PNA-FISH; monitoring; water systems

1. Introduction

Legionella pneumophila is a bacterial species of public health relevance, known for its capacity to persist and proliferate in aquatic environments [1–3]. Its resilience under a range of physical and chemical conditions allows it to colonize various water systems (e.g., cooling towers and plumbing systems), posing risks in the community, as well as in industrial and healthcare settings [4,5]. Infections caused by *L. pneumophila* can lead to a severe respiratory illness, known as Legionnaires' disease, underscoring the need for reliable monitoring strategies that enable early detection and a rapid response [6].

Conventional diagnostic techniques, such as culture-based assays (e.g., ISO 11731:2017) [7] and polymerase chain reaction (PCR) (e.g., ISO 12869:2019) [8] remain standard for *L. pneumophila* detection. However, these techniques are often constrained by their dependence on skilled personnel and lengthy processing times [9,10]. Furthermore, culture-based methods rely on colony-forming units (CFUs) for quantification, which can underestimate bacterial populations due to viable but non-culturable (VBNC) cells [11]. These limitations across

conventional techniques have driven the development of alternative molecular detection strategies with the intent to provide operational simplicity, and high specificity and sensitivity, suitable for point-of-care monitoring and field applications [12–14].

Peptide Nucleic Acids (PNAs), synthetic molecules that mimic DNA, offer strong and specific binding to bacterial rRNA sequences [13] and have shown promise in microbial detection applications, with relevance for *Legionella* detection in water and biofilm samples in industrial systems [15,16]. PNA-based probes are particularly well-suited for visual detection strategies such as fluorescence in situ hybridization (PNA-FISH) [17–19], colorimetric assays [20,21], and biosensors [22,23], significantly reducing the time between sampling and results. Furthermore, their adaptability to portable and miniaturized formats [24–27] positions them as promising tools for on-site diagnostics and real-time surveillance. The application of PNA probes in visual detection offers rapid and accurate pathogen identification, even at low bacterial concentrations, with potential for multiplexing and real-time analysis.

As part of efforts to expand the utility of PNA probes for rapid environmental pathogen monitoring, a new PNA probe was developed for the detection of *L. pneumophila*.

Although PNA probes targeting *Legionella* spp. and *L. pneumophila* have been previously reported [15,28], we designed a novel PNA probe targeting the 16S rRNA gene of *L. pneumophila* to reflect the updated genetic diversity of *L. pneumophila* and its closely related species. Advances in sequencing technologies and the continuous availability of publicly available 16S rRNA gene data over recent years have revealed greater intraspecies variability and potential for off-target hybridization lacking in earlier designs. The specific *L. pneumophila* probe developed here, and another previously reported probe by Wilks et al. (2006), PLPNE620 [28], were subjected to comparative in silico analyses to assess hybridization efficiency and overall suitability for molecular diagnostic applications and integration into future field-ready monitoring procedures.

The new probe was optimized for fluorescence performance through adjustments in hybridization conditions and imaging parameters to ensure maximum specificity and sensitivity. Probe efficiency and limit of detection were determined and corroborated by colony-forming unit (CFU) counts, and standard analysis protocols were tested to assess probe performance. This work aims to support the integration of this new PNA probe into routine laboratory procedures, contributing to improved *L. pneumophila* monitoring and outbreak prevention.

2. Material and Methods

2.1. Strain and Growth Conditions

In this study, a total of 17 strains of *Legionella pneumophila* and 37 non-*Legionella pneumophila* bacteria were tested. All the strains listed in Table S1 (see Supplementary Material) were obtained from different collections, such as bacterial collection from Laboratório de Microbiologia do Departamento de Saúde Ambiental Porto do Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA-DSA ASMIP), Chalmers University of Technology, and Professor Manuel Simões (LEPABE) [29]. *Legionella* strains were grown on standard buffered charcoal yeast extract agar (BCYE)supplemented with L-cysteine and ferric pyrophosphate, and incubated at 37 °C for 2 to 4 days. Non-*Legionella* strains were grown on tryptic soy agar (TSA) (3% (*w*/*v*) tryptic soy broth and 1.5% agar) at 37 °C for 24 h, except *Pseudomonas fluorescens*, which was incubated at 30 °C.

2.2. In Silico PNA Probe Design

Available alignment programs and 16S rRNA databases were used to design a specific probe for *L. pneumophila*, as described by Teixeira et al. (2021) [30].

286 *L. pneumophila* target sequences and 65 non-target sequences were carefully selected from the ARB Silva database [31]. The selection was based on specific quality criteria, including sequences with a length greater than 1200 bp and high/quality scores for sequence, alignment, and pintail (>90% for all). Regions of interest were subsequently identified using MEGA-X and aligned with ClustalW [32] The sequences were also evaluated for a high GC content and a low number of consecutive self-complementary nucleotides.

Theoretical specificity and sensitivity were calculated using the ProbeCheck program available in the ARB Silva database) [33] and the values were determined as described by Nacher-Vazquez et al. (2022) [15]. Specificity was determined using the equation nLs/(TnL) × 100, where nLs represents the number of non-*Legionella* sequences did not align with the probe, and TnL corresponds to the total number of non-*Legionella* sequences analysed. Sensitivity was determined as Ls/(TLs) × 100, in which Ls represents the number of *Legionella* sequences successfully detected by the probe, and TLs refers to the comprehensive total of *Legionella* strains available in the databases. The selected sequence was synthesized (Eurogentec, Seraing, Belgium) and labeled at the N-terminus with AlexaFluor®594 via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker.

2.3. Hybridization Conditions

Hybridization experiments were performed as previously described [17,18,34]. Briefly, suspensions of 1 × 10^8 cells mL^{-1} of *L. pneumophila* serogroup 1, ATCC™ 33152, and *L. pneumophila* serogroup 2–15 were dispensed in 8 mm well slides (Marienfeld, Lauda-Königshofen, Germany) and allowed to air dry. Following that, the cells were permeabilized and fixed, with 30 µL of 4% (*w*/*v*) paraformaldehyde dispensed in the wells at room temperature, followed by 50% (*v*/*v*) ethanol, and an incubation for 10 min each. After that, slides were covered with 20 µL of hybridization solution containing 10% (*w*/*v*) dextran sulfate, 10 mM NaCl, 30% (*v*/*v*) formamide, 0.1% (*w*/*v*) sodium pyrophosphate, 0.2% (*w*/*v*) polyvinylpyrrolidone, 0.2% (*w*/*v*) Ficoll, 5 mM disodium EDTA, 0.1% (*v*/*v*) Triton X-100, 50 mM Tris-HCl (pH 7.5) (Sigma-Aldrich, Sintra, Portugal), and 200 nM of the PNA probe (Eurogentec, Belgium). The slide wells were covered with coverslips, protected from the light, and incubated for 60 min at different temperatures (55 °C, 57 °C, 59 °C, 60 °C, 61 °C, and 63 °C) to evaluate the signal-to-noise ratio. Following hybridization, the slides were transferred to a copling jar containing prewarmed washing solution, containing 5 mM Tris base, 15 mM NaCl, and 1% (vol/ vol) Triton X (pH 10) (Sigma-Aldrich, Sintra, Portugal) for 30 min. The samples were allowed to air dry, mounted with a drop of nonfluorescent immersion oil, and covered with coverslips.

Slides were kept in the dark for up to 24 h before microscopy observation. After optimizing the hybridization conditions, the probe was applied to other *L. pneumophila* and non-*L. pneumophila* strains listed in Table S1 (See Supplementary Material) to evaluate the probe's specificity and sensitivity.

2.4. Evaluation of PNA-FISH on Artificially Contaminated Water Samples

The detection of *L. pneumophila* in water samples typically requires a concentration step, achieved either by centrifugation or by filtration, depending on the nature of the water sample [7]. The suitability of PNA-FISH after filtration was evaluated as described by Nácher-Vázquez et al. (2022) [15]. Briefly, 50 mL of sterile water was artificially inoculated with *L. pneumophila* serogroup 1, ATCC™ 33152, at different concentration values from 10^3 to 10^8 CFU mL^{-1} using a standard filtration system with White Nuclepore™ (Whatman™) membranes with a diameter of 47 mm and a pore size of 0.22 µm.

It was hypothesized that the number of cells identifiable by PNA-FISH was proportional to these concentrations. After filtration, two protocols were applied: direct detection on the membrane and cell elution.

For direct detection, the membranes were air-dried and then sequentially treated for 10 min each at room temperature with 4% (*w*/*v*) paraformaldehyde followed by 50% ethanol (*v*/*v*). Next, each membrane was air-dried on a glass slide. For hybridization, 60 µL of hybridization solution (pH 7.5), containing 200 nM of the PNA probe, was applied to the membrane and spread with a coverslip, and incubated in the dark for 60 min. Following incubation, the membranes were carefully removed from the slide and transferred to a petri dish, which was also protected from light. The petri dish was previously filled with a pre-warmed washing solution (pH 10), and the filters were incubated for 30 min. Finally, the samples were allowed to dry, mounted with a drop of non-fluorescent immersion oil, and covered with coverslips. The slides were stored in the dark for no more than 24 h before microscopic visualization.

For the elution test, the membranes containing adherent cells were agitated at 270 rpm for 20 min in a Falcon tube with 5 mL of sterile distilled water to resuspend the cells. Then, the sample was centrifuged at 3000× *g* for 20 min, and the supernatant was carefully removed. The pellet was resuspended in 100 µL of sterile distilled water, placed on a microscopic slide, and air-dried. Finally, the standard PNA-FISH protocol was applied as described in Section 2.3. To confirm the results, CFU counts were performed in all experiments.

2.5. Colony Forming Unit (CFU) Quantification

To confirm the bacterial concentration in the artificially contaminated samples, CFU counts were performed in parallel with PNA-FISH. Aliquots (100 µL) of the appropriate serial dilutions were spread-plated onto BCYE agar plates. The plates were incubated at 37 °C for 3 to 4 days, after which colonies were counted to determine the number of CFU mL^{-1}.

2.6. Microscopy Visualization

An epifluorescence microscope Nikon Eclipse 80i (Japan) with a NikonDS-Fi1 (Izasa, Japan) camera and a filter sensitive to the Alexa Fluor 594 molecule linked to the PNA probe (excitation 590 nm; emission 618 nm) was used for microscopy visualization. The other filters in the microscope that are not sensitive to the probe fluorescence signal were also used for autofluorescence control. In each experiment, a no-probe control was

processed in parallel, following all the same steps described above, except for the absence of the probe during the hybridization step. All images were acquired using NIS-Elements B.R. 3.2 (Nikon, Japan) software with a magnification of ×100.

2.7. Image Analysis

For each experimental condition, three independent fields of view were acquired and analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) [35]. Images were segmented using the Otsu thresholding method, followed by particle/object identification. Segmentation was followed by particle/object identification, after which the mean fluorescence intensity of each segmented object was quantified. Data are reported as mean fluorescence intensity (AU). All analyses were performed under identical settings to ensure consistency and reproducibility across samples.

2.8. Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). Results were compared using one-way ordinary ANOVA followed by Tukey's multiple comparisons test in GraphPad Prism 8.4.3® (GraphPad Software, CA, USA). All tests were performed with a 95% confidence level.

3. Results and Discussion

3.1. PNA-FISH Optimization

For probe design, the initial selection of potential probe regions was based on an alignment of 16S rRNA gene sequences from *L. pneumophila* and its closest phylogenetic relatives, as well as other bacteria commonly found in aquatic environments. The primary criterion for selection was the presence of significant sequence variability between *L. pneumophila* and the non-target strains. From these candidate regions, the probe with the optimal performance demonstrating the best compromise between the number of detected targets and undetected non-targets was identified. The selected probe has the sequence N-terminal-CTGTATCGGCCAT-C-terminal, targeting the 16S rRNA between positions 1247 and 1260 of the *Legionella pneumophila* subsp. *pneumophila* JCM 7571 (Accession number AB594755; SILVA database).

The impact of evolving genomic data on probe performance was assessed by conducting a comparative in silico analysis between PLPNE620, originally published in 2006 [28], and the newly designed probe developed in this study. At the time of its publication, PLPNE620 was not accompanied by reported theoretical sensitivity and specificity values, likely due to the limited availability of comprehensive sequence data. In contrast, the current availability of large, curated 16S rRNA gene databases, such as SILVA and NCBI, enabled us to retrospectively evaluate the PLPNE620 probe under the same conditions used for our newly designed probe. Interestingly, both probes exhibited equivalent theoretical sensitivity (approximately 99%) and specificity (100%) (Table 1) when analyzed against the expanded sequence databases. However, the new probe benefits from being designed using a significantly broader and more diverse representation of *L. pneumophila* strains and related species. This may provide greater confidence in its diagnostic robustness and highlights the importance of periodically re-evaluating and updating molecular tools as genomic resources evolve. In addition, although PLPNE620 demonstrated good theoretical performance, Wilks et al. (2006) [29] reported a positive signal when the probe was tested against *L. quinlivanii* and *L. longbeachae* serogroup 1, also known to be a human pathogen, which may compromise its specificity in practical applications. In contrast, the newly designed probe did not target any *Legionella* non-*pneumophila* species (please see results for specificity and sensitivity). Nonetheless, both probes show suitable theoretical performance and can be considered effective tools for *L. pneumophila* monitoring applications.

The hybridization performance of the designed PNA probe was then assessed using 30% formamide [15] at various temperatures (55 °C, 57 °C, 59 °C, 60 °C, 61 °C, and 63 °C) (Table 2). The hybridization temperature, at which a nucleic acid is allowed to anneal to its complementary target sequence by base-pairing (A-T, G-C), is a critical parameter that determines probe-target binding specificity and signal-to-noise ratio, by minimizing non-specific interactions. This temperature depends on several parameters, such as the probe length, GC content, and the accessibility of the target sequence on the three-dimensional structure of the rRNA [36,37].

Table 1. Theoretical evaluation of PNA probes for the specific detection of *L. pneumophila*.

Target Gene	Sequence (5′–3′)	Length (bp)	GC (%)	Sensitivity [a] (%)	Specificity [a] (%)	Reference
16S rRNA	CTG ACC GTC CCA GGT	15	66.7	99.4 (95% CI, 99.3–99.4)	100.0 (95% CI, 99.9–100.0)	[28]
16S rRNA	CTG TAT CG GCC AT	13	54.0	99.8 (95% CI, 99.3–99.4)	100.0 (95% CI, 99.9–100.0)	This work

[a] The theoretical determination of sensitivity and specificity was performed based on Almeida et al. (2010) [33] using the ARB Silva database (accessed in September 2025).

The results showed that the probe signal was detected at all tested temperatures except 63 °C, while the strongest and most distinct signal, with the highest signal-to-noise ratio, was observed at 60 °C, indicating it as the optimal hybridization temperature (Table 2, Figure 1). Around the optimal temperature, there is a range where the duplex can still form (55–59 °C), though stability and specificity may vary, with some non-specific binding happening. At higher hybridization temperatures (61 and 63 °C), there is some signal loss, which is in agreement with [15,38], which also shows that duplexes may be more unstable at higher temperatures, resulting in weak or no detectable signal. These results were also corroborated by the mean fluorescence intensity, which was significantly higher at 60 °C compared to the other temperatures tested (Figure 1).

The quantitative analysis of fluorescence intensity is detailed in Table 2. As visually corroborated by Figure 1, statistical analysis confirmed that the mean fluorescence intensity at 60 °C was significantly higher than at all other tested temperatures (p-value < 0.01), identifying it as the optimal hybridization condition. Lower temperatures resulted in moderate signal intensities, while no signal was detected at 63 °C.

Table 2. Optimization of PNA-FISH hybridization conditions and resulting signal intensities.

Temperature (°C)	PNA-FISH Outcome	Mean Fluorescence Intensity (AU)
55	+	23.20 ± 2.50
57	+	23.63 ± 2.11
59	+	23.05 ± 2.37
60	++	29.83 ± 0.92
61	+	25.56 ± 0.40
63	-	ND

(+) Intermediate signal-to-noise; (++) Strong signal-to-noise; (-) Negative; ND—Not Determined.

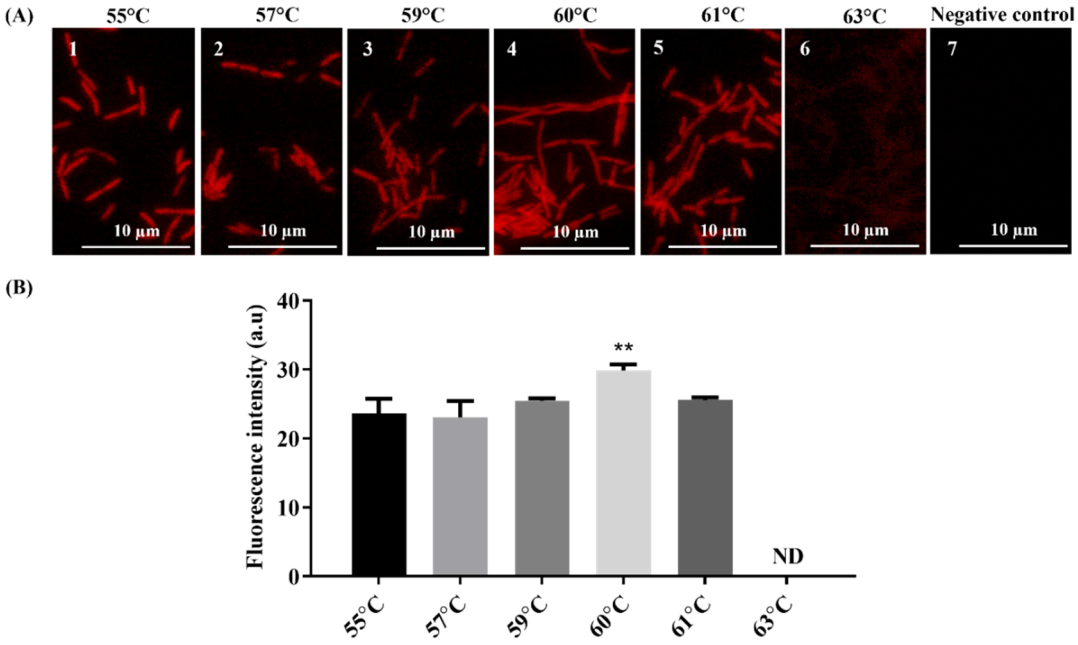

Figure 1. PNA-FISH probe optimization. (**A**) Epifluorescence images of *L. pneumophila* serogroup 1, ATCC™ 33152, at different hybridization temperatures: 55 °C (**1**), 57 °C (**2**), 59 °C (**3**), 60 °C (**4**), 61 °C (**5**), 63 °C (**6**), and negative control (**7**). Images were obtained with equal exposure times. (**B**) Mean fluorescence intensity quantified across all tested temperatures. Error bars represent standard deviation. ** Statistical differences observed between 55 °C and all other temperatures tested (** p-value < 0.01). (ND—Not Determined).

Following the optimization step, the PNA probe's specificity and sensitivity were evaluated. The probe was tested against a panel of 17 *L. pneumophila* strains, including isolates from serogroups 1 and 2–15 to confirm its sensitivity, and 37 non-*L. pneumophila* bacterial species representing diverse genera such as *Acinetobacter*, *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus*, and *Stenotrophomonas* to assess its specificity (Table S1, see Supplementary Material). As shown in Figure S1 (see Supplementary Material), the probe successfully detected every *L. pneumophila,* demonstrating 100% sensitivity. It also demonstrated 100% specificity, as no signals were detected from any non-target bacteria. Control experiments further confirmed the absence of autofluorescence in samples without the probe and showed no non-specific signals in other fluorescence channels.

3.2. PNA-FISH on Artificially Contaminated Water Samples for Monitoring Protocol Assessment

Culture remains the reference method for *L. pneumophila* detection and enumeration, as established in ISO 11731:2017 and adopted by regulatory frameworks across Europe [7]. Its main advantage lies in the low theoretical limit of detection, which under optimized conditions may reach as little as 1–20 CFU per 100 mL [39]. Furthermore, culture enables the recovery of isolates, which is essential for epidemiological research and antimicrobial susceptibility testing [40]. Nevertheless, this technique is limited by the slow growth rate of *L. pneumophila*, requiring up to 14 days for final confirmation, and by its inability to detect cells in a viable but non-culturable (VBNC) state, thereby potentially underestimating the true bacterial load in environmental samples [39,41].

In contrast, PNA-FISH offers a rapid, molecular alternative that targets rRNA within intact cells. Although this technique exhibits a limit of detection of approximately 10^3 cells/mL [13,42,43], largely dictated by the resolution and sensitivity of microscopy equipment, it provides the advantage of rapid detection compared to conventional culture methods, significantly reducing the time to decision-making. Moreover, PNA-FISH can detect *L. pneumophila* irrespective of its cultivability, thereby providing a more comprehensive picture of microbial presence in systems where stress conditions may drive cells into the VBNC state [11,44,45]. Its high specificity, determined by the probe design, also supports reliable identification at the genus or species level [17,42].

In bacterial examinations, standard methodologies commonly employ two membrane filter-based sample preparation protocols: (i) direct placement of the filter onto culture media, or (ii) concentration followed by elution. To ensure methodological consistency, the PNA-FISH assay was evaluated under both preparation schemes.

The PNA-FISH method was applied to tap water samples artificially contaminated with *L. pneumophila* at concentrations ranging from 10^8 to 10^3 CFU mL^{-1} and compared with culture-based CFU enumeration. Concentrations below 10^3 CFU mL^{-1} were not assessed, as explained above, this value may correspond to the established limit of detection (LOD) of the PNA-FISH assay. After the filtration process, the method successfully detected the bacteria with a limit of detection of 10^3 CFU mL^{-1} directly on the membrane and 10^4 CFU mL^{-1} on the eluted samples, as demonstrated in Figure 2 and Table 3. The presence of bacteria was verified by CFU enumeration at all tested concentrations; colony counts decreased from ~100 CFU per plate at the 10^8 to 10^5 CFU mL^{-1} concentrations to ~40 CFU per plate at 10^4 to 10^3 CFU mL^{-1} concentrations, indicating dilution effects and the decreased cell count. However, the PNA-FISH method consistently detected bacteria when applied directly on the membrane across the tested concentration range. These results imply that some cells may have been lost or were less detectable during sample processing, especially in eluted samples.

Table 3. Detection of *L. pneumophila* in filtered tap water by PNA-FISH directly on the membrane and from eluted cells, and traditional culture.

Artificially Inoculated Tap Water			
CFU mL^{-1}	**PNA-FISH Outcome**		**Traditional Culture**
	Membrane	**Eluted Cells**	
10^8	+	+	+
10^7	+	+	+
10^6	+	+	+
10^5	+	+	+
10^4	+	+	+
10^3	+	-	+

(+) Positive result for PNA-FISH (rod-shaped bacteria displaying bright red fluorescence) or traditional culture; (-) Negative result for PNA-FISH (absence of signal or intensity indistinguishable from background).

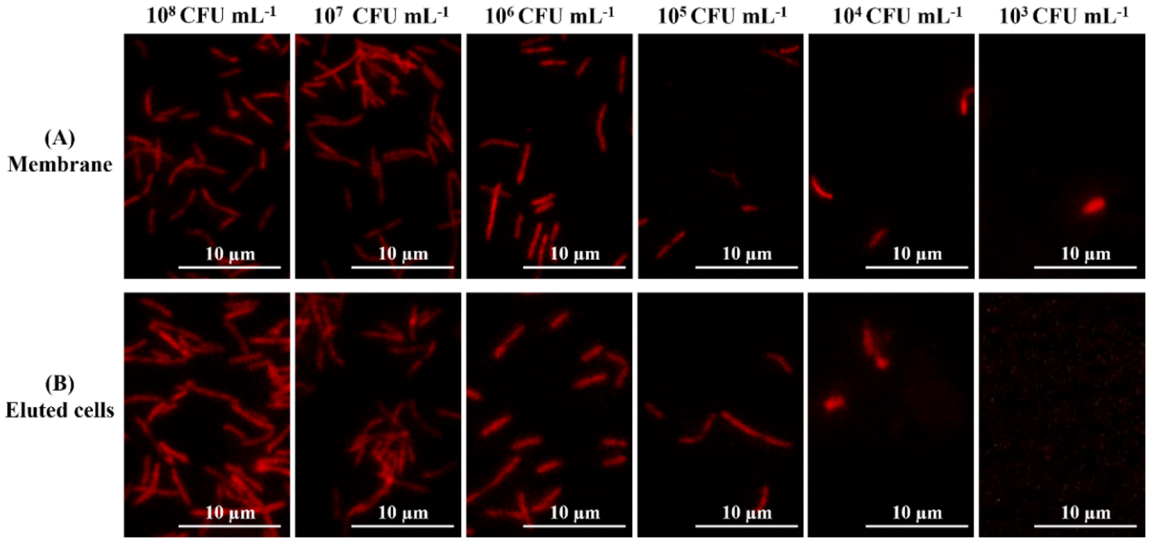

Figure 2. (**A**,**B**) PNA-FISH analysis of artificially contaminated tap water samples containing *L. pneumophila* serogroup 1, ATCC 33152, at concentrations ranging from 10^8 to 10^3 CFU mL^{-1}. (**A**) PNA-FISH images of bacteria captured directly on the membrane. (**B**) PNA-FISH images of bacteria from eluted cells. All images were obtained with equal exposure times for direct visual comparison.

It is important to acknowledge, however, that while culture methods theoretically have a limit of 1–20 CFU per 100 mL [39], microscopy-based methods like PNA-FISH are typically limited to a higher LOD (~10^3 CFU mL^{-1} to 10^4 CFU mL^{-1}) [13,42,43]. Furthermore, as the current validation was performed in artificially contaminated tap water, additional testing is required in complex environmental matrices (e.g., cooling tower water), where background autofluorescence could pose challenges. Future studies should also address the validation of the probe's performance on cells in the Viable but Non-Culturable (VBNC) state to evaluate potential changes in cell wall permeability associated with environmental stress.

Given these results, PNA-FISH should not be viewed as a replacement for culture in compliance monitoring, but rather as an early-warning tool that can be integrated into existing surveillance frameworks. By enabling the rapid detection of *L. pneumophila* cells at levels of potential concern, PNA-FISH can trigger timely preventive actions while awaiting confirmatory culture results.

4. Conclusions

This study successfully validated a novel 16S rRNA-targeted PNA probe for the specific identification of *L. pneumophila*. The method demonstrated 100% specificity and effectively detected the pathogen in water samples with a time-to-result significantly shorter than traditional culture. Although the limit of detection is higher than that of culture-based methods, PNA-FISH offers a valuable early-warning capability for rapid risk assessment. Future research should focus on validating this probe in diverse real-world water systems (e.g., cooling towers) to assess background interference and on optimizing the protocol for the detection of VBNC cells. Furthermore, integration with automated platforms, such as flow cytometry, could further enhance throughput and sensitivity for routine environmental monitoring.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/2512301430461498/JMHD-25100111-Supplementary-Materials.pdf. Figure S1: Specificity and sensitivity of the PNA-FISH probe for *L. pneumophila* serogroup 1, isolate 3 (A); L. pneumophila serogroup 2-15 (B); *L. micdadei* (C), and (D) *E. coli*. Images were obtained with equal exposure times. Table S1: List of strains used in this study. Specificity and sensitivity test results for PNA-FISH probe at 60 °C with 30% formamide.

Author Contributions

A.B.: visualization, investigation, writing—original draft preparation; M.N.-V.: visualization, investigation; D.G.: supervision, editing; C.A.: conceptualization, supervision, editing; N.F.A.: supervision, editing; L.C.: conceptualization, supervision, writing—original draft preparation, editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

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Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable.

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Conflicts of Interest

Given the role as Editorial Board Member, Nuno F. Azevedo had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal. The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

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