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Reliable detection and control of *Legionella pneumophila* in wastewater

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Reliable detection and control of *Legionella pneumophila* in wastewater

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Summary

Introduction

Legionella and growth conditions

Legionella pneumophila is the most prevalent pathogenic *Legionella* species and the main cause of Legionnaires' disease, a severe pneumonia that can occur after inhalation of contaminated aerosols. The bacterium is naturally present in freshwater but becomes a public-health concern when it grows in man-made water systems such as drinking water installations, cooling towers and wastewater treatment plants (WWTPs). Growth is promoted by favourable conditions, particularly temperatures around 25–42°C and nutrient availability. These temperatures are common in biological WWTPs, especially in warm and aerated process steps. According to a RIVM report from 2019, industries with nutrient-rich wastewater include the food industry, the paper and wood industry, the petrochemical industry, rendering plants, and municipal wastewater treatment plants with a warm sidestream (for nitrogen removal in the digestate coming from the sludge digestion). Based on the latest insights and operational experience, however, this list can be expanded to include other industries, such as the textile industry, manure processing facilities, and companies operating mesophilic digestion processes.

Legionella situation in the Netherlands and control measures

The number of registered patients with Legionnaires' disease has increased in the Netherlands over the past decade. This trend has intensified the need for improved monitoring and control of *Legionella* in water systems. In conventional water systems, *Legionella* control relies on risk assessment, routine monitoring, and the implementation of control measures. In WWTPs, however, control measures are more difficult to apply because biological treatment depends on microbial activity and often operates under conditions that may also support *L. pneumophila*. Aeration can further increase the risk by producing aerosols that contain *Legionella* bacteria and can travel considerable distances depending on weather conditions.

L. pneumophila control and risk management in WWTPs

Although WWTPs are increasingly recognised as possible sources of *L. pneumophila* growth and transmission, mandatory Dutch measures remain limited. Current and upcoming guidance (Environmental Activities Decree, 'Besluit Activiteiten Leefomgeving') mainly recommends risk assessments, monitoring and intervention when elevated concentrations are detected. Covers, enclosures and air treatment can reduce aerosol dispersion, but they do not remove the growth conditions in the wastewater process. The project therefore focuses on improving detection, risk assessment and practical control of *L. pneumophila* in wastewater systems.

Current *L. pneumophila* detection and cultivation methods

Reliable detection is essential for assessing public-health risks and deciding on control measures at WWTPs. The current standard, ISO 11731:2017, is culture-based and thus detects culturable cells, and is officially standardized for wastewater, but has important drawbacks. It requires 7–10 days of incubation and is strongly affected by background microorganisms in wastewater. To reduce overgrowth on plates, samples often need dilution, which raises the practical detection limit and can cause false-negative results at concentrations still relevant for risk assessment.

Project scope and objectives

The TKI project "Reliable detection and control of *Legionella pneumophila* in wastewater", of which the results are reported here, aims to improve the monitoring, risk assessment, and control of *L. pneumophila* in wastewater systems. The project is divided into three phases.

- Phase 1 focused on evaluating and comparing alternative, faster detection methods for *L. pneumophila* in wastewater against the standard ISO 11731:2017 method, including protocol optimization.
- Phase 2 addressed further optimization of the alternative detection methods, including the assessment of wastewater pre-treatment methods, and explored alternative strategies for *L. pneumophila* control and (risk) management in WWTPs.
- Phase 3 aims to translate the knowledge generated into practice by developing an online knowledge platform. This platform will centralize information on *L. pneumophila* in wastewater, including detection methods, control strategies, and practical experiences, making it accessible to relevant stakeholders.

Phase 1: Reliable detection of *L. pneumophila* in wastewater

Phase 1 compared ISO 11731 with four commercially available methods that were adapted for wastewater: Legiolert (IDEXX), MICA Advance (Diamidex), rqmicro.COUNT (rqMicro) and QIAcuity dPCR (QIAGEN). Dilution series and six weeks of WWTP monitoring showed that Legiolert, MICA and dPCR generally detected *L. pneumophila* at lower concentrations than ISO, and thus were more sensitive, and could provide earlier warning. In the six-week monitoring, the alternative methods detected *L. pneumophila* at lower concentrations compared to the ISO at the communal WWTP in Leuven, detected low-level presence at Sitech when ISO remained negative, and confirmed high concentrations at the meat-processing WWTP in Boxtel.

Legiolert and MICA were more sensitive than, and correlated with the ISO method, although they often reported lower concentrations in wastewater, possibly because the required pretreatment steps (including decantation or centrifugation) removed particle-associated *L. pneumophila*. dPCR was also more sensitive, but measured gene copies rather than culturable cells and therefore cannot yet be converted reliably to cfu/L. rqmicro.COUNT was not yet suitable for wastewater because of weak correlations, suspected off-target detection and technical problems such as clogging.

Overall, Legiolert, MICA and QIAcuity dPCR are the most promising methods for wastewater application, mainly because they are more sensitive than ISO, yield faster results and may thus support earlier intervention. However, further comparison to the ISO method is needed: larger datasets across different wastewater types and wastewater from different locations are needed to confirm detection limits, understand matrix effects, improve protocols and define action thresholds that are comparable to the current ISO-based cfu/L values.

Phase 2: *L. pneumophila* control measures

Control of *L. pneumophila* in WWTPs is primarily a risk-management challenge rather than a simple disinfection problem. Biological WWTPs can combine favourable growth conditions, including warm water, nutrients, biofilms, protozoan hosts and aerobic treatment, with process steps that generate aerosols. The most relevant factors that determine the probability of *Legionella* growth are wastewater type, process temperature and pH. Factors that are important with regard to the probability of exposure are type of aeration systems, sludge handling and effluent reuse together with the distance to nearby populations. Industrial WWTPs treating food, paper, wood, petrochemical or rendering-related

wastewater are at high risk because they often operate at elevated temperatures and contain nutrient-rich wastewater streams.

For practical implementation, the most feasible measures often reduce exposure rather than eliminate *L. pneumophila* at the source. These include selecting aeration systems that produce fewer aerosols, covering aeration tanks, extracting and treating air under covers, using respiratory protection during high-risk activities, avoiding high-pressure cleaning, and setting up a management plan with regular monitoring and clear action levels.

Current *L. pneumophila* control tools for WWTPs are mostly reactive and focused on containment or worker protection. Predictive modelling, larger datasets and better links between wastewater concentrations and aerosol risks will give more information for management plans and thus preventive actions to be taken, and can be used to evaluate which measures truly reduce risk under different WWTP conditions.

Phase 2: sample pre-treatment for detection of *L. pneumophila* in wastewater

Different pre-treatment of wastewater of two industrial WWTPs were tested to improve the performance of three alternative *L. pneumophila* detection methods (QIAcuity dPCR, rqmicro.COUNT and MICA). These sample pre-treatment strategies were adapted from the already available Legiolert pretreatment protocol that was successful in enabling accurate reads in wastewater and were evaluated for their suitability in other rapid detection methods.

Pre-treatment effects were method-specific and did not generally improve detection. Centrifugation reduced visible microbial overgrowth on MICA Advance membranes, which may improve readability, but its influence on quantified *L. pneumophila* concentrations remained unclear. Centrifugation reduced reaction interference and improved signal quality for QIAcuity dPCR, but it also significantly lowered the number of detected *L. pneumophila* DNA copies, suggesting that cells or DNA may be associated with suspended solids and are removed with the pellet. Sonication alone did not significantly improve detection, and in combination with centrifugation it did not recover the apparent loss of *L. pneumophila*. rqmicro.COUNT showed no meaningful response to the tested pre-treatments, and positive blanks suggested off-target detection or autofluorescence.

Although centrifugation led to lower *L. pneumophila* concentrations measured by Legiolert, MICA and dPCR compared with ISO, the ISO method itself remains vulnerable to especially overgrowth and has a high detection limit. It is recommended to test alternative DNA extraction kits and PCR additives for dPCR, optimize acid or heat treatments for MICA, and investigate off-target signals in rqmicro.COUNT. Larger, matrix-diverse datasets are needed before wastewater pre-treatment protocols can be standardized or used confidently for routine monitoring and model input.

Phase 2: Algorithm for predicting *L. pneumophila* in WWTP

The ‘Liquisens approach’ was developed for an industrial WWTP to predict *L. pneumophila* risk and support earlier warning than periodic laboratory testing alone. The model combined historical ISO culture measurements with other data on flow, pH, oxygen and operational indicators. Elevated *L. pneumophila* concentrations were linked to the MBBR-based biological treatment stage which has favourable conditions for *L. pneumophila* growth. Factors such as pH, organic-load indicators, sludge-related variables and production-related events were identified by the model to be predictors of *L. pneumophila*

growth. Although the dataset was limited, the case study showed that process data can help distinguish low-, moderate- and high-risk situations and identify parameters associated with increasing risk.
A predictive model is envisioned to be used in addition to the regulatory monitoring of *L. pneumophila* in wastewater and *Legionella* risk management plans. A possible early warning for *L. pneumophila* could be followed by more intense *Legionella* monitoring to confirm this. A predictive model supports proactive management and may act as an early warning system before *Legionella* concentrations become critical. Further validation of the model is needed with larger datasets and more *L. pneumophila* results.

Phase 3: online knowledge database

An online knowledge platform was developed to improve access to information on *L. pneumophila* in wastewater treatment systems. The platform, www.legionellainafvalwater.nl, brings together fragmented knowledge and project findings for operators, industrial users, consultants, researchers, and regulatory authorities. It is intended to be a flexible basis for future content updates.

A summary of the Hydroscope knowledge document and information generated within the project were included in the website. Using generative AI the technical material was adapted into accessible web content, which was subsequently reviewed and validated. The platform's content, structure, and usability was further refined based on comments of the project group.

The website is available in both Dutch and English. The platform is intended to support continued knowledge sharing and dissemination of project results beyond the project's completion.

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1 Introduction

1.1 *Legionella* and growth conditions

Legionella is a genus of Gram-negative bacteria comprising over sixty species, many of which pose significant public health risks. The genus was first identified following an outbreak of severe respiratory illness at the American Legion convention in Philadelphia in 1976 (Fraser et al., 1977). Among these sixty species, *Legionella pneumophila* is the most prevalent and well-studied pathogen (World Health Organization, 2007).
Infections caused by *L. pneumophila* manifest primarily as Legionnaires' disease, a severe form of pneumonia, or Pontiac fever, a milder, flu-like illness (World Health Organization, 2007). While many infections remain asymptomatic, vulnerable populations, including the elderly and immunocompromised individuals, are at increased risk of developing severe disease. Transmission occurs predominantly through inhalation of contaminated aerosols generated from water systems (Liu and Shin, 2019; Vance et al., 2015).
L. pneumophila is naturally present in freshwater environments but becomes a significant concern when it colonizes and proliferates in man-made water systems such as drinking and hot water installations, cooling towers and wastewater treatment plants (WWTPs) (NASEM, 2019; Vance et al., 2015; World Health Organization, 2007; van den Berg et al., 2023; Pijnacker et al., 2024). Growth is strongly influenced by environmental conditions, particularly temperature. The optimal growth range for *Legionella* lies between 25 and 42 °C, although *L. pneumophila* can survive and potentially grow at higher temperatures (Bourdon et al., 2019). Temperatures within this range are commonly encountered in engineered water systems, thereby facilitating colonization.

1.2 *Legionella* situation in the Netherlands and control measures

The number of registered patients with Legionnaires' disease has increased in the Netherlands over the past decade. This trend has intensified the need for improved monitoring and control of *Legionella* in water systems. In the Netherlands, *Legionella* control plans are an essential tool for managing risks in installations such as drinking water systems and cooling towers. These plans typically include risk assessments, routine monitoring, and the implementation of control measures (Lodder et al., 2019; World Health Organization, 2007). Key strategy includes maintaining water temperatures in man-made water systems outside the optimal range for *Legionella* growth (30 - 38 °C) or even outside the growth range (25 - 45 °C). In The Netherland the following water temperatures are maintained: above 60 °C for hot water storage and below 20 °C for cold water distribution. Additionally, preventing water stagnation during extended periods of time and enforcing regular cleaning and maintenance protocols are critical measures for minimizing the risk of *L. pneumophila* proliferation (Bourdon et al., 2019).

1.3 *L. pneumophila* control and risk management in WWTPs

In addition to temperature, nutrient availability plays an important role in *Legionella* proliferation. Industrial and municipal biological WWTPs have therefore been increasingly recognized as a potential source of *Legionella* growth and transmission. In particular, biological WWTPs with aeration operating at elevated temperatures (e.g., 30–38 °C) (Pijnacker et al., 2024; van den Berg et al., 2023; Vermeulen et al., 2021). But also low-risk downstream municipal biological WWTPs that receive water contaminated with *L. pneumophila* pose a risk. Depending on the type of aeration, aerosols generated above the aeration tanks of WWTPs may contain substantial concentrations of *Legionella* (up to 3300 CFU/m³) and can travel considerable distances, potentially causing infections far from the original source (Blatny et al., 2008). Figure 1 provides a schematic representation of a WWTP with a graph visualizing the risk potential over the different wastewater treatment steps (chapter 5).

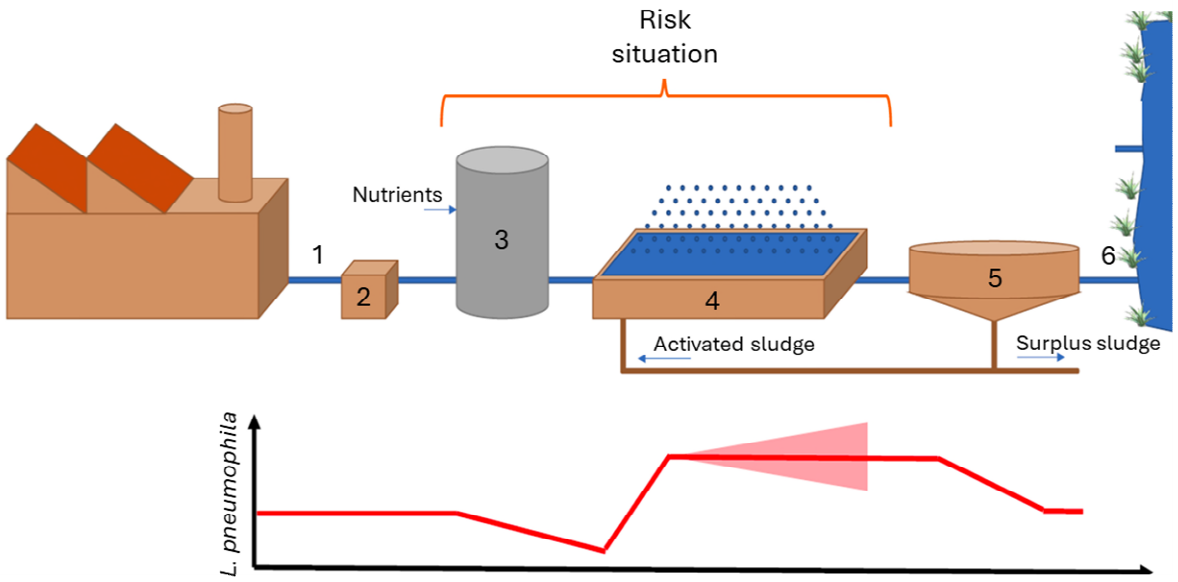


Figure 1-1. Typical hotspots for L. pneumophila in industrial wastewater treatment systems. The L. pneumophila growth and dissemination risk increases in the aerated activated sludge step where the conditions promote its growth and aeration leads to its spread through aerosols (chapter 5). 1 – Effluent, 2 – Pre-filter, 3 – Anaerobic reactor, 4 – Aeration tank, 5 – Sludge separation, 6 – Effluent.

Despite the recognized risk, there are currently no mandatory precautionary measures in place in the Netherlands to prevent the spread and growth of *L. pneumophila* from WWTPs. So far, guidance has been limited to recommendations for high-risk WWTPs, advising regular monitoring of *L. pneumophila* concentrations and implementing appropriate prevention and control measures when elevated levels are detected (Lodder et al., 2019; World Health Organization, 2007; Oesterholt & Hollebekkers, 2022). While it is planned that regulations for WWTPs will be included in the Environmental Activities Decree (Besluit Activiteiten Leefomgeving), these will be comparable to those for cooling water systems, with a primary focus on carrying out a risk assessment and drawing up a management plan when risks are identified, with no concrete measures yet in place.

Given that the risk of *L. pneumophila* growth in artificial water systems increases under specific conditions (e.g., temperature), at the moment, the key strategy includes maintaining water temperatures as much as possible outside of *L. pneumophila* optimal range, which is rather challenging in some WWTP biological systems that depend on temperatures optimal for microbial growth (e.g. for Anammox bacteria). As a preventive measure for spreading of *L. pneumophila* within the aerosols, so far, the implementation of an aeration tank cover or enclosure and/or air treatment is recommended, which indeed limits the spreading but does not resolve the issue at its core (Kanters, 2022).

1.4 Current *L. pneumophila* detection and cultivation methods

Reliable detection of *L. pneumophila* is essential for risk assessment and the implementation of and monitoring of the effect of appropriate control strategies. Currently, the standard method for detection is the culture-based ISO 11731:2017 method (ISO, 2017). While widely accepted, this method has several limitations. Firstly, the incubation period is relatively long, 7 to 14 days, which delays timely intervention in high-risk situations. Secondly, the method relies on the ability of *Legionella* to grow on selective agar media, thereby excluding viable but non-culturable cells.

A major limitation arises when analyzing complex matrices such as wastewater. In these samples, high concentrations of background microorganisms often lead to overgrowth on selective plates, obscuring *Legionella* colonies and complicating detection (Heining et al., 2023; van den Berg et al., 2023). To mitigate this issue, samples are frequently diluted. However, each dilution step increases the detection limit significantly. For example, a tenfold dilution can raise the detection limit to approximately 20,000 CFU/L, while a hundredfold

dilution may increase it to 200,000 CFU/L (Oesterholt & Hollebekkers, 2022). As in practice such dilutions are often required for wastewater and sludge samples from WWTPs (Kanters, 2022), the current culture method may lead to false negatives at concentrations that are still relevant for public health, limiting the reliability of risk assessments (Oesterholt & Hollebekkers, 2022). These limitations highlight the need for faster and more robust detection methods for *L. pneumophila* in complex water matrices.

1.5 Alternative *L. pneumophila* detection methods

In recent years, several alternative detection methods for *L. pneumophila* have been developed and commercialized, primarily for use in drinking water systems but also more challenging water systems such as cooling water. However, their applicability to more challenging matrices such as wastewater has not yet been fully established.
This TKI project "Reliable detection and control of *Legionella pneumophila* in wastewater" aims to evaluate a range of these alternative methods. Four detection techniques representing different analytical principles were tested for their suitability to detect and quantify *L. pneumophila* in industrial and communal wastewaters: two alternative culture-based methods (Legiolert from IDEXX; and MICA from Diamidex), a flow cytometry-based method (rqmicro.COUNT from rqmicro), and a molecular method based on digital PCR (dPCR on QIAcuity system from QIAGEN).
These methods offer potential advantages, including shorter analysis times and improved sensitivity. However, as they are currently optimized for relatively clean water matrices, such as drinking water and cooling tower water, their performance in wastewater requires further validation and optimization.

1.6 Project scope and objectives

The TKI project "Reliable detection and control of *Legionella pneumophila* in wastewater", of which the results are reported here, aims to improve the monitoring, risk assessment, and control of *L. pneumophila* in wastewater systems. The project is divided into three phases.

- Phase 1 focused on evaluating and comparing alternative, faster detection methods for *L. pneumophila* in wastewater against the standard ISO 11731:2017 method, including protocol optimization.
- Phase 2 addressed further optimization of the alternative detection methods, including the assessment of wastewater pre-treatment methods, and explored alternative strategies for *L. pneumophila* control and (risk) management in WWTPs.
- Phase 3 aims to translate the knowledge generated into practice by developing an online knowledge platform. This platform will centralize information on *L. pneumophila* in wastewater, including detection methods, control strategies, and practical experiences, making it accessible to relevant stakeholders.

The TKI project "Reliable detection and control of *Legionella pneumophila* in wastewater" is a collaborative project involving knowledge institutions (KWR Water, CEW), consultancy firms (NORMEC Kalsbeek, Hydroscope, Rehydro), technology providers (IDEXX, QIAGEN, rqmicro, Diamidex, Liquisens, Innosieve Diagnostics), and end users (GETEC Park Emmen, VITO, Waterstromen, Circle Wastewater Services Services, Tyson).

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2 Phase 1. Reliable detection of *Legionella pneumophila* in wastewater

2.1 Summary

The number of registered patients with Legionnaires' disease has increased in the Netherlands over the last ten years. One possible contamination source for *Legionella pneumophila*, the dominant cause for Legionnaires' disease, are wastewater treatment plants (WWTP). The current detection method for *L. pneumophila* in wastewater is a cultivation method (ISO 11731). The analysis time of the ISO method is 7-10 days, and the detection limit is high ($1 \times 10^5 - 1 \times 10^6$ cfu/L). A faster, more sensitive method could be an improvement for public health.

Four different methods designed for detecting *L. pneumophila* in drinking water had been adapted to detect *L. pneumophila* in wastewater and were tested in this study: Legiolert from IDEXX, rqmicro.COUNT from rqmicro, MICA from Diamidex, and Qiacuity dPCR from QIAGEN. Dilution series of wastewater with high *L. pneumophila* numbers into 0.22 µm filtered wastewater were done with all four methods and the ISO method. Additionally, three warm partial streams of WWTPs were sampled for six weeks and measured with all methods.

The results from the dilution series showed that the Legiolert (125x – 12500x dilution), MICA (125x – 12500x dilution), and Qiacuity dPCR (5x - 125000x dilution) gave a positive result for *L. pneumophila* at higher dilutions than the ISO method (1x – 12500x dilution). During the weekly monitoring of the WWTPs, location one was negative for *L. pneumophila* in weeks 1-3 and positive in weeks 4-6 with the ISO method. All four adapted methods did detect *L. pneumophila* in weeks 1-3 as well as in weeks 4-6. Location two was negative for the ISO method throughout the experiment, but the adapted methods sporadically detected *L. pneumophila*, mostly around their lower limit of detection. Location three was positive with all methods during the six weeks.

The Legiolert (if potential outliers were removed), MICA, and dPCR significantly correlated with the ISO method. The ISO method, Legiolert, MICA, and dPCR all significantly correlated with each other. The rqmicro.COUNT either did not correlate with the other methods or only weakly.

The MICA and Legiolert have a deviation from the ISO-method results that were less clear in previous work with easier water matrices. It is possible some signal is lost when using wastewater as a matrix. Testing a larger test set of WWTP samples could give further insight into this, and if it is indeed a real effect, the protocol might need some adjustments. The rqmicro.COUNT protocol needs further optimization before being applied to wastewater. Two initial steps could be to add the acid treatment to the pre-treatment to potentially improve performance and verify that there is no off-target detection as that could not be done in this study. Although the dPCR did do quite well, it will be important to collect more data to perhaps be able to convert the gene copy number into an estimation of viable *L. pneumophila* cells.

Overall, the new methods were able to detect *L. pneumophila* at lower concentrations than the ISO method during the WWTP monitoring and showed that they might be able to give an earlier warning as to the presence of *L. pneumophila*.

2.2 Introduction

2.2.1 Introduction

The number of registered patients with Legionnaires' disease has increased in the Netherlands over the last ten years. *Legionella pneumophila*, the dominant cause for Legionnaires' disease, mainly occurs in aquatic environments when the water temperature reaches between 25 and 45°C. Such temperatures can occur in several different man-made water systems such as drinking water installations, cooling towers, and wastewater treatment plants (WWTP), which all have been shown to be a possible source of *L. pneumophila* contaminations (NASEM, 2019; van den Berg et al., 2023; Pijnacker et al., 2024). In the Netherlands, a *Legionella* control plan is an important tool to control *Legionella* in these water systems. To effectively control *Legionella* multiple measures such as risk analysis, monitoring, and implementation of control measures in the various water systems is essential.

However, the cultivation method for *Legionella* (ISO 11731:2017), that is currently prescribed for all water matrices has several downsides like: only *Legionella* species that can grow on the selective agar medium are cultivated and the analysis time is 7-10 days. The biggest downside is that the growth of other microorganisms on the selective agar is high for dirtier water samples, such as those from cooling towers and WWTPs. The resulting bacterial colonies or fungal growth makes the small *Legionella* colonies difficult to detect. The only solution is to dilute the water sample, including the interfering microorganisms, which results in a higher detection limit. With a ten times dilution, the detection limit can already increase to 20,000 CFU/l and with a 100 times dilution this increases further to 200,000 CFU/l (Oesterholt & Hollebekkers, 2022). The experience shows that such dilutions can be necessary to analyze wastewater or the sludge at wastewater treatment plants (Kanters, 2022). This means that with the current method it is hard to estimate the impact of *L. pneumophila* on public health as the method could result in many false negatives for concentrations that could already impact public health (Oesterholt & Hollebeekers, 2022). Thus, an alternative reliable detection method for *L. pneumophila* is an essential tool to get insight in the numbers of *L. pneumophila* in WWTPs and use this information to estimate the risk of their plant on public health, to decide whether and which control measures they might want to implement and to test whether these control measures indeed lowered the number of *L. pneumophila* to acceptable levels.

Recently, a number of different alternative detection methods for *L. pneumophila* in drinking water have been commercialized. However, it has not yet been established whether these methods are also suitable to detect *L. pneumophila* in a more challenging water matrix such as wastewater. Within phase one of this TKI-project these alternative methods are tested and compared to the standard culture method (ISO 11731) to establish whether these alternative methods could be a potential replacement for the standard culture method. The alternative methods included in the project work according to different principles. There are two alternative cultivation methods: Legiolert from IDEXX and MICA from Diamidex, a flowcytometry method: rqmicro.COUNT from rqmicro, and a molecular method: dPCR from QIAGEN.

2.2.2 Goal

The overall goal of phase 1 in this TKI project is to determine whether the new alternative methods are applicable with the wastewater matrix. Because the protocols for these alternative methods are made for drinking water, the following subgoals were identified:

- Optimize the protocols for wastewater as they are currently optimized for drinking water
- Compare the new methods with the current ISO method and determine the limits of detection
- Make a preliminary assessment of whether the new methods could be used for routine monitoring of *L. pneumophila* at WWTPs.

2.3 Materials and methods

2.3.1 Setup

Phase 1 was split into two experiments i) attempting to determine a detection limit through dilution series and ii) testing the methods for monitoring WWTPs.

2.3.1.1 Bacterial strain

For the experiments where *L. pneumophila* was dosed, the serogroup 1 strain ATCC33152 was used.

2.3.1.2 Cultivation and hardening of *L. pneumophila*

L. pneumophila was cultivated on MWY plates at 37°C until fully grown and the colonies were suspended in physiological buffered saline (PBS). The concentration was determined using flow cytometry according to KWR protocol LMB-071. A hardening step was first applied to the resuspended colonies in PBS as it was noted by the partners that transferring *L. pneumophila* cells directly from a cultivation medium to wastewater can be too strong of a shock and lead to the death of the cells. The hardening protocol functions as an acclimatization step to lower this shock and leave the cells alive upon transfer to the wastewater. Hardening was done according to the protocol provided by IDEXX. In short, cells were added to 7 mL of a hardening buffer solution (Table 2-1) to obtain an optical density of 0.1 at 600nm. The suspension was placed at 36°C for 20-24 hours.

Table 2-1. Hardening buffer composition

Chemical	Concentration g/L
Magnesium sulfate, anhydrous	0.481
Calcium chloride, dihydrate	0.059
Ferrous ammonium sulfate	0.020
Sodium monohydrogen phosphate	0.355
Potassium dihydrogen phosphate	0.340
Sodium citrate	0.090

2.3.1.3 Experiment 1: Dilution series

Dilution series were performed to compare the new methods to the ISO method under controlled lab conditions and to determine the limits of detection for the different methods using wastewater as the matrix. For experiment 1, two different setups were used. Initially wastewater was spiked with a known quantity of *L. pneumophila* sg1 (ATCC 33152) that was cultivated on BCYE agar plates after which the cells were hardened as described in section 2.3.1.2. Then 1×10^6 cfu/L cells were added to a wastewater sample from a location with historically no or low levels of *L. pneumophila*. A dilution series was made in PBS and each dilution was measured with all five methods (Table 2-2). The dilution series was based around a reported theoretical detection limit for the various new methods at 100-1000 cfu/L in drinking water. As it was expected that the detection limit in wastewater might be higher than for drinking water, most of the dilution series was put at higher concentrations than 100 cfu/L.

Table 2-2. Setup for the dilution series using wastewater spiked with cultivated L. pneumophila.

Dilution step	Dilution	Spiked *L. pneumophila* concentration
Start	Spiked wastewater	1×10^6 cfu/L
10x	10x dilution	1×10^5 cfu/L
5x	50x dilution	2×10^4 cfu/L
5x	250x dilution	4,000 cfu/L
5x	1,250x dilution	800 cfu/L
5x	6,250x dilution	160 cfu/L
5x	31,250x dilution	32 cfu/L
Control	Unspiked wastewater	0 cfu/L

As this initial setup did not result in usable results as *L. pneumophila* was not detected or at much lower concentrations than spiked (see section 2.4.1.1), the setup was adjusted. For the final three dilution series (250 to 31,250 x dilution) of a wastewater with historically high *L. pneumophila* levels was used as a spike into a wastewater from which all cells were removed (Table 2-4). Cells were removed by centrifuging the wastewater sample down for 5 minutes at 3000g, followed by filtration over a 1.2 µm and then 0.22 µm membrane. As the exact *L. pneumophila* concentration in the wastewater that was used as a spike was not known on forehand, an estimated concentration was determined based on the historic data from that location. This gave an estimated lower and upper limit of what could be spiked (Table 2-3). The dilution series was designed in such a way that in either case a usable result in terms of determining the detection limit should be obtained. Each dilution was measured in triplicate with each method. The average and standard deviation were used in this report.

Table 2-3. Setup for the dilution series using high L. pneumophila wastewater. Limits are based on the variability of the L. pneumophila concentrations that have been measured in the high L. pneumophila wastewaters.

Dilution step	Total dilution	Lower limit *L. pneumophila* that could be spiked (cfu/L) and the resulting dilutions.	Upper limit *L. pneumophila* that could be spiked (cfu/L) and the resulting dilutions.
Start	NA	1×10^5/L	1×10^8/L
5x	5x	2×10^4/L	2×10^7/L
5x	25x	4,000/L	4×10^6/L
5x	125x	800/L	8×10^5/L
10x	1,250x	80/L	8×10^4/L
10x	12,500x	8/L	8,000/L
10x	125,000x	0.8/L	800/L
Filtered wastewater (Control)	NA	0	0

Table 2-4. Wastewater used for the dilution series spiked with high L. pneumophila wastewater.

Dilution series	Spiking wastewater	Wastewater used for diluting
Dilution series 1	Industrial, Boxtel	Industrial, Boxtel
Dilution series 2	Communal WWTP Ede	Communal WWTP Ede
Dilution series 3	Industrial WWTP Ede	Circle Wastewater Services, Geleen

2.3.1.4 Experiment 2: Monitoring of WWTPs

The monitoring of the different WWTPs was performed to compare the new methods to the ISO method in a practical setting. Three different WWTP's (Table 2-5) were sampled weekly during 20-Feb-2024 to 16-Apr-2024, resulting in six samples per WWTP (Table 2-6). A mix of one communal and two different types of industrial WWTPs was deliberately selected to cover a broader range of practical WWTP. Also, two of these plants had a history of positive *L. pneumophila* samples and one did not to investigate whether the methods would perform similarly for these different WWTPs. Samples were taken from the aeration basin and for each sample *L. pneumophila* concentrations were determined using all five methods. Wastewater samples at Leuven were taken by the local operator and shipped on ice to KWR on the same day. Samples at Circle Wastewater Services and Boxtel were sampled by Hydroscope and shipped on ice to KWR on the same day.

Table 2-5. WWTP's sampled during the monitoring experiment

Location number	Location	Type	Historic *L. pneumophila* levels
Location 1	Leuven	Communal	Moderate (sometimes positive detections)
Location 2	Circle Wastewater Services, Geleen	Industrial (chemical)	Low (very rarely a positive detection)
Location 3	Boxtel	Industrial (meat)	High (often positive detections)

Table 2-6. Sampling scheme WWTP monitoring

Location	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Location 1	20-Feb-24, duplicate	27-Feb-24, triplicate	12-Mar-24, duplicate	26-Mar-24, triplicate	09-Apr-24, duplicate	16-Apr-24, triplicate
Location 2	Not sampled	27-Feb-24, triplicate	12-Mar-24, duplicate	26-Mar-24, triplicate	09-Apr-24, duplicate	16-Apr-24, triplicate
Location 3	20-Feb-24, duplicate	27-Feb-24, triplicate	12-Mar-24, duplicate	26-Mar-24, triplicate	09-Apr-24, duplicate	16-Apr-24, triplicate

2.3.1.5 *Legionella pneumophila* detection methods

2.3.1.5.1 ISO 11731

Determining the number of *L. pneumophila* and other *Legionella* species in the spiked or diluted wastewater samples was done according to NEN-EN-ISO 11731 using the protocol for matrix C (wastewater) for wastewater samples and matrix A for drinking water samples, according to KWR-protocol LMB-080. The samples were treated with a heat-shock and acid treatment as prescribed. Afterwards the samples were incubated on a selective medium (Buffered Charcoal Yeast Extract Agar (MWY) or Glycine-vancomycin-polymyxin-cycloheximide agar (GVPC)). The agar plates were incubated for 7-10 days at 36°C. The colonies were confirmed to be *L. pneumophila* or *L. non-pneumophila* through PCR according to KWR-protocol LMB-050.

2.3.1.5.2 Legiolert

Legiolert is a detection method based on cultivation in a nutrient rich medium. A specific substrate is added to the medium that when metabolized by *L. pneumophila* results in an excreted product that creates a brown color in the medium. The medium with the sample, at different dilutions, to be analyzed is poured in a tray with 96 wells and cultivated at 37°C for 7 days. After cultivation, wells positive for *L. pneumophila* can be counted due to

the color change, allowing for a quantitative determination of viable and culturable *L. pneumophila* in the sample.
Experiments with the Legiolert were performed according to the manual provided. There were two pretreatment protocols, one for drinking water samples and one adapted for wastewater samples. The adapted protocol for wastewater has the following pretreatment steps: Mixing through inversion, 9 min of ultrasonic treatment in a 35W, 44kHz water bath, and centrifugation at 1000g for 5 minutes. Finally, the supernatant is then placed in a 50°C water bath for 30 min. and cooled back to ambient temperature before proceeding with the standard Legiolert protocol. Unless noted the pretreatment protocol for wastewater samples was used. For samples that were processed according to the drinking water protocol the results are labelled as simple pretreatment.

2.3.1.5.3 MICA

MICA is a plate-based cultivation method. Before plating a specific substrate is added to the sample. This substrate can be metabolized by *L. pneumophila* when *L. pneumophila* grows on the plate medium. The produced metabolite from the specific substrate remains associated with the bacterial cell during growth. The plates are incubated during 48 hours at 37°C. After this cultivation period a fluorescent compound is used that binds to the metabolite which was produced from the specific substrate added before cultivation. Subsequently, the *L. pneumophila* cells can be identified as fluorescent colonies, allowing for an automatic quantitative determination of viable and culturable *L. pneumophila* in the sample. Experiments with the MICA were performed according to the manual provided. During the study the provided pretreatment protocol changed from version 1 to version 2. For version 2 the pH treatment was increased in volume from 5mL in version 1 to 20 mL in version 2 and the maximum filtration volume of the samples was reduced to 2mL. Version 2 of the pretreatment protocol was implemented for the second set of dilution series (see 2.3.1.3) and from week 4 onwards for the monitoring of the WWTP's (see 2.3.1.4).

2.3.1.5.4 rqmicro.COUNT

The rqmicro.COUNT is a flowcytometry-based detection method. Magnetic beads coated with specific antibodies were added to the sample. The antibodies bind to *L. pneumophila* cells in the sample, also binding them to the magnetic beads. Then the sample is run by a magnetic field to separate the magnetic beads from the rest of the sample. Finally, detection is done through flowcytometry, allowing for automatic quantitative determination of *L. pneumophila* cells in the sample. Experiments with the rqmicro.COUNT were performed according to the manual provided.

2.3.1.5.5 Qiacuity dPCR

The Qiacuity dPCR is a DNA-based molecular detection method. DNA is extracted from a sample and a mastermix for the PCR reaction is prepared with specific primers for *L. pneumophila*. This mastermix is then added to the dPCR plate with approximately 26000 partitions per well. After the PCR reaction is finished the number of positive partitions in a well is determined, allowing for a quantitative determination of the gene copies of *L. pneumophila* in the sample. DNA extraction for the dPCR was done with the QIAGEN DNeasy® PowerWater® Kit, according to the provided protocol. The dPCR was subsequently used according to the manual provided with the Qiacuity machine.

2.3.1.6 Statistical analysis

For data analysis the log10 values of the *Legionella* concentrations, obtained with the different methods, were used, unless stated otherwise. QQ-plots and the Shapiro-Wilson test showed that data was normally distributed. To compare the alternative methods with the ISO method, a Pearson correlation analysis was performed using only samples that had a positive result for both the ISO method and the alternative method to avoid skewing the results if one method had far less detections. As there were a few datapoints that seemed like potential outliers, the data was further assessed. Potential outliers were determined by calculating the Cook's distance (Cook, 1977) of each datapoint and defined as a Cook's distance > 4/n and > 3x the average Cook's distance of the

included samples. Both the correlations with and without outliers are reported as comparison. Differences between methods were tested for significance using the pairwise two-sample t-test. Analyses were done in RStudio version 2022.07.2+576. For the correlation matrix the package ggcorrplot was used, using Spearman correlation to work with data that was not normally distributed (as non-detects were also used in the analysis). Here non-detects were included because the number of samples for which every method had a positive detection was too limited (n = 7). The other figures were made using the ggplot2 package.

2.4 Results

In Tables I-1 in Appendix I all the results obtained during the project are presented in table form. For some samples, some of the applied methods resulted in an error or the sample could not be analyzed. An overview of all these errors/missing samples, with the reason for the detection failure as far as it could be determined, are shown in Table I-2 in Appendix I.

2.4.1 Experiment 1-1: Dilution series spiked with cultivated *L. pneumophila*

2.4.1.1 Results dilution series with cultivated *Legionella pneumophila*

In the initial setup of the dilution series, two dilution series were performed with *L. pneumophila* cultivated as described in section 2.3.1.2. The combined results are shown in Table 2-7. When spiking was done with a final concentration of 1×10^6 cfu/L concentration of *L. pneumophila,* an issue could already be seen with the ISO method not properly detecting the *L. pneumophila* spike in the samples. Similarly, the MICA (pretreatment version 1) and Legiolert also did not detect *L. pneumophila* or in concentrations multiple orders of magnitude lower than the concentration in the spiking solution, which was measured with FCM. Although the rqmicro.COUNT method did detect the presence of *L. pneumophila* in these dilution series, there was no decrease detected with this method when *L. pneumophila* was further diluted, indicating that also with the rqmicro.COUNT something was not performing as expected. The dPCR was the only method that showed a decrease in *L. pneumophila* concentrations roughly in line with the dilutions until the signal was lost between 800 and 4,000 cells per liter.

These results seem to show that the cultivation methods have the most difficulties in detecting *L. pneumophila* as they generally did not give a positive detection at all, indicating perhaps a shift from culturable bacteria to VBNC (Viable But Not Culturable) or dead bacteria soon after spiking. No experiments were performed to confirm the VBNC and/or dead shift. This was the case even when the wastewater from one location was spiked twice at different moments. These inconsistent testing results make it also difficult to quickly resolve the issue and, consequently, the approach of spiking with cultivated *L. pneumophila* was not continued.

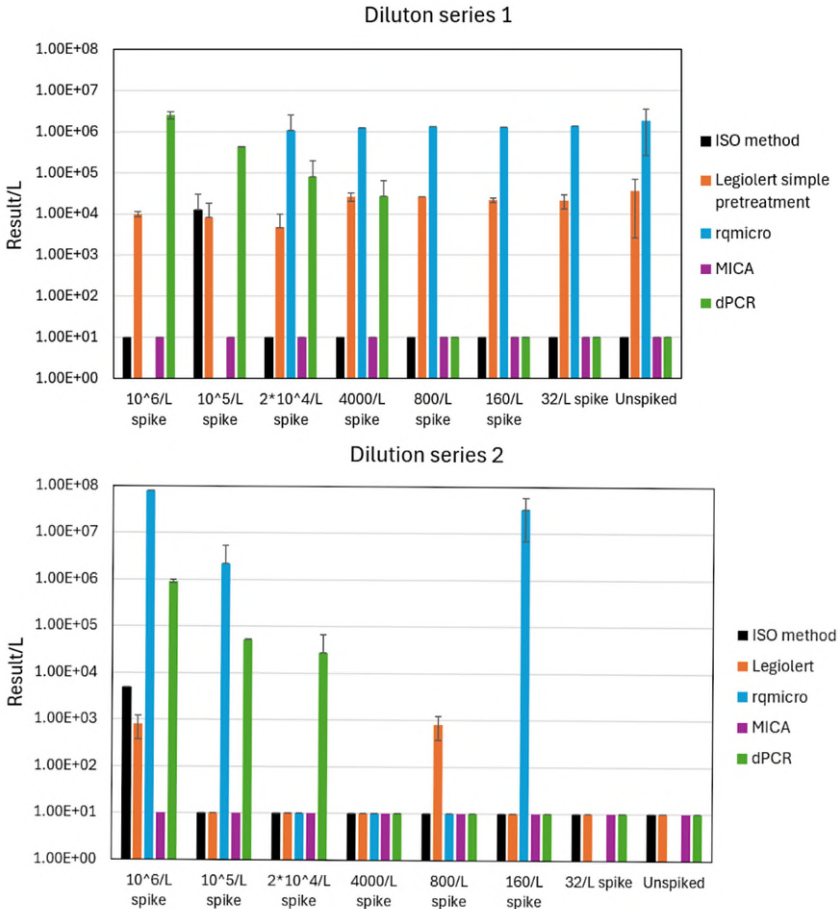


Table 2-7. Measurement data for all methods for both dilution series using cultivated L. pneumophila as a spike in wastewater with historically low L. pneumophila numbers. MICA pretreatment version 1 was used.

2.4.2 Experiment 1-2: Dilution series spiked with high *L. pneumophila* wastewater

Some results are compared to the theoretical detection limit. This is the concentration that would be reported if one cell is detected across the replicates combined.

2.4.2.1 Dilution series 1

An overview of the results of the first dilution series is provided in Table 2-8. The ISO method was only able to detect *L. pneumophila* in the wastewater used for spiking with a result of 6.7×10^5 cfu/L. It did not detect *L. pneumophila* in any of the dilutions, despite the concentration being above the theoretical detection limit, which was determined on the result from the spiked wastewater. Legiolert and MICA both detected *L. pneumophila* up to and including the 125× dilution, although of these two only the Legiolert showed lower concentrations after each dilution. The dPCR detected *L. pneumophila* up to and including the 5× dilution. The rqmicro.COUNT detected *L. pneumophila* in all dilutions, but also in the filtered wastewater, which does not contain *L. pneumophila*.

If the assumption is made that the ISO method's determination of *L. pneumophila* counts is accurate and the concentration of the undiluted wastewater is corrected for the appropriate dilution step, it is possible to translate the detection of the other methods to ISO cfu/L equivalents. This results in the following *L. pneumophila* concentrations being successfully detected by the other methods (as cfu/L equivalents of the ISO method): 5.3×10^3 cfu/L for Legiolert and the MICA, 1.3×10^5 cfu/L for the dPCR, and 5.3 cfu/L for the rqmicro.COUNT. The rqmicro.COUNT is unlikely to be reliable as it also detected *L. pneumophila* in the negative control.

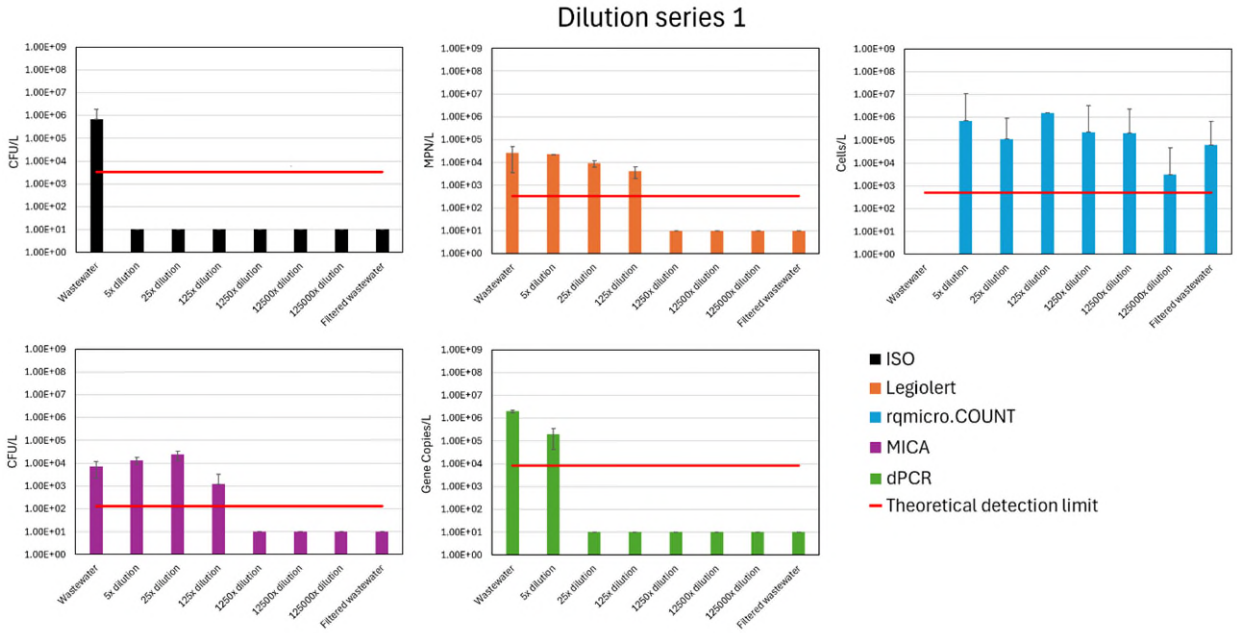


Table 2-8. L. pneumophila concentrations for each method for dilution series 1, using high L. pneumophila wastewater as a spike. The theoretical detection limit is the concentration of one cell detected across the replicates. Shown is the average of triplicate measurements of each dilution ± standard deviation. MICA pretreatment version 2 was used.

2.4.2.2 Dilution series 2

An overview of the results of the second dilution series is provided in Table 2-9. The ISO method was only able to detect *L. pneumophila* up to and including the 25× dilution with the spiking wastewater giving a result of 6.0×10^5 cfu/L. The Legiolert and MICA both detected *L. pneumophila* up to and including the 125× dilution, although of these two methods only the Legiolert data showed decreasing *L. pneumophila* numbers with increasing dilution and the MICA did not detect the 5× dilution. The dPCR detected up to and including the 12,500× dilution and showed results in line with a dilution series, although it missed the 1,250× dilution. The rqmicro.COUNT did not

detect *L. pneumophila*. If the assumption is made that the ISO method's determination of *L. pneumophila* counts is accurate and the concentration of the undiluted wastewater is corrected for the appropriate dilution step it is possible to translate the detection of the other methods to ISO cfu/L equivalents. This translation results in the following *L. pneumophila* concentrations successfully detected by the other methods (as cfu/L equivalents of the ISO method): 6.3×10^{3} cfu/L for Legiolert and the MICA, 6.3×10^{1} cfu/L for the dPCR. The number for the dPCR is far below the theoretical limit for cfu/l (one cell in all replicates combined), but gene copy determinations can be higher than cfu as determined by cultivation methods due to multiple gene copies in one cell and the possible detection of DNA from dead and/or viable but non-culturable cells.

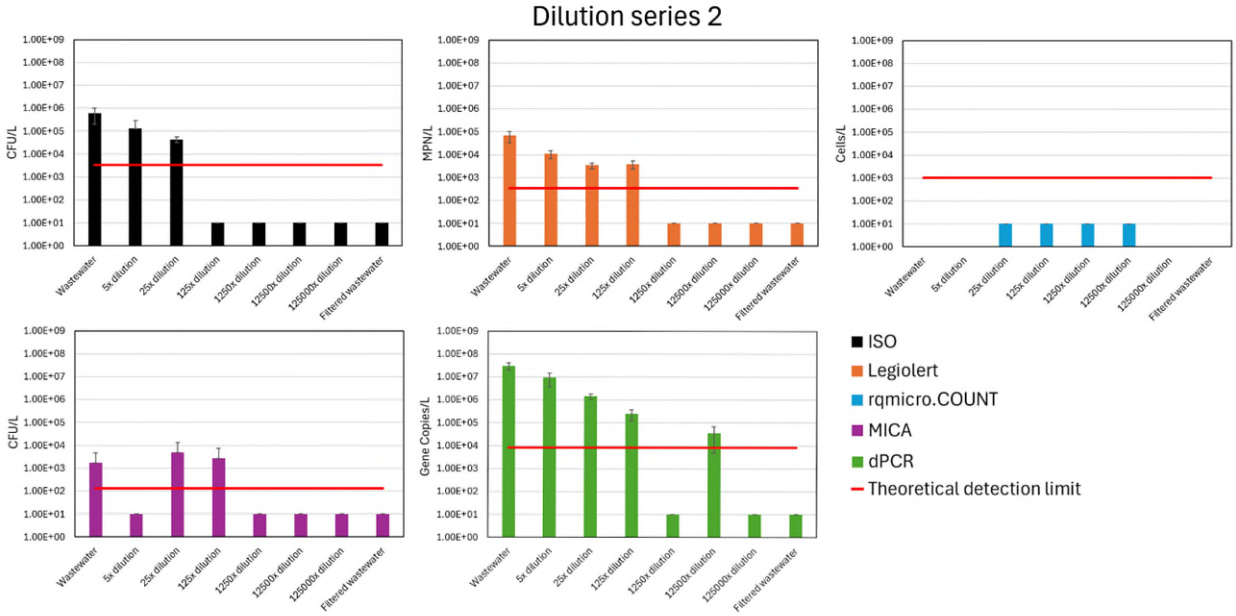


Table 2-9. L. pneumophila concentrations for each method for dilution series 2, using high L. pneumophila wastewater as a spike. The theoretical detection limit is the concentration of one cell detected across the replicates. Shown is the average of triplicate measurements of each dilution ± standard deviation. MICA pretreatment version 2 was used.

2.4.2.3 Dilution series 3

An overview of the results of the fourth dilution series is provided in Table 2-10. The ISO method was able to detect *L. pneumophila* up to and including the 12,500× dilution with the spiking wastewater giving a result of 2.2×10^7 cfu/L. Legiolert and MICA both also detected *L. pneumophila* up to and including the 12,500× dilution. Legiolert showed decreasing *Legionella* concentrations with increasing dilution across the entire dilution series. MICA missed the 5× and 25× dilution but did show decreasing *Legionella* numbers from the 125× dilution. The dPCR detected up to and including the 125,000× dilution and showed in general results in line with the dilution series, although the 25× dilution gave an increase compared to the 5× time dilution, which is inaccurate. The rqmicro.COUNT did not detect *L. pneumophila*. If the assumption is made that the ISO method's determination of *L. pneumophila* counts is accurate and the concentration of the undiluted wastewater is corrected for the appropriate dilution step it is possible to translate the detection of the other methods to ISO cfu/L equivalents. This translation results in the following *L. pneumophila* concentrations successfully detected by the other methods (as cfu/L equivalents of the ISO method): 1.2×10^3 cfu/L for Legiolert and MICA, 1.2×10^2 cfu/L for dPCR. The *L. pneumophila* number for the dPCR is again far below the theoretical limit for gene copies, which can be possible as explained at dilution series 2.

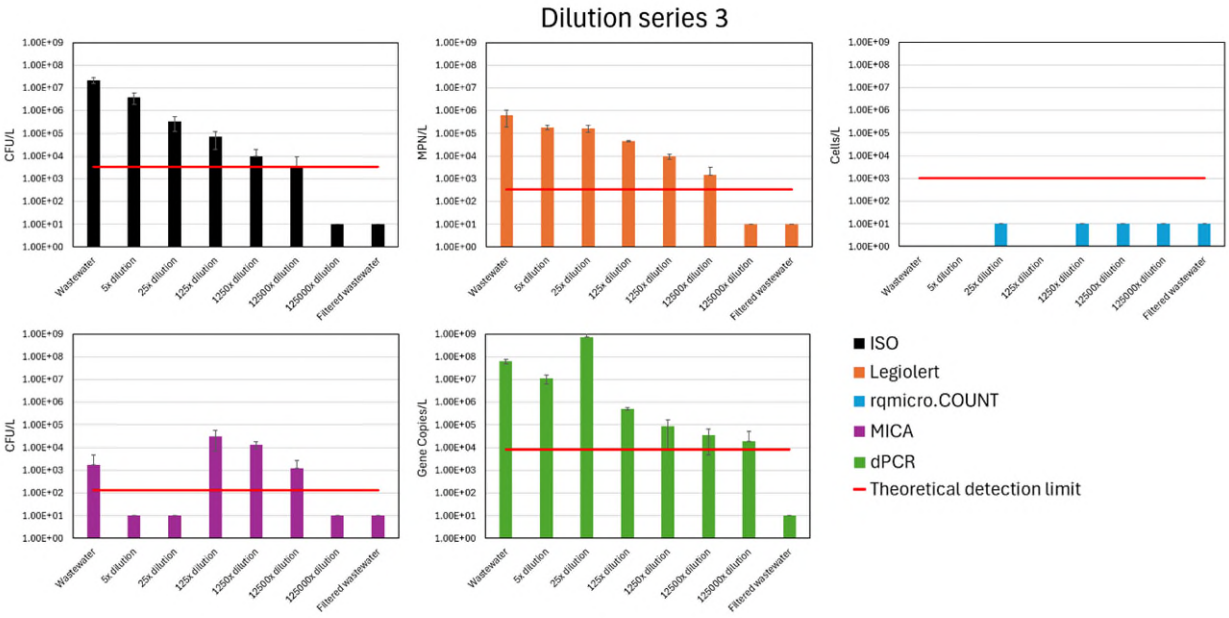


Table 2-10. L. pneumophila concentrations for each method for dilution series 3, using high L. pneumophila wastewater as a spike. The theoretical detection limit is the concentration of 1 cell detected across the replicates combined. Shown is the average of triplicate measurements of each dilution ± standard deviation. MICA pretreatment version 2 was used.

2.4.3 Experiment 2: Monitoring of full scale WWTPs

The monitoring of the full scale WWTPs was performed to compare the new methods to the ISO method in a more practical setting. The overview of the results of experiment 2 is presented by location for each detection method below. Theoretical detection limits varied due to adjustments in used dilutions over the experiment and the use of duplicate and triplicate measurements depending on the measurement week. The theoretical detection limit is the concentration of 1 cell detected across the combined replicates.

2.4.3.1 Location 1: communal WWTP Leuven

An overview of the results of location 1 is given in Table 2-11. The ISO method did not detect the presence of *L. pneumophila* during weeks 1-3, whereas the four other methods did show the presence of *L. pneumophila* and in general well above their theoretical detection limits. In weeks 4-6 the ISO method detected *L. pneumophila* in the samples. In week 4 this was still close to the detection limit (concentration 4× higher than the theoretical detection limit), but in week 5 and 6 the concentrations were higher, at around 1×10^6 cfu/L. The other methods also detected *L. pneumophila* in weeks 4-6, except for the MICA in week 6. Only the dPCR matched with the current ISO method in terms of reporting an increase in *L. pneumophila* concentrations in weeks 5 and 6. It seems likely that *L. pneumophila* was present during all six measurement weeks at location 1, but at concentrations too low for the ISO method to detect in week 1-3. The positive detection results from the Legiolert ranged from 8.2×10^3 to 2.4×10^6 MPN/L, from the rqmicro.COUNT from 1.7×10^6 to 4.9×10^8 cells/L, from the MICA from 2.7×10^3 to 1.9×10^4 cfu/L, and from the dPCR from 1.4×10^5 to 8.6×10^5 gc/L

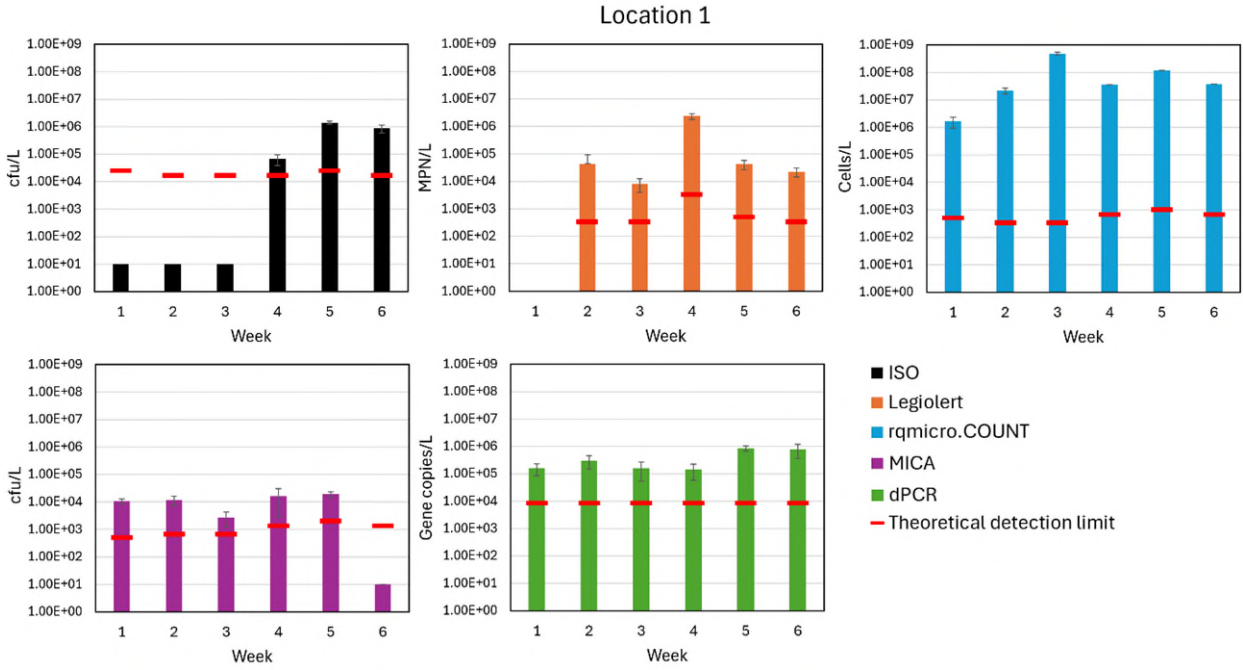


Table 2-11. L. pneumophila numbers for each method at location 1: WWTP Leuven. The theoretical detection limit is the concentration of one cell detected across the replicates. Shown is the average of duplicate or triplicate measurements at each week ± standard deviation. MICA pretreatment version 1 was used in weeks 1-3, version 2 in weeks 4-6.

2.4.3.2 Location 2: Industrial WWTP of Circle Wastewater Services

An overview of the results of location 2 is given in Table 2-12. There was no sample analyzed in week 1 with any of the methods. The ISO method did not detect the presence of *L. pneumophila* in any of the measurement weeks, whereas the other methods detected the presence of *L. pneumophila*: Legiolert in weeks 3-6, rqmicro in weeks 2-4 and 6, MICA in week 6, and dPCR in weeks 2, 3, and 6. For Legiolert, MICA, and dPCR, all the positive samples had *L. pneumophila* numbers close to the theoretical detection limits. The rqmicro.COUNT reported *L. pneumophila* multiple orders of magnitude above the theoretical detection limits (2.4×10^5 – 9.2×10^6 cells/L). Based on the combined results of all the methods, it seems likely that *L. pneumophila* was present during all the measurement weeks, but that the concentration was low and around the detection limit of most methods, but below the detection limit of the ISO method.

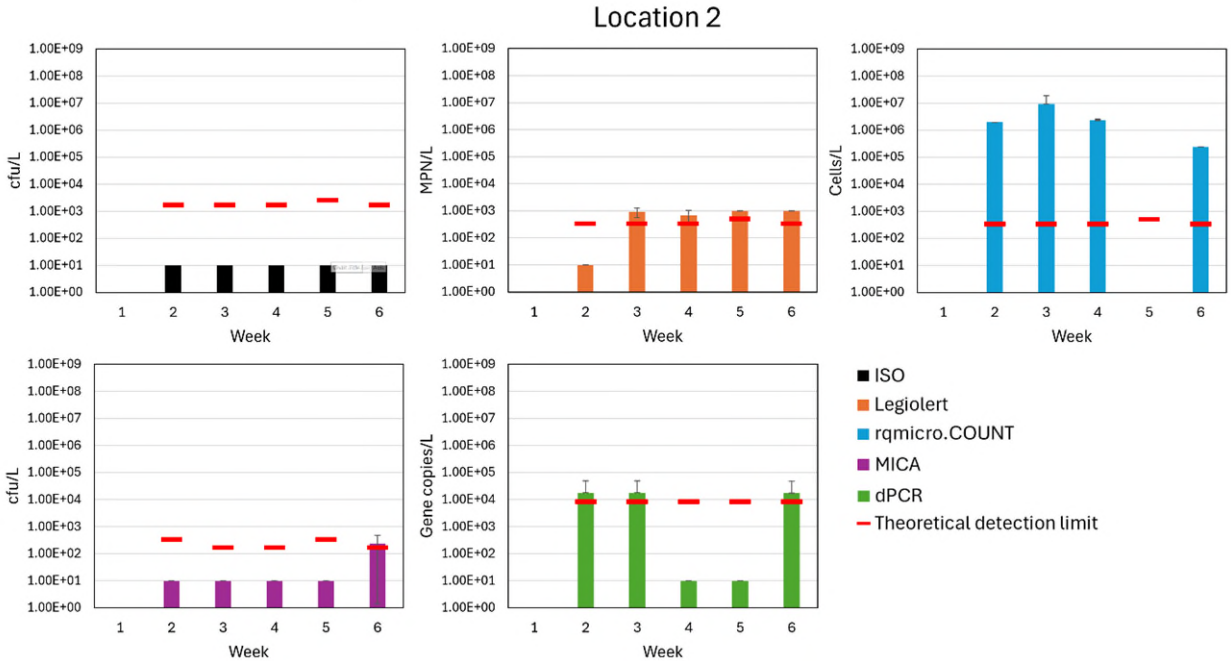


Table 2-12. L. pneumophila numbers for each method at location 2: WWTP Circle Wastewater Services. The theoretical detection limit is the concentration of one cell detected across the replicates. Shown is the average of duplicate or triplicate measurements at each week ± standard deviation. MICA pretreatment version 1 was used in weeks 1-3, version 2 in weeks 4-6.

2.4.3.3 Location 3: industrial WWTP of Boxtel

An overview of the results of location 3 is given in Table 2-13. The ISO method detected *L. pneumophila* in all measurement weeks. The concentrations also increased over weeks 1-4 and then stayed stable at a high level between 2×10^7 – 1×10^8 cfu/L. The other four methods also detected *L. pneumophila* during each measurement week. The MICA matched best with the results from the ISO method in terms of the pattern of the measured concentrations of *L. pneumophila* over the measurement weeks (increasing values from weeks 1-4, from 7.2×10^2 to 6.1×10^5 cfu/L and then stable at around 1.6×10^6 cfu/L). The dPCR did not show the clear increase in week 3 observed with ISO and MICA, but there is still a division in *L. pneumophila* numbers where in weeks 1-3 numbers (1.9×10^6 – 9.0×10^6 gc/L) are lower than in weeks 4-6 (2.9×10^7 – 2.0×10^8 gc/L). The pattern of concentration of *L. pneumophila* measured with the Legiolert and rqmicro.COUNT did not match the ISO method. With Legiolert ranging from 4.9×10^3 to 2.7×10^5 MPN/L and rqmicro.COUNT from 3.8×10^5 to 1.8×10^8 cells/L

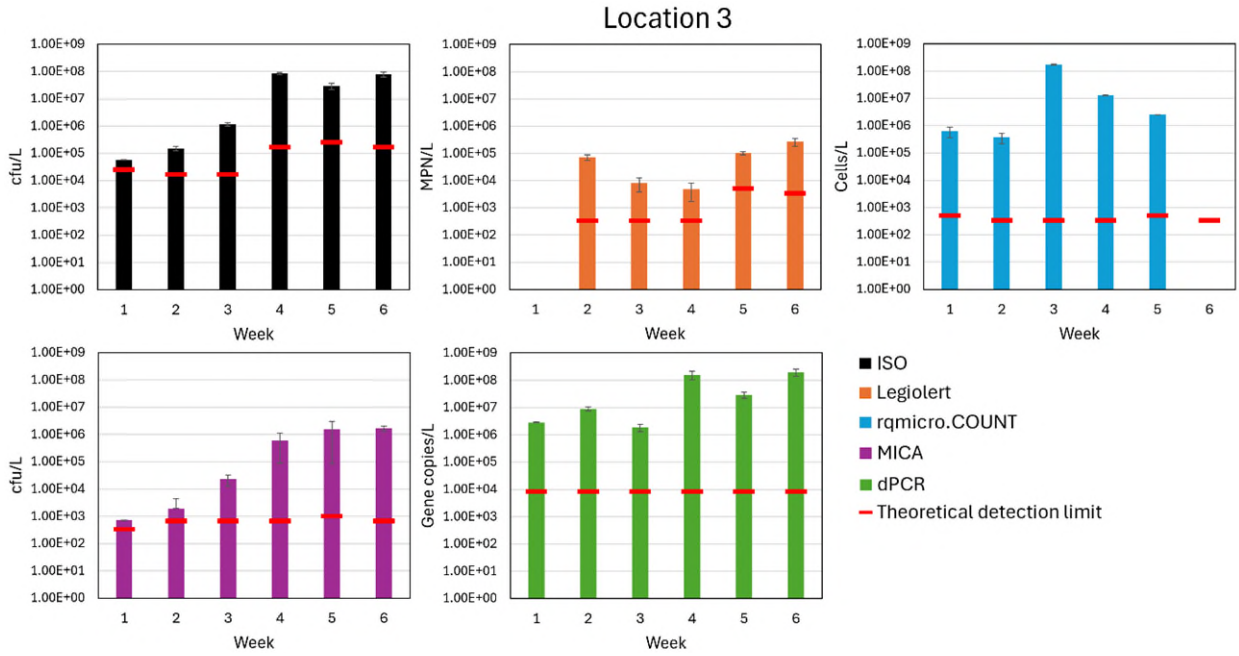


Table 2-13. L. pneumophila numbers for each method at location 3: WWTP Boxtel. The theoretical detection limit is the concentration of one cell detected across the replicates. Shown is the average of duplicate or triplicate measurements at each week ± standard deviation. MICA pretreatment version 1 was used in weeks 1-3, version 2 in weeks 4-6.

2.4.3.4 Combined results

The ISO method is currently the standard method for detecting *L. pneumophila* in wastewater and, therefore, it is worthwhile comparing the results of the alternative methods to those from the ISO method to see if they correlate. Correlations were only determined on datasets including the samples with positive results for both methods unless stated otherwise. However, this does sometimes lead to a low number of samples, as the detection limit of the ISO method is high.

2.4.3.4.1 Legiolert

There were 18 samples in experiments 1 and 2 that had positive values for both the ISO and Legiolert method. The *L. pneumophila* numbers observed with the ISO or Legiolert method were not significantly correlated with each other ($p>0.05$). Removing two potential outliers from the data did result in a significant correlation ($p<0.001$) with R^2 of 0.58 (Table 2-14). The two potential outliers were from week 4 samples of location 1 and 3 (experiment 2).

Table 2-14.Correlations between ISO method and Legiolert. Positive samples are samples where both methods detected L. pneumophila

Dataset	R^2 value (Pearson)	p value	# of samples
Positive samples	N.A.	> 0.05	18
Potential outliers removed	0.58	< 0.001	16

The correlation between the ISO method and the Legiolert are presented in three graphs in Table 2-15, one graph with all samples, one graph with only samples with positive *L. pneumophila* detection with both methods, and one graph with positive samples where potential outliers were removed. Comparing the correlation line (with possible outliers removed) with the dotted one-on-one line, it can be seen that the results from the ISO method are higher than with the Legiolert and that this difference seems to increase as the concentration of *L. pneumophila* rises. This means that the ISO-method detected higher *L. pneumophila* numbers (expressed in cfu/l) than the Legiolert method (expressed in mpn/l). However, the difference changes based on the amount of *L. pneumophila* in the sample. The difference in *L. pneumophila* numbers obtained with Legiolert and ISO was statistically significant ($p < 0.001$).

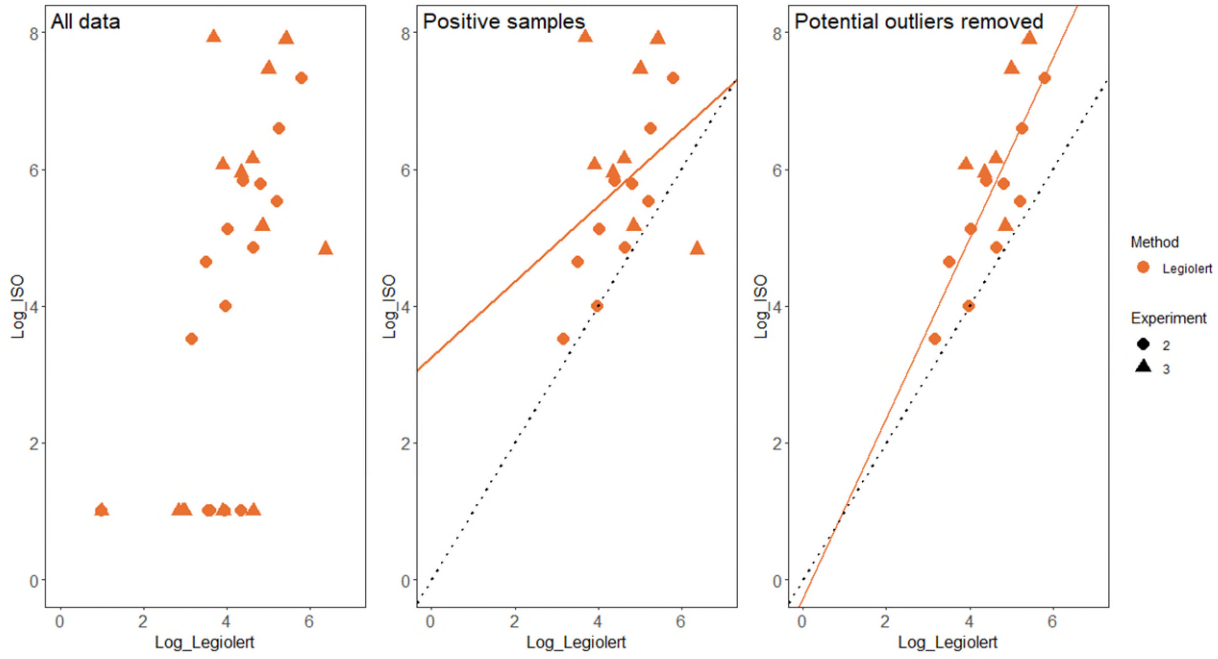


Table 2-15. The L. pneumophila numbers obtained with Legiolert plotted against the L. pneumophila numbers obtained with the ISO method. Left to right: All data points between experiments 1 and 2 (data with no positive detection of L. pneumophila was set to a log value of 1); only samples with a positive L. pneumophila detection with both methods; samples with positive detection with both methods with potential outliers removed based on Cook's distance. The closed line is the plotted correlation line according to Pearson correlation formula, it was not attempted to ascertain a correlation based on the 'all data' graph. The dotted line is the one-to-one line.

2.4.3.4.2 rqmicro.COUNT

There were eight samples in experiments 1 and 2 that had positive values for both the ISO method and the rqmicro.COUNT. All these were from experiment 2 and did not result in a significant correlation between the two methods (Table 2-16). Based on the cut-off criteria used for Cook's distance no potential outliers were identified.

Table 2-16. Correlations between ISO method and rqmicro.COUNT. Positive samples are samples where both methods detected L. pneumophila

Dataset	R^2 value (Pearson)	p value	# of samples
Positive samples	N.A.	> 0.05	8

Two graphical variations between the ISO method and the rqmicro.COUNT are presented in Table 2-17, one graph with all samples and one graph with samples with positive *L. pneumophila* detection for both methods.

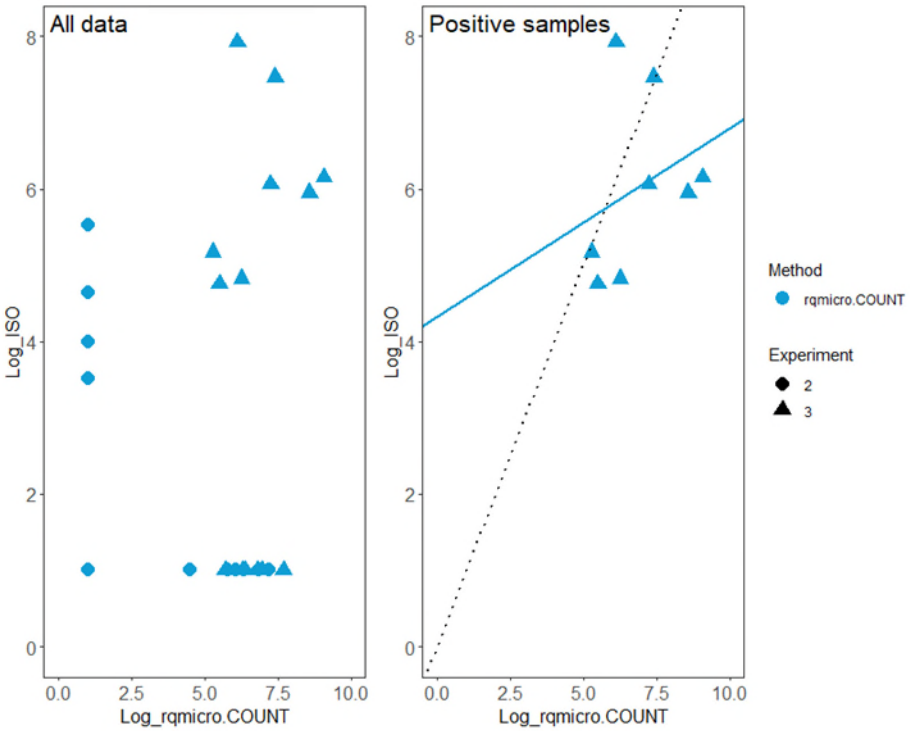


Table 2-17. The L. pneumophila numbers obtained with rqmicro.COUNT plotted against the L. pneumophila numbers obtained with the ISO method. Left to right: All data points between experiments 1 and 2 (data with no positive detection of L. pneumophila was set to a log value of 1); only samples with a positive L. pneumophila detection with both methods. The closed line is the plotted correlation line according to Pearson correlation formula; it was not attempted to ascertain a correlation based on' the all' data graph. The dotted line is the one-to-one line.

2.4.3.4.3 MICA

There were 15 samples in experiments 1 and 2 that had positive values for both the ISO and MICA methods. The *L. pneumophila* numbers obtained with ISO and MICA were significantly correlated ($p<0.05$), with an R^2 of 0.41. Removing one potential outlier increased the R^2 value to 0.64 (Table 2-18). The removed point was the 5× dilution from the final dilution series (dilution series 3 of spiking with high *L. pneumophila* wastewater; experiment 1).

Table 2-18. Correlations between ISO method and MICA. Positive samples are samples where both methods detected L. pneumophila

Dataset	R^2 value (Pearson)	p value	# of samples
Positive samples	0.41	< 0.01	15
Potential outlier removed	0.64	< 0.001	14

Three graphic variations between the ISO method and the MICA are presented in Table 2-19, one graph with all samples, one graph with samples with positive *L. pneumophila* detection for both methods, and one graph with positive samples as well but with potential outlier removed. Comparing the correlation line (with possible outlier removed) with the dotted one-on-one line, it can be seen that the results from the ISO method are consistently higher than with the MICA and this difference seems consistent at increasing concentrations of *L. pneumophila.* This means that the ISO-method detected higher *L. pneumophila* numbers (expressed in cfu/l) than the MICA method (also expressed in cfu/l) and based on the correlation formula the ISO method gives results 1.3 log higher *L. pneumophila* numbers than the MICA method. The difference between the MICA and ISO method was statistically significant ($p < 0.01$).

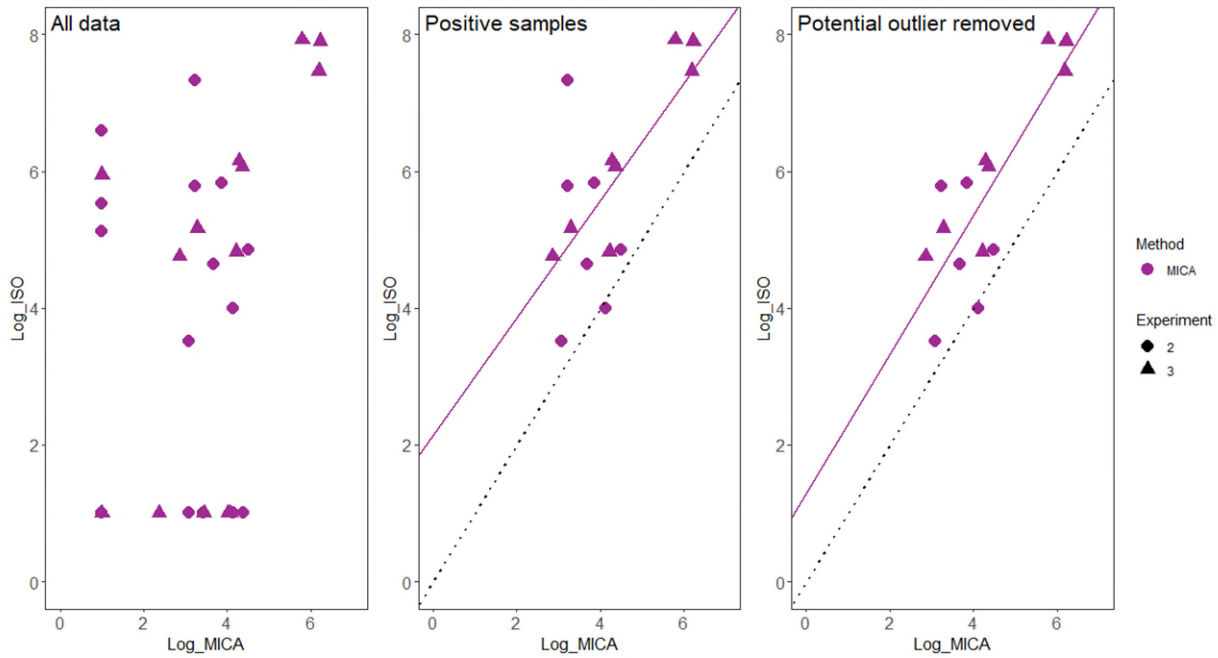


Table 2-19. The L. pneumophila numbers obtained with MICA plotted against the L. pneumophila numbers obtained with the ISO method. Left to right: All data points between experiments 1 and 2 (data with no positive detection of L. pneumophila was set to a log value of 1); only samples with a positive L. pneumophila detection with both methods; samples with positive detection with both methods with the potential outlier removed based on Cook's distance. The closed line is the plotted correlation line according to Pearson correlation formula, it was not attempted to ascertain a correlation based on 'the all' data graph. The dotted line is the one-to-one line.

2.4.3.4.4 dPCR

There were 19 samples in experiments 1 and 2 that had positive values for both the ISO method and the dPCR. The *L. pneumophila* numbers obtained with both methods showed a significant correlation ($p<0.05$) with an R^2 of 0.51. Removing one potential outlier increased the R^2 value to 0.70 (Table 2-20). The removed outlier was the 25× dilution from the final dilution series (dilution series 3 of spiking with high *L. pneumophila* wastewater; experiment 1).

Table 2-20. Correlations between ISO method and MICA. Positive samples are samples where both methods detected L. pneumophila

Dataset	R^2 value (Pearson)	p value	# of samples
Positive samples	0.51	< 0.001	19
Potential outlier removed	0.70	< 0.001	18

Three graphical variations between the ISO method and the dPCR are presented in Table 2-21, one graph with all samples, one graph with samples with positive *L. pneumophila* detection for both methods, and one graph with positive samples as well but with a potential outlier removed. Comparing the correlation line (with possible outlier removed) with the dotted one-on-one line, it can be seen that the results from the ISO method are consistently lower than with the dPCR and this difference seems consistent at increasing concentrations of *L. pneumophila*. This means that the dPCR method detected higher *L. pneumophila* numbers (expressed in gc/l) than the ISO method (expressed in cfu/l). This was expected as previous work has also shown that gene copy numbers for *L. pneumophila* are higher than cfu numbers, probably because *L. pneumophila* contains multiple gene or genome copies (Wullings & Heijnen, 2017). Besides that, dPCR also detects dead or viable but non-culturable cells (VBNC). The difference between the dPCR and ISO method, with the dPCR yielding results 0.85 logs higher based on the correlation formula was not statistically significant ($p > 0.05$).

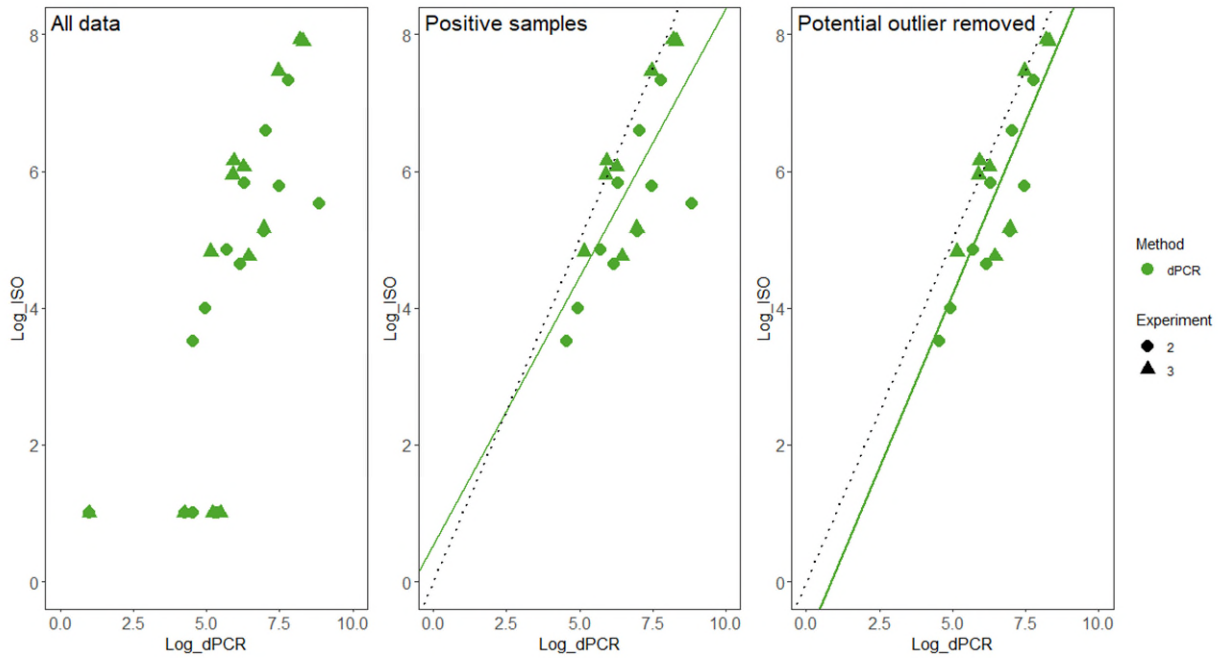


Table 2-21. The L. pneumophila numbers obtained with dPCR plotted against the L. pneumophila numbers obtained with the ISO method. Left to right: All data points between experiments 1 and 2 (data with no positive detection of L. pneumophila was set to a log value of 1); only samples with a positive L. pneumophila detection with both methods; samples with positive detection with both methods with the potential outlier removed based on Cook's distance. The closed line is the plotted correlation line according to Pearson correlation formula, it was not attempted to ascertain a correlation based on 'the all' data graph. The dotted line is the one-to-one line.

2.4.3.4.5 Correlation between methods

A correlation matrix between all methods was generated with a subset of the data from experiments 1 and 2, whereby only samples where all five methods had a result were included (errors and missing samples removed). This resulted in 30 samples within the subset. For a non-detect, but otherwise valid measurement, the theoretical detection limit was used as an input. Another subset of the data was made and that included only data where all methods had a positive *L. pneumophila* detection. However, this subset had a mere of seven samples available, which was too small to use for correlation analysis. Table 2-22 gives a visual representation of the correlation matrix. Significant correlations were found between all combinations of methods except for combinations of the rqmicro.COUNT with either Legiolert or MICA. The significant correlations ranged from very weak (R^2 value of 0.31) between the data from the ISO method and rqmicro.COUNT, to a relatively strong correlation (R^2 value of 0.75) between the data from the ISO method and dPCR. The R^2 values here differ from those presented before due to the inclusion of results with a non-detect at the detection limit whereas they were not included in the earlier analyses.

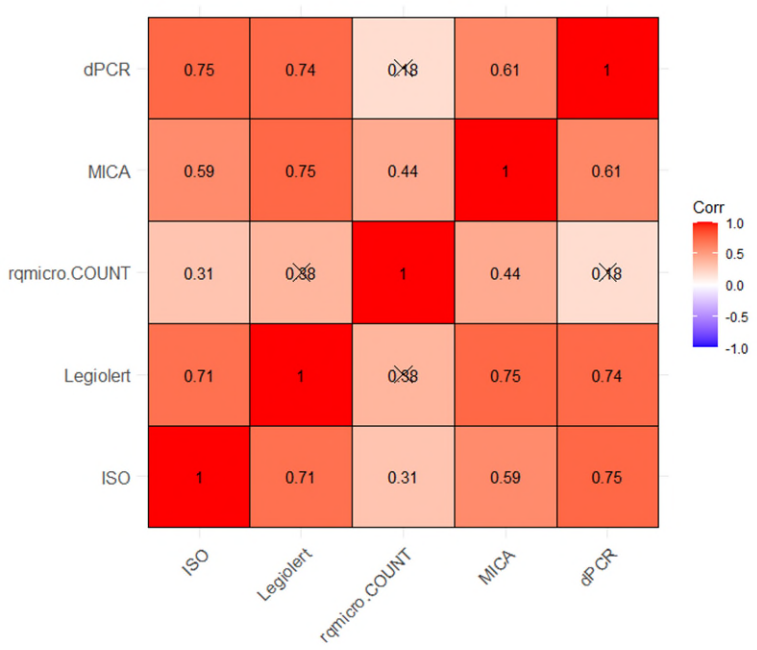


Table 2-22. Correlation matrix for the correlation between L. pneumophila numbers obtained with the five methods (Spearman correlation). Data included were all samples from experiments 1 and 2 where all methods gave a result (non-detects set at the theoretical detection limit). Displayed numbers are the R^2 values. Crosses indicate non-significant correlations ($p > 0.05$)

2.5 Discussion

2.5.1 Dilution series

When looking at the results of the dilution series with the high *L. pneumophila* wastewater as a spike, it should first be noted that the ISO method was able to detect lower concentrations of *L. pneumophila* during dilution series 2 and 3 than the observed detection limit (roughly between 2×10^4 - 2×10^5 cfu/L) in practice (Oesterholt & Hollebekkers, 2022). The most important factor responsible for the high detection limit of the ISO method in wastewater is other microorganisms that can grow on the plates and thereby leaving *L. pneumophila* either not visible or uncountable on the plates. In the dilution series experiments, wastewater was filtered, thereby removing all microorganisms and, thus, also the ones that grow as disturbing flora on the agar plates of the ISO method.

In the three dilution series that ended with usable results, the Legiolert, MICA, and dPCR detected *L. pneumophila* to either the same concentration or lower concentration than the ISO method. So, although it is not a perfect representation of the full-scale WWTP situation, it is likely that the alternative methods indeed have a lower detection limit for *L. pneumophila* in wastewater than the ISO method. The observed detection limit of the Legiolert and MICA over the different dilution experiments was also relatively consistent. When calculating back from the ISO method detection, the detection limit for both methods varied between 1.2×10^3 to 6.3×10^3 cfu/L ISO method equivalent. The *L. pneumophila* numbers in some of these measurements were very close to the theoretical detection limit and had negative replicates, meaning that *L. pneumophila* could have potentially been missed if only a single measurement is done. Nevertheless, even the top of the range would represent a very clear improvement of the detection limit compared to the current ISO method. Although again, the methods could have benefitted from the wastewater filtration during the experimental setup as discussed above for the ISO method. The detection limit for the dPCR was more variable ranging from 6.3×10^1 to 1.3×10^5 cfu/L ISO method equivalent. More variation could perhaps be expected as the ratio between viable cells and

gene copies, and the number of dead cells, could theoretically be different between locations. The upper part of the dPCR detection range would represent a small improvement in detection limit compared to the ISO method, whereas the lower part of the range would represent an exceedingly low detection limit.

The Legiolert and dPCR results consistently showed a dilution series pattern, as the detected *L. pneumophila* numbers went down as the dilution series progressed. This was not observed for most MICA results, although in dilution series 3 a dilution pattern with the MICA was observed as well for the final four dilutions. It could be that the MICA struggled to reliably quantify the higher concentrations of *L. pneumophila* in the first dilutions, or that there was another source of interference in the samples with a less diluted spike. However, it was not within the scope of the project to determine the exact cause of this possible interference.

Finally, the rqmicro.COUNT did not give reliable results with the dilution series samples. In dilution series 2 and 3 it did not detect *L. pneumophila,* perhaps partially due to errors that happened during the analyses of samples with higher concentrations of *L. pneumophila*. One of the issues was that the magnetic beads got stuck in the channel in several measurements, resulting in failed measurements. In dilution series 1 rqmicro.COUNT did detect *L. pneumophila* in all the dilutions, but due to detection of *L. pneumophila* in the *L. pneumophila* free negative control, it is difficult to conclude whether the results from the other dilutions were accurate.

2.5.2 WWTP monitoring

In general, the alternative methods were able to detect *L. pneumophila* at lower concentrations than the ISO method. At location 1, the alternative methods already detected around 10^4 to 10^5 *L. pneumophila* cells or gene copies per liter of wastewater before the ISO method detected *L. pneumophila*. At location 2, various methods detected *L. pneumophila* in various weeks, whereas the ISO method could not detect *L. pneumophila* in these wastewater samples.

The ISO method started to detect *L. pneumophila* at a concentration of around 10^5 cfu/L, which is in the lower range of what is conventionally found (Oesterholt & Hollebekkers, 2022). Despite this relatively good detection limit for the ISO method in this project, the alternative methods were more sensitive and detected lower concentrations of *L. pneumophila* in drinking water.

Although the rqmicro.COUNT also detected *L. pneumophila* in the wastewater, some doubts about the accuracy of the concentrations came up during interpretation. The results for location 2 and 3 are similar to each other in terms of measured concentrations except for week 3 when a *L. pneumophila* concentration of 10^8 cells/l was found. The ISO, Legiolert, MICA and dPCR method showed clearly higher concentrations of *L. pneumophila* in wastewater from location 3 compared to wastewater from location 2, with wastewater from location 3 having the highest and wastewater from location 2 having the lowest *L. pneumophila* concentrations of the three locations measured. In contrast, the rqmicro.COUNT results did not observe this difference in *L. pneumophila* in the wastewater from these two locations. As the other methods encompass both culture and DNA techniques, it is likely that the *L. pneumophila* concentrations in wastewater from location 3 were indeed higher than in wastewater from location 2.

The results obtained roughly match with the historic *L. pneumophila* levels observed at the sampled WWTPs and thus line up with the expectations. In particular, the site at location 3 (industrial WWTP of Boxtel, meat-processing plant) had also high levels of *L. pneumophila* in the past. It is known that WWTPs that process wastewater from the food industry have a higher chance of high levels of *L. pneumophila,* especially when it's related to processing animal products (Kanters, 2022).

2.5.3 Correlations between the methods

Despite the small size of the combined dataset, a significant correlation between the various combinations of the ISO method, Legiolert, MICA, and dPCR was found. During the previous analyses and discussion, the assumption was made that the ISO method reports accurate *L. pneumophila* counts, although with a relatively high detection limit compared to the other methods. As the *L. pneumophila* numbers obtained with these four methods correlate significantly with each other, it seems that this assumption is valid as otherwise it would be expected that the other methods would not correlate with the ISO method and (potentially) correlate with each other instead. The only exception is the rqmicro.COUNT method, which deviates from the other methods as it either does not significantly correlate with them or significantly with only a weak relation. rqmicro.COUNT has a different underlying method (flow cytometry) compared to the others (culture or PCR) however as the results are different from both cultivation and PCR methods and do not match the 1:10 or 1:5 dilution steps, the rqmicro.COUNT results appear less reliable with the currently used protocol.

During this study, the Legiolert showed an increasing deviation from the ISO method results at higher concentrations of *L. pneumophila*. At higher concentrations the Legiolert returns increasingly lower results than the ISO method.

The results obtained with the MICA also showed consistently lower *L. pneumophila* concentrations than the ISO method. It was also noted that the MICA had some issues with the higher concentrations of *L. pneumophila* during the dilution series, where it was unable to differentiate well between the initial and later dilutions in the dilution series. In contrast, it did show increases during the weekly monitoring of wastewater from full-scale plants that were in line with the ISO method and dPCR. It, thus, appears that MICA handles higher concentrations of *L. pneumophila* in practice better than it did during the dilution series.

The rqmicro.COUNT appears to have less reliable results compared to the other methods. Due to detection of *L. pneumophila* in the negative control and poor differentiation between the WWTPs with low and high *L. pneumophila* concentrations, it might be that the method is hindered by some off target detection. No experiments to substantiate this hypothesis were performed within the scope of this study. The rqmicro.COUNT also had the highest numbers of error measurements. This mainly happened due to the clogging of the channel in the plates during measurements. Some actions were taken to limit this clogging (e.g. using higher diluted samples as input), which did improve the success rate, but did not fully solve the problem. A further suggestion by rqmicro of using an acid treatment of the samples could not be implemented in time for this study but it would be worthwhile investigating it as an option for further optimization of the protocol.

The dPCR performed quite well in both the dilution series and the routine monitoring of the WWTPs with a notable outlier found in dilution series 3. Naturally the detection of gene copies does not result in a one-to-one translation to cells as cells can have multiple gene copies and the dPCR also detects dead cells. If the ratio between the dPCR and ISO method is consistent a conversion factor might be established. Unfortunately, in this study the ratio between the dPCR result and the ISO-method was neither consistent between the three monitoring locations, nor between monitoring weeks. This again emphasizes the difficulty in reliably translating dPCR result to a colony forming unit count. A larger dataset is likely required to have a chance of finding a reliable conversion factor, although it might not be possible to find a reliable constant conversion factor.

2.5.4 *L. pneumophila* serogroups

There are 15 different serogroups of *L. pneumophila* of which serogroup 1 is most often identified from patients, which is mainly caused by the fact that the most applied *Legionella* test for patients, the urine antigen test, only detects serogroup 1 (NASEM, 2019). Although serogroup 1 is most often isolated it is not the only serogroup that can cause infections in humans (NASEM, 2019). *L. pneumophila* detection in water is generally done for all serogroups and not specifically for serogroup 1. The ISO method can, in principle, detect all *L. pneumophila* serogroups. Similarly, the alternative methods tested in this project also can, in principle, detect the presence of

all *L. pneumophila* serogroups (ISO 11731:2017; Checa et al., 2021; Passot et al., 2023; Streich et al., 2024; Wilson et al., 2003), although this was not verified for detection in wastewater within this project. Additionally, the rqmicro.COUNT and dPCR methods can, next to detecting all *L. pneumophila* serogroups, also specifically detect *L. pneumophila* serogroup 1 (Keserue et al., 2021; Mérault et al., 2011). This option was not used in this project, as it was beyond the scope of our study, but could be interesting should clients desire to make a separation between serogroup 1 and serogroup 2-15. Although the ISO method, Legiolert and MICA do not distinguish between serogroups, serotyping can still be performed on positive detections to determine the serogroups present in a sample by utilizing the *L. pneumophila* cells cultured during the method.

2.5.5 Comparison with earlier studies

For the four alternative methods, the protocols for the detection of *L. pneumophila* in wastewater were new adaptations from the protocol that was initially developed for detecting *L. pneumophila* in drinking water. These adapted protocols had not been fully optimized at the start of this study and, therefore, it is likely that further improvements and optimization of the adapted protocols can still be made. Some of the methods tested in our study have also been tested on other non-potable water matrix, albeit mostly not wastewater.

The Legiolert method was compared to the CDC method for non-potable water, although these were not wastewater samples. The CDC method is similar to the ISO-11731 method although only an acid treatment was employed (Rech et al., 2018). A similar number of positive samples was identified with both methods. The relationship between the *L. pneumophila* concentrations obtained with these two methods was different from this study. In that study the MPN results of the Legiolert tended to give higher *L. pneumophila* numbers than the CFU count from the CDC method, whereas in our study the ISO-11731 tended to give higher CFU counts than the MPN numbers from Legiolert.

The MICA detection of *L. pneumophila* has also been compared to the ISO-11731 method for cooling tower waters using spiked samples (Passot et al., 2023). Both methods obtained similar results, although those of the MICA seemed more consistent. Unlike the results from our study, the ISO-11731 method did not seem to give consistently higher CFU results than the MICA method.

The use of PCR methods has been most extensively tested for *L. pneumophila* detection as an alternative for the ISO-11731. However, the use of wastewater as a matrix has been less well studied. In an older study more *L. pneumophila* positive WWTP's were found with qPCR than with the ISO method. Although this was done with normal qPCR instead of dPCR. However, the principle of qPCR and dPCR is comparable and thus it is interesting to discuss the results. Depending on the location, a higher gene copy count was found or a higher CFU count (Lund et al., 2014). In our study the gene copy counts were generally higher than the CFU counts.

A study comparing Legiolert, qPCR, and the ISO-11731 method has also been done, however the majority of samples were potable water samples and no clear results on the non-potable water samples are presented (Monteiro et al., 2021).

Although detection of *L. pneumophila* in wastewater samples in this study went well for most of the methods, it can be noted that the *L. pneumophila* numbers obtained with Legiolert or MICA were often lower than those from the ISO 11371 method, which contrasts with earlier work with cleaner water matrices. It is possible some signal is lost, but the improved detection limit over the ISO method still allows for detection at lower *L. pneumophila* concentrations.

2.6 Conclusions and recommendations

2.6.1 Conclusions

Legiolert, MICA, and dPCR had a lower detection limit for *L. pneumophila* in wastewater than the ISO method in the dilution series. Legiolert and dPCR also showed *L. pneumophila* results in line with the expected pattern of a dilution series.

The alternative methods were also able to detect *L. pneumophila* at lower concentrations in wastewater than the ISO method during the WWTP monitoring. As a result, these alternative methods will give an earlier warning than the ISO-method when *L. pneumophila* starts to become present in wastewater treatment plants.

When potential outliers were removed, *L. pneumophila* numbers determined with Legiolert, MICA, and dPCR significantly correlated with the ISO method, but *L. pneumophila* numbers quantified by the ISO method were higher than those quantified by Legiolert or MICA.

The ISO method, Legiolert, MICA, and dPCR all significantly correlated with each other, whereas the rqmicro.COUNT did not or only weakly correlate with the other methods.

2.6.2 Recommendations

The dataset in this phase was relatively small. For a proper validation of the new methods as a replacement for detecting *L. pneumophila* in WWTP's through NEN-EN-ISO 16140-6:2019 a larger monitoring program should be set up to monitor various WWTP's with different types of wastewater sources over a longer period.

Based on the results from this study, the dPCR, MICA, and Legiolert have best potential for further work towards implementation in practice.

The *L. pneumophila* numbers obtained with MICA and Legiolert were lower than those obtained with the ISO-method, which was not observed in previous work with cleaner water matrices. A hypothesis for an underestimation by the Legiolert and MICA may be that some signal is lost when using the wastewater matrix. A possible hypothesis for why the ISO method may overestimate *L. pneumophila* numbers is that the result according to ISO requires applying a, sometimes, large dilution factor. This introduces some uncertainty into the results when counts from the plates are low, which may explain part of the difference. However, neither was verified in this study. A larger test set of WWTP samples could give further insight into this and if these differences remain, further research is required to determine whether Legiolert/MICA underestimate *L. pneumophila* numbers or whether the ISO method overestimate *L. pneumophila* numbers.

The rqmicro.COUNT protocol needs further optimization before being applied to wastewater. Two initial steps could be to add an acid treatment to the pre-treatment to potentially improve performance and verify that there is no off-target detection as that could not be done in our study.

Although the dPCR did do quite well in quantifying *L. pneumophila* in wastewater samples, it will be important to collect more data to investigate whether a constant and reliable ratio exists between gene copy numbers per liter and cfu per liter, that can be used to translate the dPCR results to cfu/l values.

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3 Phase 2: Overview of *Legionella* control measures in WWTPs (literature study)

3.1 Summary

Legionella are bacteria that thrive in water. They can grow in water installations such as drinking water networks, cooling towers or Wastewater Treatment Plants (WWTPs). Because it can be dangerous to human health when breathed in through aerosols, these bacteria are one of the main concerns of public health organizations. WWTPs are sources at risk because they gather favorable conditions for *Legionella* growth and processes that can generate aerosols.

As part of phase 2 of the TKI *Legionella* project aiming to determine the suitability and reliability of techniques to control *Legionella*, a literature study was conducted to collect all the information needed to manage *Legionella* in WWTPs. Interviews with all the partners involved in the project were conducted in this context to get their knowledge and their expectations.

First, regulations about *Legionella* in the European Union and most precisely in the Netherlands were reported. A regulation about *Legionella* in WWTPs should be applied in the Netherlands from 2027. Then, scientific knowledge about growth conditions of *Legionella*, its hosts, and the illnesses it causes if inhaled as well as the functioning of wastewater treatment plants can be found.

Furthermore, different types of measures to control *Legionella* are reported: preventive ones and curative ones. They are classified into 2 categories: measures to limit growth of *Legionella* in water and measures to limit spread of aerosols with *Legionella* by air. These include, among others, the modification of operating parameters like temperature to no longer be in the range of favourable conditions for *Legionella* growth and the disinfection of the influent to avoid the entrance of the bacteria in the process. To limit aerosols formation, the type of aerator is a key parameter. And to avoid their spread, it is possible to cover the tank. However, most of the existing measures target the reduction of aerosol dispersion instead of growth control. It is indeed difficult to get rid of *Legionella* in WWTPs without affecting the efficiency of the process itself. Further research, especially experimental one, is needed to determine the effectiveness of most of these methods.

Since *Legionella* are very resistant and difficult to eliminate, it is better to prevent it by regular cleaning and maintenance of the wastewater treatment plant. Making a risk assessment is also essential to better anticipate problems and have an idea of measures to take in case of *Legionella* occurrence. There is still a lot of knowledge gaps concerning *Legionella* in wastewater treatment plants remaining in the report. Recommendations are written at the end of the report and the report can be a support to design experiments.

3.2 Introduction

Legionellosis is an infectious disease caused by inhaling the *Legionella* bacteria. It can lead to more or less severe symptoms: from a mild febrile illness “Pontiac fever” to a potentially fatal form of pneumonia called “Legionnaires' disease” [1]. Some factors like high age, underlying illness, immunosuppression, or smoking increase the risk of developing a severe form of the disease. Men are more often affected then women (factor 2.5).

Cases of Legionnaires' disease have doubled in the last decade and have reached a rate of 1.9 cases per 100 000 people (8 372 cases) in 2020 in the European Union. The notification rate in The Netherlands outperforms the European average with 461 cases in 2020 (2.6 per 100 000 population) [2].

Legionella pneumophila grows in water between 20 and 50°C and in stagnant water. It is common to find the bacterium in tap water, swimming pools, wet cooling towers or WWTPs. The risk of *Legionella* comes from aerosolization of those waters and subsequent inhalation of these aerosols by humans [3].

In the Netherlands, approximately 80 WWTPs over 776 in total have an increased risk of growth and spread of *Legionella* [4].

3.2.1 Partners’ perspective

As part of this literature study, most of the partners have been interviewed (see appendices) to get their expectations and their knowledge on the topic. These companies specialize in different fields, including management of WWTP plants (Circle Wastewater Services, GETEC, Tyson Foods, Waterstromen), consultancy companies (Normec), some of them having their own WWTP. There are also detection technology suppliers (Idexx, Rqmicro, QIAGEN) and knowledge institutes (VITO, Hydroscope, KWR).

From a general point of view, they are aware of the risks of *Legionella* in WWTPs and know the key parameters that influence *Legionella* presence and spread, namely the temperature, the aeration and the covering. However, concrete actions to take remain confusing for them. Some of the WWTP partners do not have preventive *Legionella* management plans and express reserves about the effectiveness of the measures suggested by institutions. Furthermore, it is difficult to modify parameters without affecting the processes or killing the biomass needed for the biological steps.

Therefore, their expectations of this project are to get more understanding about *Legionella* behaviour in WWTPs and above all, real effective measures to control *Legionella*. Furthermore, they would also like more transparency between companies and universal procedures, as well as enhanced cooperation.

3.2.2 Goals

Since *Legionella* bacteria are very persistent microorganisms, getting rid of them is difficult. Therefore, controlling them is mandatory. The aim of this literature study is to have a global view on the different means of control of *Legionella* in WWTPs. To do so, there are two viable methods: limiting the growth of the bacteria or limiting the spread of it through aerosols.
Those methods must be combined with control methods to prevent the growth of *Legionella*.

3.2.3 List of abbreviations

- AD: Anaerobic Digestor
- Aerobe: Aerobic Bacteria
- AMvB: Announced new administrative order
- Anammox: Anaerobic Ammonium Oxidation

- AS: Activated Sludge
- ASP: Activated sludge process
- BDD: Boron-doped diamond
- BNR: Biological Nitrogen Removal
- BOD: Biochemical oxygen demand
- CBOD: Carbonaceous Biochemical Oxygen Demand
- CFU: Colony-Forming Units, which estimates the number of viable cells in the sample
- COD: Chemical oxygen demand
- CW: Constructed wetland
- DAF: Dissolved Air Flotation
- DFA: Direct fluorescent antibody
- DHW: Domestic Hot Water
- DO: Dissolved Oxygen
- LCFA: Long Chain Fatty Acid
- MBBR: Moving bed biofilm reactor
- MBR: Membrane reactor
- MHS: Municipal Health Service
- MHS: The Municipal Health Service
- MPN: Most probable number
- OPS: Operational Priority Substances
- PCR: Polymerase Chain Reaction
- PHB: Poly-3-Hydroxybutyrate
- RASCI: Responsible, Accountable, Support, Consulted, Informed
- RI&E: Risk inventory and evaluation
- SBR: Sequencing batch reactor
- SS: Suspended solids
- SZW: The Ministry of Social Affairs and Employment
- UASB: Upflow Anaerobic Sludge Blanket
- UV: Ultra violet
- VBNC : Viable But Non-Culturable
- VFA: Volatile Fatty Acid
- Wabo: Environmental Law General Provisions Act
- WSP: Waste Stabilize Pond
- WWTP: Wastewater Treatment Plant

3.3 Regulation

3.3.1 The European Union

There is not any specific regulation in the European Union or in separate countries regarding *Legionella* in wastewater treatment systems. However, there are still guidelines about *Legionella* in domestic water systems. For information purposes, the following paragraph focuses on guidelines published by The European Working Group for *Legionella* Infections to get a rough idea of concentration limits that could be harmful. Although many countries have guidelines or standards for the control of *Legionella* in water systems and to prevent Legionnaires' disease, having uniform regulations is essential to facilitate the implementation of measures.

3.3.1.1 The European Working Group for *Legionella* Infections (EWGLI)

The European Working Group for *Legionella* Infections published technical guidelines for both cold and hot water installations, as well as public and private institutions. Critical concentrations of *Legionella* and associated measures are given, as shown in Table 3-1 [5].

*Table 3-1. Critical concentrations of L. pneumophila according to EWGLI. * CFU – Colony Forming Units*

Concentration limit		Measure
Between 1 000 and 10 000 CFU/L	<20% of tested samples	Resampling necessary If, after resampling, a similar number of samples are infected, it is recommended to take measures to lower the concentration
	>20% of tested samples	Obligation to take measures to decrease concentration
More than 10 000 CFU/L		Resampling necessary Take immediate action to lower concentration (for example disinfection)

The measures given to limit the growth of *Legionella* can be divided into 3 different methods:

1. Monitoring properly the domestic hot water system (DHW).
2. Water temperature control. Having storage tank with sufficiently high temperature (At temperatures below 20°C, the bacteria become dormant but remain viable for months. The bacteria grow best at temperatures between 20°C and 45°C, with an optimum range around 35°C - 41°C. Beyond 45°C, pasteurization starts, and higher temperatures will eventually kill the organism. The time needed to double the number of *L. pneumophila* cells in water is less than half a day at 41°C, and at 70°C, 90% of *L. pneumophila* die in less than a minute).
3. Limitation of water stagnation.

Those concentrations and measures are for information purposes only, each country is free to respect these concentrations or to define others. This situation leads to important differences in *Legionella* regulations from one European country to another on concentration limits and target groups. However, the measures taken by the country usually respect the 3 methods mentioned above.

3.3.2 The Netherlands

3.3.2.1 Lack of official measures and standards

The government has taken measures concerning the protection of workers from *Legionella* and to ensure the quality of drinking water or wastewater installations. Standards have also been implanted to normalize the counting of *Legionella* bacteria. However, there is still no official legislation regarding *Legionella* in wastewater or in the air. This situation should change in 2027 with more dedicated measures for wastewater treatment plants in the Environmental Activities Decree (Besluit Activiteiten Leefomgeving).
However, there are still regulations which can be applied to *Legionella* in WWTP, but they are focused on the security of workers and not on the facility itself, as mentioned below:

- The Working Conditions Decree Articles 4.84 to 4.102 are about paying attention to biological agents, such as *Legionella*. The measures taken if employees are exposed to it are the following [6]:
  - "Remove the source where possible.
  - Where possible, taking technical measures to prevent or reduce workers' exposure to biological agents.
  - Where it appears that technical measures have insufficient effect, organizational measures must be taken.
  - Personal protective equipment should be used as a last resort."

- The determination of the number of *Legionella* bacteria in water must be done following NEN-EN-ISO 11731 (2017), which was adopted by the European Committee for Standardization in May 2017 and effective in The

Netherlands since the 1st of January 2019 with the purpose of having a more standardized way to count *Legionella* in water [7]. The main points are the following:

- "A larger volume of water should be taken, namely 500 ml instead of the current 250 ml. The sample pots will therefore logically double in size.
- Under the current standard, each *Legionella* sample must be used within 24 hours. We are talking about the time of sampling and the start of the analysis process in the laboratory. Under the new standard, that period will be 48 hours.
- The number of comparison variants is expanded from 2 to 3. This third step concerns an acid treatment. The effect of this extra step means a labour-intensive extra step for the lab technician, which affects the process, but also the maximum volume.
- The number of plates required for drinking water samples is reduced. For process water and water from cooling towers, extra plates must be used.
- The lab technician is obliged to carry out an interim assessment. The purpose of this additional assessment is to determine whether any interfering flora are detected on the plate. All plates must be removed from the oven and assessed individually."

3.3.2.2 Nonofficial institutions

3.3.2.2.1 Wastewater installations

- According to RIVM (Rijksinstituut voor Volksgezondheid en Milieu, which is the National Institute of Public Health and the Environnement in The Netherlands), WWTPs should test for the presence of *Legionella* and take preventive and control measures [4].

- The Ministry of Infrastructure of Water Management, with the help of KWR, released a guide for *Legionella* prevention in biological wastewater treatment installations [8] with a:
  - "step-by-step plan for carrying out a *Legionella* risk assessment, from inventory to a functioning management plan.
  - overview of (one-off) corrective measures.
  - overview of periodic control measures to remove or limit *Legionella* risks from the WWTP".

- The Environmental Management Act [9] does not set specific requirements for *Legionella* prevention regarding wastewater treatment. However, if a *Legionella* contamination has been reported as an "unusual occurrence", the municipality can carry out an action plan and therefore add customized regulations to prevent *Legionella*. The competent authority may impose a penalty payment if the measures are not followed.

- Environmental permits are given by the Environmental Law Act (Wet algemene bepalingen omgevingsrecht; WABO). It can be modified according to article 2.31 for the following purposes [6]:
  - "Technical developments.
  - New environmental insights.
  - To prevent serious adverse effects on the physical living environment.
  - Announced new administrative order (AMvB)."

Therefore, if installations must be implemented to prevent *Legionella*, the environmental permit can be modified.

3.3.2.2.1.1 STOWA (Foundation of Applied Water Research)

STOWA's missions are to develop, collect, distribute and implement applied knowledge for an effective and efficient water management in The Netherlands. The organization gives advice to limit the spread and the growth of *Legionella* [10]:

- To sample the water in the warm partial flow installation at least once a month and to analyse it for *Legionella.*
- At high concentrations of *Legionella* in the water phase (≥ 1 000 000 CFU/litre), it is important to determine *Legionella* in the air.

- To tighten the personal protection of employees (and visitors) by wearing a face mask or face mask in the vicinity of the relevant sub-current installation.
- To switch off the ventilation of the partial flow treatment for some time before people are present in/around the installation.

3.3.2.2.1.2 KWR

KWR's mission is to generate knowledge to ensure water quality. Based on the "Handreiking *Legionella* preventie in AWZI" document, written by KWR [8], a new official regulation for *Legionella* in WWTPs should be published in 2027 in The Netherlands. Among other things, it will include the obligation for a WWTP that has a biological treatment operating at a temperature above 25°C to redact a risk assessment.

3.3.2.2.2 Air

3.3.2.2.2.1 VLA (Association of Suppliers of Air Technical Devices)

According to VLA, *Legionella* are part of "Group 3: high harmfulness". Therefore, they have established a scale to evaluate the concentration of *Legionella* in the indoor air: 10 CFU/m³ is good, a value between 10 and 20 CFU/m³ is moderate and a value >20 CFU/m³ is poor [6].

3.3.3 Employee's safety

Some types of workers are miners and welders.

In the Netherlands, The Ministry of Social Affairs and Employment (SZW) is responsible for the implementation of the EU directive 2000/54/EC which is about the protection of workers from risks related to exposure to biological agents at work. According to the law and the Working Conditions Decree, employers must take every measure possible to prevent or limit illness among their employees and to apply the provisions to any work involving hazardous substances or biological agents. In accordance with The Health and Safety Act, employees must fill in a survey indicating and evaluating the risks [11].

The European Agency for Safety and Health at Work recognizes WWTPs with activated sludge as possible sources of *Legionella* infections. To prevent exposure to *Legionella*, respiratory protective equipment (FFP3 mask) should be used. Furthermore, cooling towers, which are used to reduce WWTPs temperature, should be correctly maintained and cleaned following the European Centre for Disease Prevention and Control and the World Health Organization *Legionella* guidelines [12].

According to Hydroscope [6], if no high *Legionella* concentrations have been found in the wastewater, regular health and safety measures are sufficient. If there is a risk of nebulous processes:

- Aerosol places should be indicated with a symbol indicating that dust/aerosol masks should be worn (Table 3-2).



Table 3-2. Symbol indicating that dusk/aerosol masks should be worn

- Respiratory protection ought to be worn within one meter of a nebulizer source for exposures exceeding 4 minutes.
- If the exposure lasts more than 39 minutes, then respiratory protection ought to be worn outside one meter within the source.

It is wise to wear FFP3 mask as a respiratory protection for the following activities [8]:

- In the case of inspection and maintenance work or sampling under a cover placed above an aerosol-forming activity, such as an aeration station.
- During internal inspections of working installations.
- In cleaning activities of internal parts with high pressure. During cleaning, the environment must be cordoned off. Only personnel who are involved in cleaning, wear adequate protective clothing and use respiratory protection are allowed to enter the cordoned-off area. If necessary, the location must be shielded from the environment with the help of sails.

Furthermore, some measures are advised for maintenance procedures which are at risk for *Legionella* spreading:

- To turn off the aeration 15 minutes before a long-term maintenance procedure.
- According to the AI-09 health and safety information sheet, it is not recommended to use effluent for cleaning purposes.
- It is best to avoid high pressure to reduce atomization.
- For cleaning with effluent, surface water or drinking water, a mask with filter class FFP3 should be worn.
- Employers should instruct employees on how to work with biological agents and when to wear respiratory protection.
- Risk inventory and evaluation (RI&E) on biological agents should be performed.
- Periodic medical examinations should be offered to employees at risk.

3.4 Scientific information

3.4.1 About *Legionella*

The term *Legionella* includes 58 species of which about 20 are pathogenic. However, most human infections are caused by *Legionella pneumophilia*, discovered in 1976 [13].

Legionella are gram-negative bacteria, which means that they can be highlighted using the Gram staining method. They measure 2 to 20 µm [14]. They have a thin peptidoglycan cell wall surrounded by an inner cytoplasmic cell membrane and a bacterial outer membrane [15]. They are naturally present in aquatic habitats in low numbers. A more abundant presence is generally observable in human-made aquatic habitats at temperatures between 25°C and 45°C with an optimal range between 30°C and 38°C [12][16][17][18] or between 25 and 30°C to promote infection and transmission according to the National Academies of Sciences Engineering and Medicine [19]. In other studies, growth was observed between 16.5 and 64°C [19].
The growing conditions of L. pneumophila depending on the temperature are resumed in
Table 3-3.

Table 3-3. Growth and death behaviour of Legionella bacteria as a function of temperature [8]

Temperature (°C)	*Legionella* growth and dying behaviour
> 60	Die-off
50 – 60	Slow die-off
45 – 50	Slow growth possible
38 – 45	Rapid growth possible
30 – 38	Optimal growth possible
25 – 30	Rapid growth possible
20 – 25	Slow growth possible
< 20	Presence and survival possible

They can survive over a wide pH range: from pH 5.5 to 9.2 [16] with optimal growth between 6.5 and 7.5 [6] [17]. No *Legionella* was detected below a pH of 2.7 [19]. The growing conditions of *L. pneumophila* depending on the pH are summarized in Table 3-4 [8].

Table 3-4. Growing condition of L. pneumophila depending on the pH

pH	*Legionella* growth behaviour
< 5,0	No growth
5.0 – 6.5	Growth possible
6.5 – 7.5	Optimal growth
7.5 – 9.0	Growth possible
> 9.0	No growth

Under laboratory conditions, *Legionella* are nutritionally fastidious, requiring specific additions to standard nutrient media to grow. *Legionella* will not multiply on most common laboratory media and require supplementation of a range of amino acids which they are incapable of synthesizing. *Legionella* are auxotrophic for several amino acids, namely L-arginine, L-cysteine, L-isoleucine, L-leucine, L-methionine, L-threonine and L-valine [20]. A single elimination of one of those amino acids results in a depression or absence of growth [21]. If the medium is limited by those nutrients, *L. pneumophila* hijack the amino acids synthesis machinery of their hosts for self-profit [20].

Legionella grow poorly as individual, free-living cell. However, they have the ability to multiply within eukaryotic cells such as in 14 species of protozoa [1] and mainly amoeba that are associated with biofilms. *Legionella* colonize biofilms, aggregations of microorganisms that secrete extracellular polymeric substances such as polysaccharides to form a protective matrix that adheres to surfaces. The microbiological interaction with biofilms differs depending on the system conditions such as water flow rate, temperature, exposure to light, and nutrient content.

Studies of interactions between *Legionella* and protozoa has demonstrated *Legionella*'s ability to parasitize a range of protozoa and to multiply within them in the absence of any supporting nutrients. *Legionella* enter macrophages and protozoa via either traditional phagocytosis or via a mechanism known as coiling phagocytosis, in which the bacteria are internalized in a coil of the host's cell membrane. Once internalized, *Legionella* secrete proteins encoded and recruit components to protect herself from attack by the host cell. *Legionella* are able to grow in a relatively nutrient-free environment solely utilizing parasitisation as a survival mechanism. *Legionella* multiply within this protected vacuole, and when amino acid concentrations become too low to allow for continued multiplication, flagella are formed to allow the bacteria to escape the host cell and to search for a new host to parasitize. The survival of *L. pneumophila* inside an amoeba depends also on the temperature. For instance, at 15°C, the bacteria are digested by *Acanthamoeba palestinens*, whereas at 25°C they can proliferate inside. In *A. castellanni,* they can proliferate but are digested at 20°C [19]. The host provides protection against high temperature and disinfectants [6].

Under stress, *Legionella* can synthesize PHB (Poly-3-Hydroxybutyrate) as an energy source for survival in low-nutrient environments. They can maintain a vegetative state for extended periods, at least 600 days [6]. To grow, *Legionella* need iron. They can use dissolved iron or can obtain it from its host. The level of iron controls the construction and the consumption of PHB. However, a high concentration of iron Fe^{3+} in wastewater can inhibit the growth [18]. It has been observed that the addition of the iron chelator lactoferrin reduces viability of *L. pneumophila.* Moreover, ferric/ferrous ions also promote *L. pneumophila* virulence. However, excessive iron inhibits *Legionella* biofilm formation [19]. Likewise, zinc and potassium are key factors at low concentration but toxic at higher levels [18].

The presence of dioxygen at low level is also required. *Legionella* can survive with a dissolved dioxygen between 0.2 and 15 mg/L [22] but according to Wadowsky *et al.* (1985) no growth is observed below 2.2 mg/L [16] [17]. However, Caicedo *et al.* (2019) observed *Legionella* growth with a DO from 0.2 to 2.2 mg/L [12].
L. pneumophila survive in water up to 3% NaCl only at low temperature [18] [17]. At 30% salinity, the metabolic activity is maintained but the growth is inhibited.

Copper can also inhibit *Legionella* growth [19]. Indeed, Borella *et al.* (2005) showed that a copper concentration higher than 50 mg/L prevented colonisation of *Legionella spp* [18].

The *Legionella* life cycle switches between infectious, replicative forms and dormant cell forms. This form is called VBNC (Viable But Non-Culturable) which means that the bacteria do not grow on agar plates [19]. Under stress conditions, both *Legionella* and protozoa change their form to be more resistant.

Heterotrophic bacteria, such as *Pseudomonas spp.* and *Aeromonas spp.* may inhibit the growth of *Legionella*. These bacteria compete *Legionella* by using the same nutrients as *Legionella* or by excreting substances that kill *Legionella* bacteria. However, some strains are capable of both stimulating and inhibiting *Legionella* depending on the media [23].

Acanthamoeba castellanii is inhibited in growth by peptide. The growth of *Legionella* within this host is also inhibited [24].

3.4.2 Protozoa

Protozoa are microscopic unicellular or colonial eukaryotes, living in almost every possible habitat, mainly in aquatic or moist environments [25]. They can be divided into four groups according to their mobility: Flagellates, Ciliates, Amoebae or Apicomplexa (spore forming). However, *Legionella* develop preferentially in Amoeba (in about 20 species of Amoebae) and in some Ciliates such as Paramecium caudatum [6].

The temperature and the oxygen are the most important factors that regulate their growth. The light is also an important factor for the autotrophic forms with a negative influence of ultraviolet (UV). Active protozoa are found at temperatures between 0 and 50 °C with an optimal range for free-living amoeba between 22 and 44°C [26]. The optimal pH for these amoebae is 7.6. Protozoa are heterotrophic meaning that they require carbon in reduced form from their surrounding environment. Many protozoans respond to adverse environmental conditions by encysting: they secrete a thick, tough wall around themselves and effectively enter a state comparable to hibernation [27].

In activated sludge plants, protozoa can swim freely in wastewater, but are also part of sludge (flocs). Protozoa can grow up to 106 cells/L. It is estimated that the protozoa biomass can reach values around 250 mg/L (dry weight), which can represent more than 9% of volatile solids. Protozoa have positive effects on carbon mineralization in activated sludge. It is generally accepted that their feeding on bacteria improves the treatment, resulting in a lower organic load in the output water of the treated wastewaters. Protozoa are considered to be the most important bacterivorous grazers. Even though some protozoa (crawling ciliates and other forms) can eat flocculated bacteria, most protozoa (prevalently attached ciliates) can only graze on suspended bacteria and particles. The excretion of mineral nutrients by protozoa results in an accelerated usage of the carbon source by the bacteria [28]. It has been observed that the presence of protozoa increases the per-cell nitrification rate, probably because of the ability of protozoa to influence bacterial growth. Indeed, protozoa release inorganic and organic products into their surroundings which are mainly recycled nutrients, such as nitrogen, phosphorous, and organic carbon, but might also include stimulatory compounds that contribute to the dissolved organic carbon pool and affect the physiological state and growth of bacteria [28].

In aerobic biological treatment systems, the combination of ideal temperature (in combined systems receiving industrial effluents > 30 °C), availability of oxygen and organic nitrogen, and the presence of protozoa provide an ideal environment for the proliferation of *Legionella*.

3.5 Diseases

Legionella are responsible for bacterial infections called “Legionellosis”, which can go from Pontiac fever, a moderate illness, to a severe pneumonia also called Legionnaire's disease, which could lead to death [1].

3.5.1.1 Legionnaires’ disease

The incubation period of Legionnaires’ disease is between 2 to 10 days, and in rare cases, go up to 20 days. However, the disease can last for weeks. When not treated adequately with specific antibiotics it can be deadly, especially for people at risk. Smokers, people over 45 years old, people suffering from chronic lung disease or kidney disease, and immunocompromised people are most likely to develop Legionnaires’ disease after being exposed to *Legionella* [29]. In practice, men appear to be more susceptible than women.

Legionnaires’ disease does not have characteristic symptoms and not every organism will develop the same symptoms. Usually, patients will first feel a loss of strength and appetite. It may be accompanied with high fever and headaches. Patients may also develop a mild and unproductive cough. Moreover, about half of patients develop pus-forming sputum and one third of them blood-streaked sputum or cough up blood. Chest pain is also frequent. Furthermore, about half of the patients are affected by disorders related to the nervous system within the first week of the disease, among them: hallucinations, confusion, delirium, depression. Gastrointestinal troubles are also regular as half of the patients suffer from watery diarrhoea, and up to 30% suffer from nausea, vomiting or abdominal pains.

If the disease is being left untreated, it worsens during the first week and therefore can lead to death as the most frequent complications are respiratory failure, acute renal, shock and multi-organ failure. The long-term pathological symptoms such as persistent pulmonary and restrictive pulmonary disease for those who suffer

from severe respiratory failure may persist. Weakness, poor memory and fatigue may also last for several months. Retrograde amnesia can also occur.

Diagnosis can be done with a urinary antigen test or by taking a sample of sputum for a culture test [30]. Sputum sampling is often difficult because patients have a dry cough. The urinary antigen test is the most common test since it detects a molecule of *L. pneumophila* serogroup 1, which is responsible for about 80% of Legionnaires' disease, in urine within a day. Other species and serogroups of *Legionella* are however not detected by this method. The culture method is less used because it is slower and technically more difficult as the sensitivity highly depend on technical skills. Furthermore, a serious pneumonia from Legionnaires' disease can be confirmed by chest x-ray. Other methods such as PCR (Polymerase Chain Reaction), DFA (Direct Fluorescent Antibody) and serology may be done to test if there are *Legionella* bacteria present in the body [31].

In general, to treat patients with pneumonia, appropriate antibiotics treatment, based on the severity and host-related risk factors, is prescribed. In hospital, oxygen given through a face mask/tube in your nose or a machine to help you breathe may be given to you. There is no vaccine to prevent Legionnaires' disease.

3.5.1.2 Pontiac fever

The incubation period of Pontiac fever is between 24h and 48h. It usually lasts 2 days to 5 days. It is not deadly [1].
What differs from Pontiac fever and Legionnaires' disease is that Pontiac fever does not occur with a pneumonia: it is an influenza-like illness. It may be accompanied with a loss of strength, tiredness, muscle pain, headaches, joint pains. High fever, chills, difficult breathing and dry cough may also occur. Furthermore, like Legionnaires' disease, gastrointestinal troubles such as diarrhoea, nausea, vomiting can occur, although it is less frequent.

Diagnosis of Pontiac fever can be done with a urinary antigen test or with the culture method.

Complications rarely happen, which is why the treatment is usually supportive and aims to relieve symptoms.

3.5.2 Wastewater treatment plants

The function of wastewater treatment plant is to purify wastewater by removing pollutants from it. After being treated, water can be discharged or reused. They are essential as they are protecting human health from illnesses and pathogens, preserving the environment, and improving water management. Usually, the difference between industrial and municipal WWTP is that the organic load (usually 10 times higher in industrial settings) and temperature are higher in industrial wastewater. Usually, wastewater temperatures are above 25°C for industrial WWTPs. Also, there are some differences on nutrients concentration.

With respect to industrial wastewater, there are often (at least) two stages in the treatment [32]. Some wastewater treatment plants have also additional treatment steps and have a tertiary treatment (Figure 3-1, Table 3-5). Typically, for high load industrial wastewater, the process will consist of an anaerobic treatment followed by an aerobic treatment.

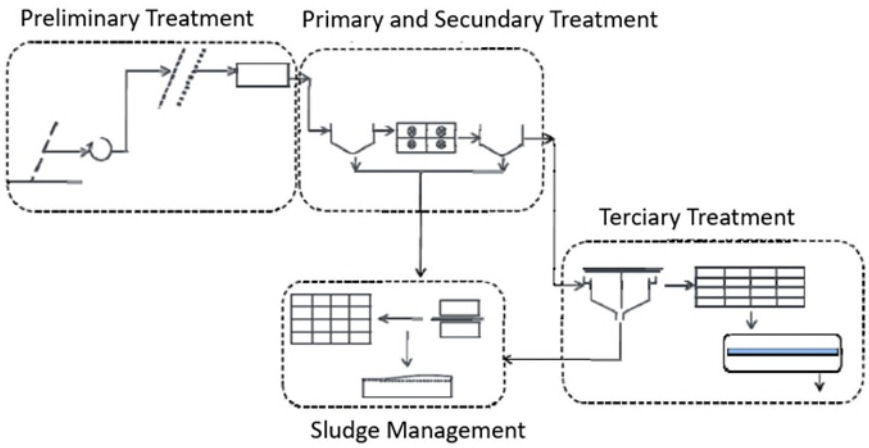


Figure 3-1. Process chain for WWTP-treatment [33]

Table 3-5. Types of industrial wastewater and how they are treated [12]

Industry	Wastewater temperature	COD wastewater/composition	Type of treatment
Wood production and paper mills	30 – 37°C	COD up to 5700 mg/L	COD < 1000 mg /L: aerobic COD > 1000 mg/L: anaerobic
Petrochemical industry	Differs considerably according to the industrial activities	Differs considerably according to the industrial activities	Combination of biological and chemical processes Cooling towers Aerated tanks
Brewery	25°C - 35°C	COD: 30 – 100 mg/L But according to ATV-DVWK, 2010: 300 – 1800 mg/L COD, 30-100 mg/L total nitrogen, 10-30 mg/L phosphorus	anaerobic (EGSB, UASB) followed by aerobic (activated sludge, fluidized bed reactor)
Food and dairy industries	20°C - 30°C	COD: 800 to 4500 mg/L Nitrogen: 10 – 110 mg/L	Activated sludge processes, moving bed biofilm reactors, sequencing batch reactor and membrane reactor, or anaerobic pretreatment combined with aerobic treatment

3.5.2.1 Preliminary treatment

Preliminary treatment is the first step of wastewater treatment, and its aim is to prepare water for purification for the following steps.

First, wastewater goes through a screen, which removes large objects, such as sticks, concrete, wood, debris, which could damage equipment or clog the pipes: this step is called screening. Then, it goes through a grit chamber.

3.5.2.2 Primary treatment

The aim of the primary treatment is to remove part of suspended solids. Indeed, when left untreated in aquatic environment, they can lead to the development of sludge deposits.

After the preliminary treatment, wastewater still contain organic, inorganic matter and suspended solids. These solids can be reduced with a Primary Clarifier, a sedimentation tank. When the speed of the flow is reduced, the solids trend to sink to the bottom and form a layer called “raw primary sludge” which will be removed by pumping and typically digested. Furthermore, during this treatment, flocculants and coagulants can be added to improve the sedimentation (Figure 3-2). Wastewater may go under an acidic or basic treatment to adjust its pH [34].

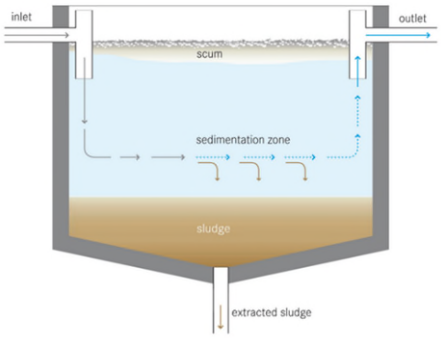


Figure 3-2. Sedimentation tank

Alternative to a primary clarifier in industrial WWTPs is often made use of a Dissolved Air Flotation systems (DAF). The DAF is aerated to float small particles. The bubbles are fine to improve attachment of solids. In case the incoming water will already contains *Legionella*, this will be a point of attention, risk area.

3.5.2.3 Secondary treatment

The aim of the secondary treatment is to remove organic matter from the water. Nutrients like nitrogen and phosphorus are also removed. Excess concentration of these nutrients can lead to eutrophication. [35].

3.5.2.4 Anaerobic Treatment

In industrial WWTPs is usually required the use of anaerobic digestion treatment. In these situations, the Anaerobic Digestor (AD) is the first step after the primary treatment.

The anaerobic treatment takes place without oxygen supply. This treatment is mainly used for wastewater containing high level of biodegradable solids that can be broken down by anaerobic microorganisms. It requires less energy than aerobic treatment (no need of aeration) and it also produces biogas (methane) which can be used for energetic purposes [36]. There are many biochemical reactions during the treatment, and they can be divided into 4 stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 3-3) [37].

- Hydrolysis: Extracellular enzymes break down cellulose, sugars, lipids, proteins and transform them into simple unites such as glucose, fatty acids, and amino acids. pH is between 5.5 and 6. The general equation of a hydrolysis is the following:

$$(C_6H_{10}O_5)n + nH2O \rightarrow nC_6H_{12}O_6 + nH_2$$

- Acidogenesis: Products from hydrolysis are degraded by acidogenic bacteria into short-chain volatile fatty acids, alcohols and ketones. CO_2, H_2, NH_3 and alcohols are common by-products. pH is between 5.5 and 6. The chemical reactions involved in acidogenesis are the following:

$$C_6H_{12}O_6 \leftrightarrow 2\ CH_3CH_2OH + 2\ CO_2$$
$$C_6H_{12}O_6 + 2\ H_2 \leftrightarrow 2\ CH_3CH_2COOH + 2\ H_2O$$
$$C_6H_{12}O_6 \leftrightarrow 3\ CH_3COOH$$

- Acetogenesis: Products of the acidogenesis and some of the long-chain fatty acids hydrolysis stage are degraded into acetate, CO_2 and H_2. The reactions involved in this stage are the following:

$$CH_3CH_2\,COO^- + 3\ H_2O \leftrightarrow CH_3COO^- + H^+HCO3^- + 3\ H_2$$
$$C_6H_{12}O_6 + 2\ H_2O \leftrightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ H_2$$
$$CH_3CH_2OH + 2\ H_2O \leftrightarrow CH_3COO^- + 3\ H_2 + H^+$$

- Methanogenesis: Methane is produced from all the intermediate products synthesized previously. Acetate and H_2 are converted into CO_2 and CH_4 by acetophilic and hydrogenophilic bacteria respectfully. Methanogenic bacteria are sensitive to pH and perform better at a pH around 7. The chemical reactions involved in methanogenesis are the following:

$$2\ CH_3COOH \rightarrow CH_4 + CO_2$$
$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$$
$$2\ CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2\ CH_3COOH$$

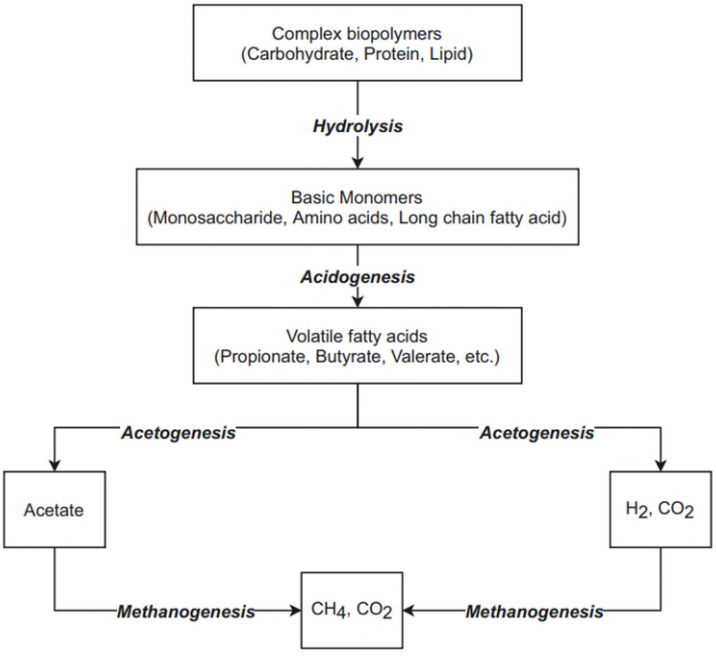


Figure 3-3. Anaerobic digestion biomass decomposition stages and key compounds

There are different types of anaerobic processes but in most WWTPs, an anaerobic step is followed by an aerobic step to degrade a wider variety of compounds. For instance:

- Upflow Anaerobic Sludge Blanket followed by activated sludge process (UASB + ASP) is a process where wastewater is distributed at the bottom of the UASB reactors and then flows through the aerated tank (Figure 3-4).

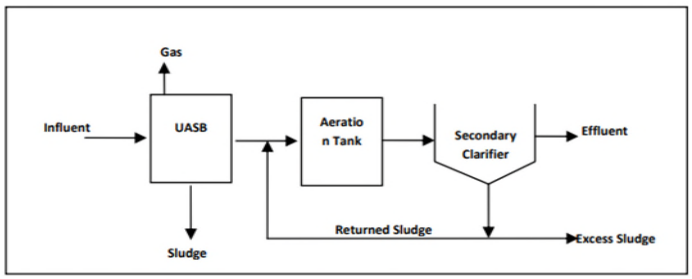


Figure 3-4. Schematic diagram of an upflow anaerobic sludge blanket process followed by ASP [33]

- Waste Stabilize Pond (WSP) is a combination of an anaerobic and an aerobic pond. Wastewater is treated in a series of ponds (Figure 3-5).

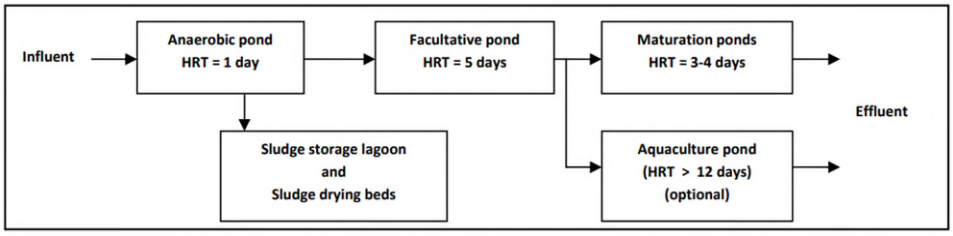


Figure 3-5. Schematic figure of a WSP [33]

3.5.2.4.1 *Legionella* in anaerobic processes

In anaerobic treatment there is no oxygen for *Legionella* to grow.

The anaerobic reactor serves sometimes as a pre-treatment for aerobic treatment. *Legionella* are aerobic bacteria (aerobic strict) but can survive without oxygen if the residence time is limited. In addition, hosts for *Legionella* may be present. The anaerobic reactor is not a risk to *Legionella* growth but might play a role due to the possible increase of nutrients during reduced functioning. It is suggested that a non-optimal condition of the anaerobic treatment contributes to a higher presence of nutrients and subsequently to a higher growth of *Legionella*, developing even faster when reaching the aerobic tank (aeration). However, to what extent the presence of amino acids and proteins plays a role, it is not yet clear.

Normally, anaerobic treatment increases the temperature in the aerobic treatment (posttreatment) and this increment of temperature could also be a dominant factor in accelerating the *Legionella* growth. Heat recovery from anaerobic effluent could play an important role in controlling the *Legionella* risk. In the WWTP design, the anaerobic temperature of the effluent could be reduced before entering the aerobic tank by using a heat pump. The benefit of this strategy regarding the control of *Legionella* should be considered when estimating the economical investment and the potential use of energy (recovered) in parallel processes. By cooling the anaerobic effluent, the temperature on the aerobic process could be kept at <25°C reducing significantly the risk. This could be considered as a potential measure to control *Legionella* growth.

3.5.2.4.2 Aerobic Treatment. Activated sludge process

The activated sludge process is the most commonly applied biological wastewater technology. In this process, a bacterial biomass suspension (the activated sludge) is responsible for the removal of organic material. Aerobic bacteria (aerobe) have an oxygen grounded metabolism, in a process known as cellular respiration. Aerobic bacteria are heterotrophs and use oxygen to oxidize organic substrates to carbon dioxide and water to gain energy (heat).

The aerobic treatment requires oxygen for their metabolic processes. Therefore, equipment that can supply oxygen and incorporate it into the wastewater is mandatory. Dissolved oxygen concentrations in activated

sludge tanks are kept around 2.0 mg/L. It is reported that in aerobic biological treatment systems, the temperature can be higher than 30°C [12]. For example, the operating parameters of Circle Wastewater Services's WWTP are the following: the pH is between 6 and 9.5 with an average of 8, the temperature is between 20 and 33°C, the DO is 2 mg/L.

Many activated sludge configurations have evolved over the years, and depending on the design an activated sludge WWPT can achieve removal of organic carbon substances and nutrients such as nitrogen (N) and phosphorus (P). In some biological wastewater treatment systems biomass grows attached to a surface. These systems are broadly defined as biofilms systems, such as trickling filters, integrated fixed film activated sludge systems, moving bed biofilm reactors, and rotating biological reactors. Microbial communities of activated sludge (AS) vary extensively based on plant operating technology, influent characteristics and WWTP capacity.

Some of the treatments will be explained below [33]. This is a non-exhaustive list.

- A trickling filter (aerobic biofilm reactor) is composed of bed stones in which wastewater goes through. Microorganisms responsible for treatment are linked to an inert packing material (Figure 3-6).

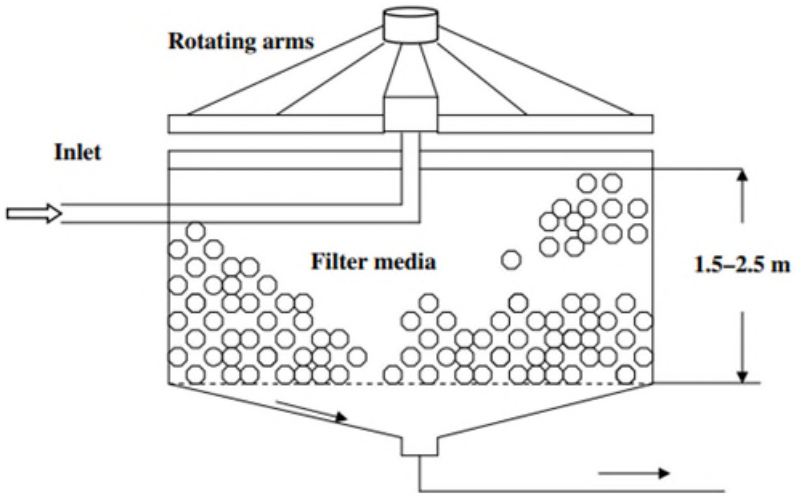


Figure 3-6. Side view of a trickling filter [38]

- Activated sludge process (ASP) is activated with billions of bacteria and microorganisms. The sludge is brought in contact with wastewater and air: therefore, it is a suspended growth aerobic process. This operation is done into an aeration tank during 4 to 6h. Then, the partially treated sewage is sent to a secondary clarifier where sludge and liquid are separated. Most of the sludge is reused and the excess sludge is sent to a digester (Figure 3-7).

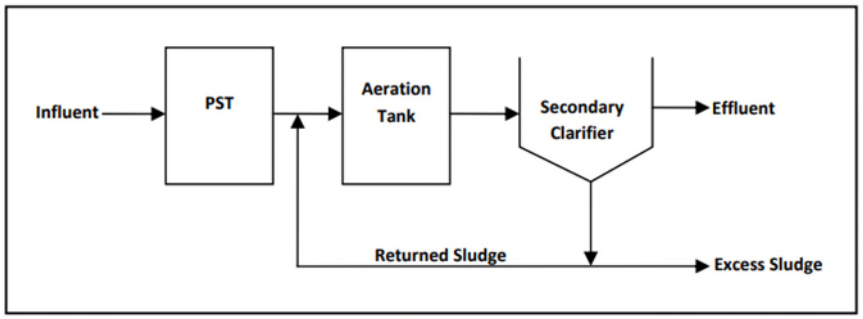


Figure 3-7. Schematic diagram of a conventional activated sludge process [33]

- Moving bed biofilm reactor (MBBR) is a process where biological mass is generated on the surface of the media, which is floating plastics in suspension (Figure 3-8).

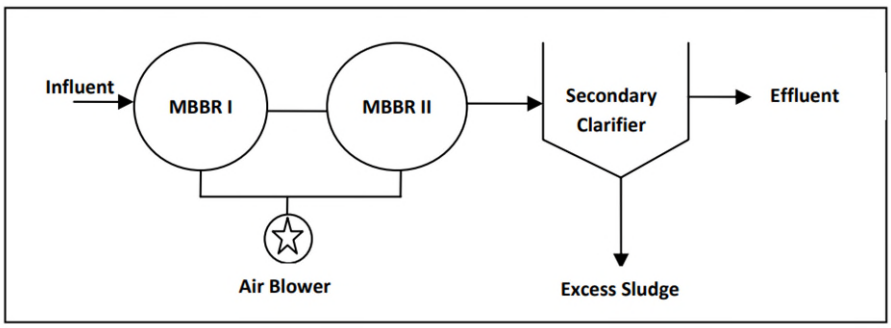


Figure 3-8. Schematic diagram of moving bed biofilm reactor [33]

- Sequencing batch reactor (SBR) is process involving an anoxic filling tank, aeration, sedimentation/clarification, decantation and sludge withdrawal (Figure 3-9).

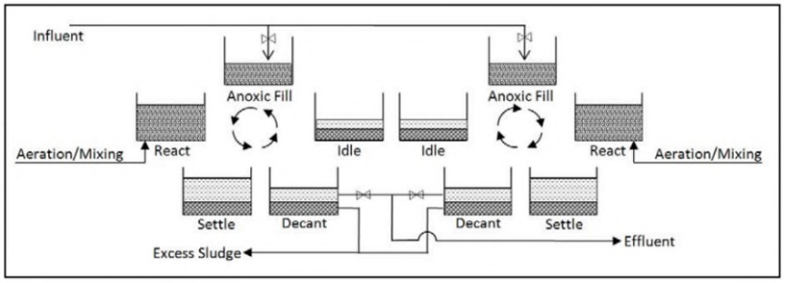


Figure 3-9. Schematic diagram of a SBR [33]

- Constructed Wetlands (CW) is a process in which submerged plants or floating islands species are used to treat wastewater. The biological process takes place at the roots of the plants.

- Membrane bioreactor (MBR) is a biological reactor containing suspended biomass. There is microfiltration membrane which separates solid from liquid (Figure 3-10).

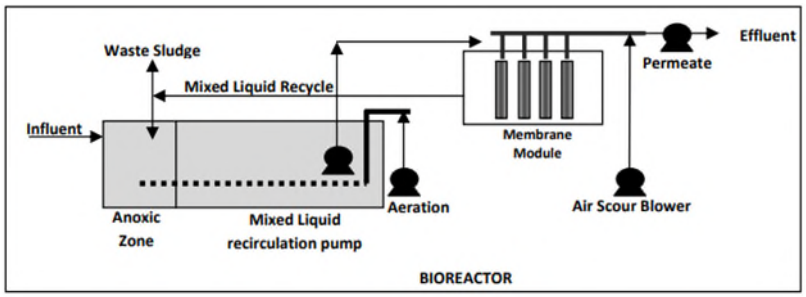


Figure 3-10. Schematic diagram of a MBR [33]

3.5.2.4.3 Biological Nitrogen Removal

Biological Nitrogen removal (nitrification/denitrification) is the process most used for nitrogen removal from wastewaters. In conventional Biological Nitrogen Removal systems, the microbial community (in the activated sludge) is exposed to different environmental conditions to accomplish COD and nitrogen removal.

During nitrification, under aerobic conditions, ammonia (NH_3) is converted to nitrate (NO_3^-). During denitrification, under anoxic conditions, NO_3^- is converted to nitrogen gas (N_2).

Biological reactors providing different conditions are usually linked in series in a WWTP, whereby the mixed liquor of wastewater and the activated sludge flows through different setup conditions to provide specific requirements for both processes.

3.5.2.4.3.1 Nitrification

Nitrification is defined as the biological or biochemical removal (oxidation) of ammonia (NH_4^+) by certain bacteria in the presence of oxygen [39] . The group of bacteria are called “nitrifiers”, “nitrifying bacteria” or “nitrification bacteria.” Two main groups of bacteria are involved in the process, Nitrosomonas and Nitrobacter. These bacteria are collectively known as nitrifiers and are autotrophic, i.e. they get their carbon source from inorganic carbon (carbonates, bicarbonates) or carbon dioxide. In nitrifying activated sludge process only 3-10% of bacteria are autotrophic (nitrifiers).

Wastewater generated by the rendering industry along with other protein-related food processing industries will generate high levels of ammonia due mainly to the biological breakdown of proteins. Amino acids are further degraded to organic compounds with the generation of ammonia.

The resulting ammonia will be in two major forms, depending upon the pH of the wastewater. Ammonia will be in the gaseous form, NH_3, at a pH higher than 9.0, and in the water-soluble form, NH_4^+ (ammonium ion) at pH lower than 9.0. The gaseous form at a higher pH is the most toxic form to humans, animals, and aquatic life. Nitrification bacteria will be interested in the NH_4^+ ammonium ion form. The chemical reactions involved are the following:

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^-$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

Optimal growth of nitrifiers is between 25 and 30°C. Approximately 4.6 mg/L (or parts per million ppm) of oxygen is required for every ppm NH_4^+ oxidized to nitrates (NO_3^-). Dissolved oxygen content of at least 2 mg/L is recommended in the plant in the aerobic zones. Rates above 4 or 5 mg/L are generally considered “overkill” and a waste of energy in a conventional setting. Depending on the bacteria, optimum pH is between 7.0 and 8.0, however, nitrification process has been observed at pH level from 6.6 to 9.7. Therefore, it is important to maintain an adequate alkalinity in the aeration tank to provide pH stability and also to provide inorganic carbon for nitrifiers. Low carbonaceous biochemical oxygen demand (CBOD) is required, a safe operating range of pH would be 7.6 to 8.2 and maintenance of a minimum total alkalinity of 50 mg/. At pH values near 5.8 to 6.0, the rates may be 10% to 20% of the rate at pH 7.0. A pH of 7.0 to 7.2 is normally used to maintain reasonable nitrification rates, and for locations with low-alkalinity waters, alkalinity is added at the water resource recovery facility to maintain acceptable pH values [39].

3.5.2.4.3.2 Denitrification

The second part is the denitrification part which is one of the key processes in biological nitrogen removal. Denitrification is the only nitrogen biotransformation process that removes nitrogen from ecosystems (essentially irreversibly). Denitrification is one of the key processes in biological nitrogen removal (BNR) from water and wastewater. Denitrifying bacteria have been identified in over 50 genera with over 125 different species and are estimated to represent 10-15% of bacteria population in water, soil and sediment [40].

Through denitrification, oxidised forms of nitrogen, such as NO_3^- and nitrite (NO_2^-), are converted to dinitrogen (N_2) and, to a lesser extent, N_2O gas. This is an anaerobic process where nitrate is converted to dinitrogen gas by denitrifying bacteria. The primary mechanism is the following:

$NH_3 \rightarrow NO_3\text{-} \rightarrow NO_2\text{-} \rightarrow NO \rightarrow N_2O \rightarrow N_{2\,(gas)}$

Heterotrophic denitrifying bacteria convert nitrate generated from autotrophic nitrification to nitrogen gas, thus removing nitrogen from wastewater. Depending on the influent characteristics, treatment configurations and operation conditions, heterotrophic denitrifying bacteria account for approximately 20–80% of all bacteria flocculated and dispersed in the activated sludge, which means that the organisms obtain their energy from organic materials built by other organisms. Therefore, the need of organic carbon as electron donor is important. The primary requirement for heterotrophic denitrifies of a carbon source will full fill two roles, growth under anaerobic conditions and stimulation of O_2 respiration under aerobic conditions, which in turn will reduce the O_2 concentration surrounding the respiring bacteria. The denitrifies among them eventually are under pressure to switch from aerobic to anaerobic respiration using NO_3 [40].

Heterotrophic denitrification consists of four consecutive reductions and the requirement of organic carbon (as electron donor) is significant. In this process, there are many different compounds used as electron donors such as methanol, acetate, ethanol, lactate and glucose. This denitrification consists of sequential reductive reactions from NO_3^- to N_2 with NO_2^-, NO and N_2O as three obligatory intermediates. Each reduction step is catalysed by at least a reductase enzyme. NO_3^- is produced by the previous nitrification process. Numerous species of heterotrophic denitrifying bacteria have been identified in activated sludge. In WWTPs, complete denitrification is likely achieved by the cooperation of denitrifying bacteria with different nitrogen oxide reduction capabilities. Generally, around 4 g of biodegradable COD is needed per gram of NO_3^- reduced. DO has inhibitory effects on the activity of the N reductases (enzymes that catalyse N reduction). However, a very limited amount of oxygen could be brought through natural surface air-liquid exchanges or flow circulation and is consumed by denitrifies immediately. Under anoxic conditions, to favor simultaneous nitrification and denitrification, DO is typically not detected but a limited amount of oxygen could be transfer through the contact of air with water surface, low concentrations can be found around 0,5 mg/L. The optimum pH for denitrification process is between 7 and 9 and the optimal temperature range is from 20 to 35°C. Denitrification is significantly inhibited at temperatures lower than 5°C. Additional external organic carbon supplementation (such as methanol or acetate) is often applied when there is an inadequate amount of organic carbon in wastewaters for removing ionic nitrogen oxides. When complete denitrification is achieved, around 75% of the influent N is removed by emitting as N_2, and 20% of the influent N is incorporated into the sludge and removed as waste sludge, with only around 5% of the influent N remaining in the effluent [40].

Anammox

Anaerobic Ammonium Oxidation (AnAmmOx) process can offer an alternative treatment of nitrogen. Anammox involves bacteria to oxidize ammonium (NH_4^+) into nitrogen gas (N_2) using nitrite (NO_2^-) as electron acceptor under anoxic condition [40] [41] [42]. Anammox bacteria requires NO_2^- as an electron acceptor, however sewage contains no NO_2^-. Therefore, a partial nitrification step is required to convert NH_4^+ into NO_2^- [43]. The main source of carbon of Anammox bacteria is CO_2. The bacteria use extracellular iron Fe(II) and Fe(III) as respiratory substrate [44] .

Five main Anammox bacteria have been discovered: "*Candidatus Brocadia*", "*Kuenenia*", "*Scalindua*", "*Anammoxoglobus*" and "*Jettenia*". Their conditions are sum up in the following Table 3-6 [40]:

Table 3-6. Physiological characteristics of anaerobic ammonium oxidation bacteria

Parameters	*Brocadia anammoxidans*	*Brocadia sinica*	*Kuenenia stuttgartiensis*	*Scalindua* sp.	*Scalindua profunda*	*Jettenia caeni*
Growth temperature (°C)	20–43	25–45	25–37	10–30	15–45	20–42.5
Growth pH	6.7–8.3	7.0–8.8	6.5–9.0	6.0–8.5	7.4	6.5–8.5
Maximum growth rate (μ_{max}, day^{-1})	0.065	0.098	0.062–0.084	0.048	ND	0.048
Doubling time (days)	10.7	7	8–11	ND	ND	ND
Biomass yield (*Y*, mmol C mmol N^{-1})	0.07	0.063	ND	0.03	ND	0.056

The advantages of Anammox are that it produces less sludge, decreases oxygen demand, it requires less space and the emissions of CO_2 and N_2O are reduced. However, because of the low biomass yield, an efficient system for sludge retention and long start-up times are needed to achieve the biomass concentration needed [40].

Anammox bacteria have a slow growth rate with a doubling time at 30-40°C about 10-14 days and are very sensitive to changing environments. The Anammox operating temperature is between 15 and 40°C with the optimal range between 30 and 40°C. Outside this range, the reaction rate will decrease rapidly [41]. However, studies have reported the possibility of operation of laboratory scale at low temperature, between 5 and 15°C, called “cold Anammox” [45]. The optimum pH range for Anammox is between 6.7 and 8.3 [41].

Anammox and nitrification can form a relationship under oxygen-limited conditions lower than 0.5 mg/L allowing to perform one-stage partial nitrification and Anammox processes [40]. Ammonium oxidation to nitrate requires about 4 g DO per g of $N\text{-}NH_4^+$ [46]. The Anammox process is temporarily inhibited if the dissolved oxygen concentration is below 0.032 mg/L. However, Anammox can also survive in an aerobic tank over 2 mg/L of dissolved oxygen [41]. Anammox bacteria can tolerate a NaCl concentration up to 75 g/L, however, Anammox sludge cannot be applied in Anammox treatment at a salt concentration lower than 30 g/L [41]. Bacterial growth is enhanced by a Fe (II) concentration of 0.09 and 0.12 mmol/L. The first concentration is more beneficial for Anammox activity, but the second can stimulate Anammox bacteria growth. Fe (II) concentration at 0.75 mmol/L can inhibit the Anammox process [47].

3.5.2.4.4 *Legionella* in Anammox processes

The major risk associated with *Legionella* in the Anammox process is associated to the high temperature range of this process, being the same as the optimal temperature range for *Legionella* growth. The combination of high temperature and excess of aeration could significantly increase the risk for *Legionella* development. Good control of the aeriation is required to provide a good equilibrium between nitrifiers and Anammox bacteria, and with this equilibrium NH_4^+, nitrite and oxygen concentration in the water phase which are important factors affecting *Legionella* growth. Dissolved oxygen (DO) is required to oxidize sufficient NH_4^+ to nitrite (NO_2^-), but the DO concentration should be low enough to avoid the inhibition of Anammox bacteria. At high DO concentrations the activity of nitrite oxidizing bacteria is stimulated, negatively affecting the performance of Anammox bacteria in nitrogen removal. Low growth rates of Anammox bacteria helps nitrifiers to develop faster and equilibrium between partial nitrification/Anammox results affected.

Stable microbial relationship in the sludge (community) must be kept for a robust Anammox process and further control of *Legionella* development.

3.5.2.5 Tertiary treatment

The tertiary treatment is a polishing process with the aim of eliminated the existence of specific compounds or disinfecting the water.

The sedimentation tank is usually disinfected with chlorine to reduce odour and kill pathogenic bacteria with an effectiveness of 99%. Sometimes, a dichlorination step is done in order to remove excess chlorine. There are also alternatives to chlorine disinfection such as:

- UV disinfection: UV light is used to destroy microorganisms and thus sterilising wastewater.
- Ozone: Ozone is used, it is more effective than chlorine but also much more expensive and produces toxic gases. Furthermore, special equipment is required.
- Ion exchange: Usually unwanted ions are swapped with inoffensive ones such as sodium for example [36].

3.6 Risks in WWTPs

WWTPs are at risk for the spread of *Legionella* because of the way their processes work.
In drawing up this document, use has been made of the *Legionella* Prevention Guide in Biological Wastewater Treatment Plants [8]. This guide provides guidelines for estimating and controlling *Legionella* risks that may arise from the Wastewater Treatment Plant (AWZI), in order to comply with the duty of care of employees (occupational safety) and the environment (environmental safety), or with the permit regulations based on of the Environmental Management Act. *Legionella* contamination leads to legionellosis.

Legionellosis is caused by inhaling aerosols contaminated with the *Legionella* bacteria. *Legionella Pneumophila* are responsible for >90% of cases of disease, but there are also many other types of *Legionella* bacteria, about half of which are pathogenic to humans. Control measures must be taken to prevent the spread of *Legionella* by means of small water droplets (aerosols). Indeed, *Legionella* aerosols can be formed during aeration processes of wastewater [48].

3.6.1.1 Growth of *Legionella*

In aerobic biological treatment systems as well as in biofilms, there is a combination of temperature (above 30°C), availability of oxygen and nutrients (nitrogen) as well as the presence of protozoa, which are ideal conditions for the growth of *Legionella*. Indeed, the dissolved oxygen concentration in activated sludge tanks is between 0.5 to 2.0 mg/L while *Legionella* require between 0.3 mg/L and 2.2 mg/L to grow [12]. Furthermore, stagnant water is also included into the risk factors for the growth of *Legionella* [1].

3.6.1.2 Spread of *Legionella*

Legionella can be spread from a WWTP through the air by aerosols (bioaerosols) formation. Bioaerosols can be formed during different treatment steps like aeration processes, spraying during cleaning activities and via seed sludge processing, especially aerobic sludge.

Concentrations over 10^5-10^6 CFU/L in wastewater resulted in a possibility of high risk of *Legionella* in aerosols. It may not be possible to have zero *Legionella* in WWTP but to reduce the amount under these concentrations would be a potential solution [6].

Bioaerosols are airborne microorganisms and microbial constituents with a diameter from less than 1 µm up to 100 µm. They can be generated at different stages during the process, particularly in those containing moving mechanisms and aeration, such as aeration pond or via effluent [49]. Furthermore, aerosols can be formed during cleaning activities particularly when high pressure spray cleaning is used [48]. In WWTPs, 45% of the bacterial species from bioaerosols were emitted from aeration tanks [50]. Evaluation of six WWTPs to identify

the main bioaerosol sources showed that pre-treatment, biological treatment, and sludge thickening were processes which generated the highest amount of bioaerosols [51]. Settling tanks or decanters did not present a significant bioaerosol source.

These aeration processes are important vectors for *Legionella* as mist is created and it might contain *Legionella*. Indeed, aerosol particles can be large enough to carry *Legionella* bacteria and can be inhaled by humans. *Legionella* can be spread over a distance of more than 12 km [52] [53]. Most of the bacteria-carrying particles in air of WWTP had a diameter below 4.7 µm. Because of their small size, they can easily be inhaled and carried by the wind to large distances [51]. The number of airborne microorganisms increases rapidly with bubble size [51].

There are different types of aeration systems such as bubble aeration, jet aeration, surface aeration to atomization of wastewater itself. Studies on the degree of aerosol formation for these different aeration systems are suggesting the following [8]:

- In surface and point aeration, more aerosols are generally formed than in fine bubble aeration (air diffusion).
- With coarse bubble aeration, more aerosols are generally formed than with fine bubble aeration.
- In jet aeration, fewer aerosols are generally formed than in fine bubble aeration.
- When using pure oxygen, the least aerosols are generally formed.
- Surface aeration devices generate more bioaerosols compared to submerged air diffused aerators.

Results showed that aeration systems involving mechanical agitation of the wastewater, such as horizontal rotors and surface turbines, generated a larger amount of bioaerosols (between 450 and 4580 CFU/m^3) compared to air diffuser aerators (between 22 and 57 CFU/m^3) [51].

The weather has an impact on the dispersion of bioaerosols. Indeed, wind direction and speed, precipitation, atmospheric stability, gravitational settling on natural surfaces, solar radiation, ambient temperature, relative humidity, de- and re-hydration of species, oxygen concentration, concentration of particulate matters and presence of other existing co-pollutants in the atmosphere are responsible for the survival and dispersion of bioaerosols. Indeed, studies showed that bioaerosols can be transported from few meters to thousands of kilometres because of the wind speed, its direction and frequency. RIVM has developed an Operational Priority Substances Model (OPS model), publicly available, to simulate the dispersion of aerosols [54]. Depending on the weather, aerosol can spread horizontally, mainly in low air layers, or vertically to higher air layers. When there is no direct sunlight, for instance during the night or in severe cloudy weather, an increased survival rate has been observed, since evaporation is not favored. It is estimated that the large aerosols (>100 µm) can spread up to about 10 meters and the smaller ones over a distance of several hundred meters [55]. During windless periods, aerosols can accumulate above the aeration basin and are dispersed when wind comes back [6].

Rainfall can reduce the concentration of bioaerosols because of a washout phenomenon. Diurnal changes and season variation also are factors to consider. Indeed, concentration of bioaerosols are usually higher in summer than in winter: 4058.9 GIMC/m^3 in summer compared to 439.7 GIMC/m^3 [56]. Temperature and humidity are higher during summer which are factors for the development of bacteria suggesting a relation to the microbial activity of bioaerosols.

Furthermore, Karra and Katsivela (2007) [57] noticed that there was a decrease in microbial activity between 10 AM and 1 AM and an increase of microbial activity between 1PM and 6 PM which may be linked with temperature and solar radiation.

Odor treatments are used in some WWTPs. A. Ouradou *et al.* (2023) studied the effect of three odor treatment systems on bioaerosol microbial concentration and diversity from wastewater treatment plants. The results showed that after biofiltration and chemical scrubber, there is an increase in *Legionella* spp. concentration in

treated air. This is certainly the consequence of high humidity and reduced competition due to acidic pH. However, this presented a low risk because of the absence of presence of *L. Pneumophilia*. The activated carbon treatment displayed the opposite trend, with a decrease in *Legionella* in treated air [58].

Furthermore, *Legionella* may be present in high concentrations in aerobic sludge. Due to the high organic/ inorganic matter content and the large amount of disturbing flora (other microorganisms), it is difficult to determine the concentration of *Legionella* in the sludge. Anaerobic sludge is less likely to contain high concentrations of *Legionella* due to the anoxic conditions. Aerosol formation can take place during sludge dewatering and thickening processes. Modern processes are often covered. Sludge dewatering processes are often limited but they can represent a risk to employees.

3.6.1.3 Risk assessment

RIVM's research focused on known cases in the Netherlands and on cases described in the literature. Based on the research, a risk assessment has been made based on the following criteria [52] [53]:

- Type of purification: Biological treatment leads to an increased risk. Mainly in aerobic biological treatment plants.
- Type of industry: *Legionella* bacteria make high demands on their nutrition. In addition to organic compounds, which serve as a source of energy and/or carbon, iron compounds and a dozen different amino acids are needed. *Legionella* have therefore mainly been detected in wastewater from industries with a high protein and amino acid content. In concrete, food industry (meat processing and brewery), paper and wood industry, rendering plants and petrochemical plants are mentioned.
- Temperature of the process: The risk is greatest in the interval 30-38 °C, but there is also a chance of development at lower (25-29 °C) and higher temperatures (39-45 °C). Above 55 °C, the bacteria will die. The specified temperatures are to be regarded as indicative. It is further noted that in addition to the temperature of the water during the treatment process, the temperature of the wastewater to be treated and the inoculation of the sludge can also lead to a high initial concentration of *Legionella*.
- Presence of an aeration step: Formation of aerosols.

The reference document for risk assessment in the Netherland is the “Handreiking *Legionella* preventie in AWZI” (Guidance on *Legionella* prevention in WWTP) written by KWR in 2022 [8]. According to this document there are four levels of risk based on the combination of wastewater type, water temperature and presence/absence of aeration process (Table 3-7).

Table 3-7 - Risk of spreading Legionella by biological WWTP [8][53]

Type industry	Temperature (partial) process	Aeration	Airborne dispersion	Effluent dispersion
Foodstuff Paper and wood Petrochemistry Rendering plants Sewage treatment plants*	30- 38 °C	Yes	Very plausible	Very plausible
		No	Plausible	Very plausible
	25- 29 °C or 39- 45 °C	Yes	Plausible	Plausible
		No	Possible	Plausible
	<25 °C or >45 °C	Yes	Possible	Possible
		No	Not plausible	Possible

* In sewage treatment plants without a hot partial flow (<25 °C), a very high concentration of *Legionella* may be (temporarily) present if these installations receive wastewater from industries where the risk of spread is very plausible. The chance of spread is then greater than shown in this table.

In addition to the temperature, acidity is also an important factor for the growth of *Legionella*.
The following ranges apply:

- No growth: <5.0.
- Growth possible: 5.0 - 6.5.
- Optimal growth: 6.5 - 7.5.
- Growth possible: 7.5 - 9.0.
- No growth: > 9.

Based on that table (Table 3-7), four risk profiles can be distinguished in a WWTP for the chance of *Legionella* spread via the air and/or the effluent:

- Not plausible, profile risk 0: *Legionella* are not present or in very low concentration. Very low risk of exposure if no measures been taken to prevent growth and/or spread.
- Possible, profile risk 1: *Legionella* may be present, but no high concentration is expected under standard conditions. Small risk of exposure if no measures been taken to prevent growth and/or spread.
- Plausible, profile risk 2: *Legionella* growth is possible, depending on the situation to high concentrations. Risk of exposure if no measures been taken to prevent growth and/or spread.
- Very plausible, profile risk 3: High to very high concentrations in aeration tanks ($\geq 10^6$ CFU/L) and effluent ($\geq 10^4$ CFU/L). High risk of exposure if no measures been taken to prevent growth and/or spread.

The chance of *Legionella* bacteria growing in the WWTP is based on the type of wastewater (column 1) and the temperature of the wastewater (column 2). Because the temperature of the wastewater can vary, for example per season, due to weather conditions, or due to location-specific conditions, a worst-case approach must be taken when selecting the temperature range.

The presence of aeration in the WWTP (column 3) mainly determines the chance of spreading *Legionella* bacteria in aerosols via the air (column 4). The type of aeration which can play an important role in the risk, should also be considered when determining the risk profile.

The chance of spreading via the effluent (column 5) is also determined by the nature of the wastewater in combination with the temperature (column 1 and column 2).

Based on the type of wastewater and the temperature of the wastewater in the WWTP the chance of spreading *Legionella* from the WWTP is still (very) plausible even though the growth conditions for *Legionella* in the WWTP themselves are unfavorable.

The risk exposure is also related to the distance between the WWTP and the neighbourhood and has been categorized in Table 3-8 (cat. I to III).

Table 3-8. Risk categorisation for WWTPs based on the location of the WWTP [8]

Categories	Location WWTP
I (high risk)	Less than 1.5 km to a residential area or locations where many people come.
II (medium risk)	At a distance of 1.5 to 3 km from a residential area or locations where many people come.
III (low risk)	More than 3 km from a residential area or locations where many people come.

Based on the risk categorisation (wastewater type, process temperature (< 25°C) and presence of aeration) an initial risk profile can be determined for the WWTP.
A monitoring plan for the risk assessment is presented in Figure 3-11, with a step-by-step protocol must be followed. Risk assessment must be conducted every 3 years or earlier if there are significant changes in the composition of WWTP influent or the processes itself [8].

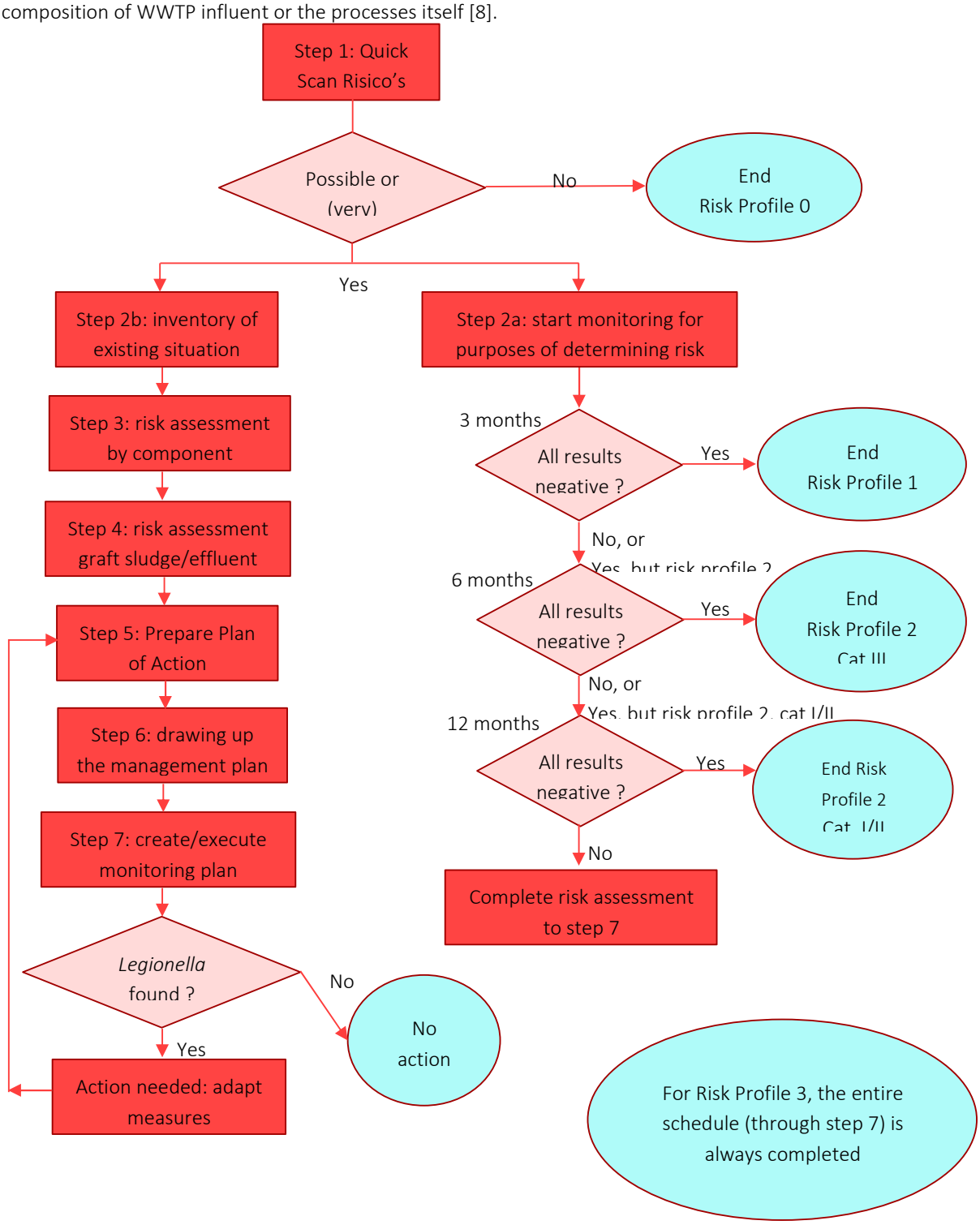


Figure 3-11. Overview of the steps to achieve risk management of Legionella in WWTPs

In Figure 3-11, Step 1 is a quick scan of the risks according to the *table 6* to determine the risk profile. This risk profile leads to a certain monitoring duration that must be maintained in step 2A. Parallel to the monitoring in route 2A, a risk assessment is done according to route 2B. This assessment is described for steps 2B, 3, 4 and 5 in the following paragraphs for the situation that is now designed.

During step 2a, the operator starts a monitoring program parallel to the risk assessment. Sampling is done monthly where aerosols are formed (aerated basins or where effluent is directly atomized).

At the same time, step 2b starts with the inventory of the existing situation considering risky components, operating conditions, and the environment.

Step 3 is the risk assessment of individual components targeted in the previous step, depending on its temperature and pH (Table 3-9). A distinction is made between aerosol-forming components (Table 3-10), components with optimal growth conditions and components with both [8].

Table 3-9. Characterisation of the individual components depending on growth conditions for Legionella

Chance of optimal growth	30-38°C and pH 5-9
Chance of growth	25-30°C or 38-45°C and pH 5-9
No chance of growth	< 25°C or >45°C or pH<5 or pH>9

Table 3-10. Characterisation of the individual components depending on aerosol formation risk

Risk of aerosol formation	Aeration via surface aerators, bubble aeration and jet aerators
No or low risk of aerosol formation	No aeration or aeration with pure oxygen

Risk assessment of sludge, discharge and reuse of effluent during step 4 considers the origin of the sludge and if necessary, presence of *Legionella* in it is tested. If the surplus sludge is reused, the chance that *Legionella* bacteria are introduced elsewhere is examined. The same work is done in case of reuse effluent, for example in cleaning work [8].

Next step is drawing up an action plan for taking corrective and control measures. Possible measures are given in the following parts of this report.

In step 6, a management plan in the long term is drawn up. This is a document that describes the operation of the WWTP and the way in which the risk of growth and spread of *Legionella* becomes composed. It should include the monitoring plan drawn up during step 7. The management plan must be regularly updated [8].

The *Legionella* management plan shall in any case contain [8]:

- A drawing or diagram with the current layout of the WWTP.
- A process description of the WWTP.
- A description of the correct and safe operation of the system.
- The outcome of the *Legionella* risk assessment carried out.
- An overview of the control measures to be taken.
- A description of all checks to be carried out at the WWTP regarding the presence of *Legionella*.
- An indication of the action levels of the physical, chemical, and microbiological parameters including the number of *Legionella* bacteria in the WWTP and/or in the effluent at the time of which improvement measures are taken, and a description of those measures.
- A monitoring plan for the sampling of water (sites, frequency, and method of analysis).
- Should the operator decide to also sample the air (sites, frequency, and method of analysis).

- A description of the measures taken during maintenances cleaning.
- An emergency procedure.
- A matrix (RASCI) that clarifies the responsibilities of relevant officers within the organization.

Last step is the drawing up of a monitoring plan to determine the effectiveness of measures taken.

The relevant parameters to measure are the flows, the temperature, the pH, the amino acids, the proteins, fatty acids, nitrogen and number of *Legionella* bacteria.
The efficiency of corrective measures is confirmed based on monthly sampling at the start. If during four consecutive sampling, no *Legionella* is found in all sample with an increased detection limit that must not exceed 2×10^5 CFU/L, the corrective action is considered as effective. If *Legionella* are detected in one or more of the samplings, it must be evaluated after six months what improvement the corrective measure has brought and whether it is sufficiently effective. If not, the measure must be revised and adjusted, or additional measures must be taken.
The effectiveness of corrective measures aimed at reducing the spread of *Legionella* from the WWTP does not have to be confirmed by sampling if the tank is fully covered with air extraction under the canopy and treatment of that air. However, the control of the effectiveness of the air treatment must be included in the management plan. In case of a not full covering or without air extraction, the effectiveness is determined by air measurements.
The effectiveness of control measures is confirmed by sampling, at the minimum with analysis of the number of *Legionella* in the aerated basin and in the effluent. The frequency of sampling depends on the risk category (Table 3-11).

Table 3-11. Minimum frequency for monitoring Legionella content. Effectiveness of control measures

Categories	Control measures
I (high risk) Less than 1.5 km to a residential area or locations where many people come.	twice a month
II (medium risk) At a distance of 1.5 to 3 km from a residential area or locations where many people come.	every quarter
III (low risk) More than 3 km from a residential area or locations where many people come.	every six months

If during consecutive sampling, all samples do not contain *Legionella*, the frequency can be reduced by two each time [8].

3.6.1.4 Monitoring

The aim of supervision and monitoring is to determine if a company possesses a high-risk WWTP and to see if the company is doing enough to identify and control the risk. The ultimate goal is to prevent the contamination of *Legionella* outside a WWTP, not to completely get rid of it. The operator's aim is to achieve the lowest possible concentration of *Legionella* [8]. Three situations can be identified and depending on it, different measures can be taken. The situations and the following measures are described with more precision in the appendix [8].

3.6.1.5 Risk assessment, management plan and monitoring plan

The risk assessment must be completed by a following a precise methodology. It includes checklists with the most relevant topics to be monitored. The latter can be found in the appendix.

3.7 Control in water

3.7.1 Preventive measures against *Legionella* growth

Measures can be taken to prevent *Legionella* growth by aiming precise source.

3.7.1.1 Sludge

Sludge is usually used in biological purification. It is important to use sludge that does not contain *Legionella*. If it is the case, the source of the sludge must be identified, as well as there might be a risk of infection [6].

Activated sludge is a great medium for the growth of *Legionella*. It is not possible to reduce the growth of the *Legionella* bacteria by reducing the sludge retention time (< 10 days) since it decreases the effectiveness of the nitrification process. Lowering the temperature of wastewater below 20°C can reduce the growth of *Legionella* but this solution might be impossible to implement because of its high energy consumption. An alternative to remove organic nitrogen from industrial wastewater would be to do an anaerobic pre-treatment before mixing the activated sludge with wastewater [59].

To prevent the risk of *Legionella*, the use of surplus sludge, potentially containing the bacteria, in other systems should be stopped [8]. The (dewatered) sludge is usually removed by truck and incinerated at sludge processing plants. Manure injection and the use of wet sludge or sludge concentrates as fertilizers in agriculture should be avoided.
During cleanup activities, sludge splashes can pose a risk to employees.

3.7.1.2 Process, operating conditions

Adjusting the type of purification itself or the operating conditions can have an influence on the performance of the biological treatment that need to be taking into account before the implementation of these measures [8].

3.7.1.2.1 Change the process

As shown previously, *Legionella* need oxygen to grow. Therefore, eliminating aerobic process steps is a way to control *Legionella*. Since sludge is nutrient rich media in presence of oxygen, there are the perfect conditions for the growth of *Legionella*. Therefore, replacing biological purification step by an air stripper to remove ammonia is an interesting alternative [6]. However, these measures are not easy to implement without affecting process efficiency.

From mesophilic to thermophilic fermentation
In the Netherlands, mesophilic digesters are common, and they operate at a temperature between 20°C to 45°C. This range of temperature favours the growth of *Legionella*. Therefore, using thermophilic digesters, which operate at least at 52°C might prevent *Legionella* [6].

Reduction of *Legionella* in nutrients additives
In order to have an effective process, nutrients can be added to adjust nutrient ratios. Since *Legionella* need particular nutrients to grow, by adjusting their ratios, it might be possible to control *Legionella* [6].

3.7.1.2.2 Temperature

Reactors usually operate at a temperature favouring the growth of *Legionella*. If possible, the temperature should be reduced below 25°C or operate at high temperature [6].
To control *Legionella*, it could be interesting to regulate the temperature between the anaerobic phase and the aerobic treatment to keep it <25°C.

3.7.1.2.3 Adjusting the WWTP

Removing pipe parts that are no longer flowing, preventing long downtime of water in basins to avoid accumulation of sludge, growth of plant, moss and biofilms by performing regular maintenance are solutions to prevent *Legionella* growth [8]. Moreover, copper can inhibit *Legionella* growth, so copper pipes can prevent the growth of *Legionella* [60]. In practice, however, this material is not used on a large scale in WWTPs, but only for drinking water networks.

3.7.1.3 Maintenance

3.7.1.3.1 Maintenance of cooling towers

Cleaning procedures are major factors in the spread and the prevention of *Legionella*. Indeed, after emptying the system, all the components in the interior should be meticulously inspected to determine the adapted treatment. The collection tank must be cleaned by removing all the sludge and debris accumulated. The water distribution system and drop separators should be cleaned as well as other accessories. After being cleaned, cooling towers should be washed and filled with clean water and chemicals, among them biocides, should be added. Once all these operations are done, the machine can be started again [61].

3.7.2 Curative measures

3.7.2.1 WWTP disinfection

An extreme measure is to empty the entire WWTP and to disinfect the basins and then restart purification. However, if the process remains the same, the plant is likely to be contaminated again [52].

3.7.2.2 Effluent/Influent treatment

Influent and/or effluent disinfection can be used as a systematic preventive measure (without having detected *Legionella* beforehand) to prevent *Legionella* from entering the WWTP. However, it is mainly a curative measure to kill *Legionella* after detection in the influent or effluent.

The reduction of *Legionella* in the effluent is only important if the effluent itself is used for aerosol forming processes such as cleaning work at the WWTP or as water in cooling tower [8]. However, the treatment of the influent is required if *Legionella* have been detected. This allows to eliminate one of the sources of contamination of the WWTP.

The effluent or/and influent can be disinfected by applying an additional purification step [8].
To reduce the amount of *Legionella* bacteria in the effluent (at the end of the purification) or to bring it below the detection limit, there are several options. Disinfection can be carried out by means of UV treatment, heat treatment, chemical disinfection or filtration (sand filtration or using membranes). P. Muraca *et al.* (1986) compared chorine, heat, ozone and UV light to kill *L. pneumophila* in a hospital water distribution system. These solutions could also be used to treat *Legionella* in the influent and effluent of WWTPs. The efficiency of these four disinfection modalities was compared using the following conditions: chlorine (4 to 6 mg/L), heat (50 to 60°C), ozone (1 to 2 mg/L) and UV light (30 000 µW-s/cm^2). The results showed that all four methods are effective to eradicate *Legionella* even if UV light and heat at 60°C are the faster methods that do not require a lot of maintenance [62]. However, this study was carried out on hospital water, not on wastewater. In practice, heating or using UV disinfection on wastewater can be more complicated and energy-intensive than with clear water.

E.L. Domingue *et al.* (1988) also compared the effects of ozone, hydrogen peroxide and free chlorine on *L. pneumophila*. Ozone was more efficient with more than 99% kill of *Legionella* in 5-min exposure to 0.10 to 0.30 µg of O_3 per mL. To kill 99% of *Legionella*, a concentration of 0.3 and 0.40 µg/mL of free chlorine was required during 30- and 5-min respectively, whereas a 30-min exposure to 1 mg/mL of H_2O_2 was needed [63].

3.7.2.2.1 Membrane filtration

Microfiltration and ultrafiltration are types of membrane filtration that can prevent bacteria and microorganisms from entering the effluent. Indeed, for *E.Coli*, a removal of 4 log was reported with this technique. It might work the same with *Legionella* bacteria. However, the maintenance of the membrane can easily be clogged with such small pores. If it is the case, the effectiveness will decrease [53].

3.7.2.2.2 Sand filtration [52]

It is possible to use a sand filter to remove micro-organisms and produce microbiologically safe water. Calvo-Bado *et al.* (2003) noticed that this method resulted in a decrease of *Legionella* at a laboratory scale. However, it is not advised to use this type of method at an ambient temperature as it can increase the risk of *Legionella* growth in the sand filter. Furthermore, these filters take up a large amount of space which is a significant disadvantage [53].

3.7.2.2.3 UV disinfection

An UV disinfection system transfers electromagnetic energy to an organism's genetic material which destroys the cell's ability to reproduce. The effectiveness of UV disinfection depends on the characteristics of wastewater, the intensity of UV radiation, the exposure time and the reactor configuration. The optimum wavelength to inactivate microorganisms is in the range of 250 to 270 nm [64]. Suspended particles can impact UV disinfection: large particles scatter UV light and shield bacteria inside their compact cores. Bacteria are usually associated with particles larger than 10µm [65]. The effectiveness of UV disinfection decreases when the water contains substances or particles that absorb UV radiation. *Legionella* located in protozoa might be protected by the protozoa and therefore not effectively be removed by this method [53].

Two types of UV disinfection reactor exist where wastewater can flow either perpendicular or parallel to the lamps [64]:

- Contact reactor: UV lamps are submerged protected by quartz sleeves.
- Non-contact reactor: UV lamps are suspended outside a transparent conduit.

Advantages [64]:

- Effective.
- No chemicals.
- User-friendly for operator.
- Fast.
- Requires less space than other methods.

Disadvantages [64]:

- Preventive maintenance program is required to control fouling of tubes.
- Turbidity and TSS can impact the efficiency.
- More expensive than chlorination.

3.7.2.2.4 Ozone treatment

The ozone reaction with COD and inactivation of *Legionella* occurred simultaneously. COD oxidization by O_3 is faster than with *Legionella*. Therefore, higher initial COD concentrations resulted in a lower inactivation rate. Higher temperature led to higher inactivation efficiency [66]. Ozone is a powerful oxidant with a redox potential of 2.07 V. Ozone is highly reactive and has a short life, therefore it cannot be stored as a gas and be transported. Thus, it is always generated on-site [67]. Ozone is usually produced by imposing a high voltage alternating current (6 to 20 kV) across a dielectric gap that contains an oxygen-bearing gas. The electrical discharge method is the most common source of energy to produce ozone. There are mainly 3 mechanisms involved in the ozone disinfection in wastewater:

- Direct oxidation/destruction of the cell wall with leakage of cellular components outside of the cell.
- Reactions with radical by-products from ozone decomposition.

- Damage to the components of the nucleic acids (purines and pyrimidines).

These are mainly caused by the decomposition of ozone in water which releases free radicals hydrogen peroxyl ($\bullet HO_2$) and hydroxyl ($\bullet OH$). After ozone is generated, it is fed into a down-flow contact chamber containing wastewater to be disinfected. The contactor allows the transfer of ozone from the gas bubble into the bulk liquid while making sure that the contact time is sufficient for disinfection. Common contactors are positive pressure injection, negative pressure (Venturi), mechanically agitated and packed tower. Off-gases from the contact chamber must be treated to destroy ozone that remains, in order to avoid its release into the atmosphere. Maintaining an optimal ozone dosage for better efficiency is therefore important.

The key process control parameters are dose, mixing, and contact time. An ozone disinfection system aims to reach the maximum solubility of ozone in wastewater, as disinfection depends on the transfer of ozone to the wastewater. The amount of ozone that will dissolve in wastewater at a constant temperature depends on the partial pressure of the gaseous ozone above the water or in the gas feed stream.

Advantages [67]:

- More effective than chlorine to destroy viruses and bacteria.
- Short contact time (10 to 30 min in general).
- No harmful by-products that need to be removed.
- After ozonation, there is no regrowth of microorganisms, except for those protected by the particulates in the wastewater stream.
- No problems for the shipping and handling since ozone is generated on site.
- Ozonation raises the dissolved oxygen (DO) concentration in the effluent which can eliminate the need to reaeration.

Disadvantages [67]:

- Requires complicated and complex materials, as well as efficient contacting systems.
- Low dosage may not effectively inactivate some viruses, spores and cysts.
- Ozone is very reactive and corrosive, therefore materials that are resistant to corrosion such as stainless steel, are required.
- Not economical for wastewater with high levels of suspended solids, biochemical oxygen demand, chemical oxygen demand or total organic carbon.
- Extremely irritating and possibly toxic.
- Cost can be high, and it is energy-consuming.

The generation of ozone requires a consequent amount of electrical power. Therefore, the maintenance of the facilities is essential to make sure that everything is working correctly.
Compared to other disinfection techniques, ozone is expensive.

3.7.2.2.5 Biocides

Some biocides can be used to disinfect the influent or the effluent [59]. However, the application of biocides in WWTPs is difficult. In fact, biocides may affect the active microorganisms necessary for plant operations (Table 3-12).

Table 3-12. Effect of biocides on Legionella in WWTPs [59]

Biocide	Observation/Comment
Biological Biocide BioMeba	Can not survive in wastewater
Bacteriophage	No supplier found
Clorious2	Negative effect on Anammox bacteria

Silver	No effect on killing *Legionella*
Hydrogen peroxide	No effect on killing *Legionella*
Chlorine dioxide	No effect on killing *Legionella*

Furthermore, the use of chemical disinfectants can lead to the formation of unwanted by-products.

A French start-up, Amobéa, has developed a chemical-free biocide called BIOMEBA, which use the properties of the amoeba *Willaertia magna C2c Maky*. This amoeba is a predator of pathogenic bacteria and other free-living amoebae and helps to control *Legionella* levels in water and biofilms. This biocide is safe for human health and the environment, currently used in cooling tower but which could be applied for other applications [68].

3.7.2.2.6 Control of protozoa

Protozoa are important reservoirs for *Legionella* as they are used as hosts, enhancing the survival of *Legionella*. It has been shown that commonly used biocides (chlorine, bromine and isothiazolinone) are effective against common isolated protozoa in cooling towers if the exposure time is long enough [69]. However, the effectiveness of biocides on protozoa highly relies on the temperature and concentration. The higher the temperature (above 30°C), the higher the time requires for disinfection will be. High temperatures in general reduce the effectiveness of biocides against protozoa. It should be noted that phosphate and molybdate additives that might be used in cooling towers inhibited bromine and isothiazolinone [6].

3.7.2.2.7 Ultrasonic treatment

Dadjour *et al. (2005)* studied the effectiveness of an ultrasonic treatment with TiO_2 to disinfect *L. Pneumophilia* from clean water. During the ultrasonic treatment of aqueous solutions, cavitational bubbles are created. When these bubbles implode, extreme temperatures and pressures are generated at the center of the collapsed bubble which leads to the dissociation of water molecules. Therefore, oxidants such as hydroxyl radicals and hydrogen peroxide are produced and kill bacteria. TiO_2 is a photocatalyst that greatly improves the disinfection process. The results were the following: a disinfection of *Legionella* in the absence of TiO_2 indicated that only 18% of the initial viable cells were killed after 30min of treatment. However, the addition of TiO_2 to the media significantly increased the disinfection process: the concentration of viable cells was reduced to 3% of the initial concentration in the presence of 1.0 g/ml TiO_2 after a 30 min treatment period. TiO_2 also increases the rate of disinfection. TiO_2 is more efficient than Al_2O_3. The disinfection process was not significantly affected by the initial cell concentrations after this period of treatment time as shown in the Table 3-13 [70]:

Table 3-13. Effects of initial concentrations of Legionella on the concentration of viable cells remaining after 30 min of treatment period in the presence of 1.0 g/ml TiO_2

Initial *Legionella* concentration (CFU/mL)	Final relative cell concentration N/No
8.0×10^2	0.002
3.8×10^3	0.031
7.3×10^5	0.004
7.3×10^7	0.011

These results are promising, therefore, it might be possible to apply it in water treatment processes by using a continuous disinfection system [70]. However, the studies have only been carried out on clean water, and more in-depth investigations are needed to assess the effectiveness of the method on wastewater and the feasibility of implementing it in WWTPs.

3.7.2.3 Electrochemical disinfection

Disinfection of water is required in many processes, such as drinking water and cooling towers. Usually, chemical and thermal methods are used to do so. Indeed, ozone and chlorine are the most common chemicals used to disinfect facilities. They have major drawbacks though: dangerous by-products can be produced, the operating costs are high, and they are ineffective on *Legionella*. Thermal treatment is difficult to use for processes with temperature limitations. These are the reasons why electrochemical disinfection can be a great alternative to these methods [71].

G. Pérez *et al.* (2010) [72] studied the effectiveness of electrochemical disinfection of the effluent of a secondary wastewater treatment plant. Boron-doped diamond (BDD) electrodes were used to remove pathogens, especially *E. coli*. BDD electrodes have many advantages such as a high overpotential for water oxidation. Therefore, many hydroxyl radicals are electro-generated, which may be responsible for production of strong oxidants (chlorine, ozone, persulfates and hydrogen peroxide). The results were promising as total inactivation of *E. coli* was reached with a current density of 120 mA/cm². However, it is not known if electrochemical disinfection could be this effective with *Legionella* disinfection in secondary stage in WWTPs.

However, Y. Delaedt *et al.* (2008) [73] studied the impact of electrochemical disinfection on *E. coli* and *Legionella Pneumophila* (10^4 CFU/mL for both) in tap water by using an electrolysis cell. It was shown that suspensions of *E. coli* were completely disinfected by electrolysis. Concerning *Legionella*, the results were not as good as there was still *Legionella* present. Furthermore, the study was lead on planktonic bacteria (free-living bacteria), but biofilms represent important sources of nutrients for *Legionella* which can therefore survive in those biofilms. *Legionella* are also able to use amoebae as hosts. Further research on the effect of electrochemical disinfection on biofilms and amoebae must be carried out to see if it is possible to avoid the possibility of *Legionella* recolonizations.

3.8 Control in air

3.8.1 Preventive measures

3.8.1.1 Type of aeration

Aeration is the process of adding air into wastewater so that microorganisms can use it to biodegrade organic materials into water and carbon dioxide. Splashing and bubble bursting that occur as a result of forced aeration in activated sludge processes are known to produce large amount of bioaerosols. The number of airborne microorganisms increases rapidly with bubble size so that the type of the aeration system greatly influences the production of aerosols.

There are different types of aeration used in activated sludge process [74] [75]:

3.8.1.1.1 Diffused Air Aeration

Compressed air is pumped through diffusers and it releases bubbles below the surface of the wastewater: the operation is therefore vertical. While these water bubbles rise to the surface, they mix with the wastewater which increases the amount of dissolved oxygen.
There are 3 types of diffused aeration systems [76] [75]:

- Fine bubble diffusers which are made of flexible membranes containing thousands of holes and produce bubbles from 1 to 4 mm in diameter. Tube diffusers have a bigger surface area. therefore, they release more bubbles per diffuser. Disc diffusers are the most used systems as they are attached to the floor of the tank. They are low-maintenance and last longer. The needle-perforated panels do not flex, and they are less efficient with deeper tanks. They also operate at high backpressure and head loss [75].

- Medium bubble diffusers which are made of perforated pipes, domes or slotted aerators and produce bubbles from 4 to 10 mm.

- Coarse bubble diffusers move a bigger quantity of water as they produce larger bubbles which enables stronger mixing. They are less sensible to fouling because they have larger pores. When wastewater has a lot of suspended solids, it is more interesting to use this type of system.

Diffused aeration systems have a lot of advantages. Indeed, they are low maintenance, durable and therefore have a low environmental impact. They are suitable for deeper tanks and a WWTP can customize the size of its diffused aeration system. They are safer and more reliable in extreme weather than mechanical aeration systems. Furthermore, they provide better surface aeration oxygen transfers and a more homogeneous mixing from bottom to top. However, they still have some flaws such as a higher capital cost. Furthermore, they are more complex and are therefore not portable. They are more sensitive to fouling because of their small pores and they are less effective in harsh environments.

3.8.1.1.2 Mechanical Aeration

Rapidly rotating shafts and propeller bladers are used which allow stronger localized mixing and higher localized dissolved oxygen concentration. The operation is usually horizontal. The 3 common types of mechanical aeration systems are the following [77]:

- Horizontal aspirators which have propellers that turn horizontally.
- Vertical aerators which have propellers that turn vertically.
- Jet aerators are a combination of horizontal and vertical propellers with injected air for a more effective aeration.

Mechanical aerations have a strong mixing and oxygen transfer. They require simple equipment, therefore, their capital costs are low, and they are portable. They have greater efficacy in very shallow tanks and in harsh environments. However, they also have some cons such as the fact that the mixing and the oxygen transfer are localized. Furthermore, they need more maintenance, have a higher operation cost and environmental impact compared to diffused air aeration systems. The aeration provided is less effective and they are not effective for very deep tanks. It is important to keep in my mind that they are sensible to weather: they become icy in cold situations and can stop working in extreme weather conditions. Surface aeration caused by mechanical aeration can have unpleasant smells. Finally, they are less safe as electrical wires in the wastewater can lead to dangerous situations [77][75].

3.8.1.1.3 Combined Aerator

It is a system where diffused air aeration and mechanical aeration are combined. An example of this system is the Dorroco aerator [78].

The following measures can be adopted:

- Adapting the type of aeration process with air [8]: fine bubble aeration leads to less aerosol formation than aeration with larger bubbles [52].
- Switching to a type of aeration with pure oxygen [8]: a smaller amount of aeration is needed for the same amount of oxygen [52].
- In aeration system in which the wastewater is mechanically moved, such as horizontal rotors and surface turbines, more aerosols are formed than in systems which use air diffusion [52].
- Using substitute surface aerators with diffusers placed at the bottom of activated sludge tanks is recommended to minimize the risk of infection [59].

3.8.1.2 Tank

It may be possible to limit aerosol dispersion into the environment by raising the basin edge, for example with tarpaulin, cloth or other means [8].

3.8.1.3 Covering

By covering the aeration basin, the spread of *Legionella* via aerosols can be prevented or limited [8] [52]. Different types of covers exist, including construction made of hard material such as fiberglass or steel, these are permanent covers. Less permanent options are made of more flexible materials such as [8] [52]:

3.8.1.3.1 Sails

Applying a sail above the aeration basins is effective if the construction is well covered and no parts remain open during aeration [8] [52]. The air released during the process must be removed in a controlled manner and free of aerosols, for instance by placing a filter [52].

3.8.1.3.2 Tent

A tent construction can also be placed above the aeration basin, but it is often difficult to seal hermetically because there are still cracks and openings affecting the efficiency [8] [52].

3.8.1.3.3 Closed systems

Placing the aeration basin in a housing [8] or concrete constructions are also possible [52].

3.8.1.3.4 Floating covers

Various floating covers are available such as hexacovers, hexaballs, hollow balls of various materials and balls half filled with water. When choosing floating covers, it is important that they continue to cover the entire aeration basin when the aeration is fully operational [52].
Hsueh Fen Hung *et al.* (2010) [79] mentioned that floating balls were effective in controlling the generation of bacteria (*E. coli*) droplets from bubbling in aeration process. An efficiency of 91% was reached by using a single layer of balls with a diameter of 1.9 cm.
Floating cover also provides more surface for biofilm formation [8].

The effectiveness increases if the air is extracted under a cover. Moreover, the air must also be treated to remove the aerosols and/or kill *Legionella* bacteria. If the air is treated, it may be useful to first dehumidify the air with cyclones, rotary scrubbers, fog filters, demisters or by condensation via cooling. For example, UV disinfection, air filters and air scrubbers can be used for air.

The efficiency of these technologies is dependent on the composition of the effluent and its physical parameters. In each situation, the most suitable technology must be studied by an expert [8]. A cover can lead to an increase in temperature which can affect efficiency. Moreover, maintenance and economic arguments play a role in the choice of the cover [52].

3.8.1.4 Air treatment

To treat the air, the simplest thing to do is to make openings in the cover and fill them with filter mats. Because of *Legionella*'s shape and size, filter mats respecting ISO 16890 class ePM1 stop a large part of the bacteria. It is also possible to use activated carbon filters to capture *Legionella* bacteria.
A more effective method is to extract the air and to treat it. To do prevent the escape of aerosols, a slight negative pressure can be created [6]. To treat the air, it is possible to use:

- UV disinfection.
- Air filters.
- Pasteurization.
- Air scrubbers: no experience is known in relation to *Legionella* prevention. Furthermore, misting can take place in air scrubbers which can lead to *Legionella* outbreaks.

3.8.1.5 Dehumidification

To prevent emissions to the environment, it is effective to purify the air and then dehumidify and/or treat it. However, air treatment methods are much more effective at low humidity. This is the reason why it is strongly recommended to dehumidify at least part of the air [6]. To do so, many techniques are available such as:

3.8.1.5.1 Cyclone

A cyclone uses centrifugal force. The air flows through a cylindrical chamber and it will have a rotational motion. Because of this phenomenon, the heavier particles are pushed towards the walls and discharged. This technique is effective for particles which have a size higher than 5 µm. Indeed, above 10 µm, the efficiency increases and between 6 and 10 µm, the efficiency is around 50% [6].

3.8.1.5.2 Spray tower/Rotary washer

Spray towers and rotary washers also use centrifugal force but coupled with the rotating atomization of washing water. Thanks to these processes, particles are dragged to the wall of the scrubber. Furthermore, a droplet separator increases the dehumidification phenomenon. The efficiency is between 70 and 99% for particles that have a size below 10 µm [6].

3.8.1.5.3 Fog filter

Fog filters are based on the principle of mechanical capture. The efficiency of the process depends on the speed of particles at which they pass through the filter. It can go up to 99%. The smaller the mesh size is, the more efficient it is for small particles, ranging from 1 to 3 µm. However, the smaller the mesh size is, the bigger the risk of clogging is. Fog filters can be used in cooling towers to prevent aerosol spreading. Thanks to this technique, up to 98% of aerosols are captured. A high velocity air is essential. Nevertheless, it is not suitable as a single barrier measure [6].

3.8.1.5.4 Condensation

Condensation appears when air flows on a cold surface for a long time. Condensation is collected and discharged. It is possible to apply nitrogen cooling to cool the surface and control the amount of condensation [6].

3.8.1.6 New WWTPs

New WWTPs design must incorporate growth- and spread-control measures in line with the risk to which the plant is exposed (*Tables 6 and 7*).

If the new WWTP has a risk profile 2 and is built less than 3 km from a residential core or with a risk profile 3, the following precautions should be taken during the system selection and design phase:

- Positioning: Choose a position on the site for the aerosol-forming components that are as far away from residential areas as possible.
- System choice: do not consider applying processes with conditions that promote the growth of *Legionella* bacteria.
- System selection: consider pretreatment of influent streams that may be highly loaded with *Legionella* bacteria.
- Design: choose a type of aeration that involves less aerosol formation, consider a raised basin edge so that wind can less easily carry the aerosols formed in the basin into the environment, consider covering the aeration basins or at least include preparations in the design that will simplify and accelerate the realization of covering in the long term. Furthermore, pipe work should be kept as short and as direct as possible. The materials used should not encourage the growth of *Legionella*. Pipes and tanks should be adequately insulated [80].

3.9 Measures in case of presence of *Legionella*

3.9.1 Action level

Action level has been established (Table 3-14). This associates the approach to follow as a function of the category of WWTP and the level of *Legionella* detected [8].

Table 3-14. Action level depending on risk category and Legionella concentration

Action Level *Legionella* (CFU/L)	Approach
Category I/II < 10 000 Category III < 100 000	No or little presence or growth of *Legionella* Values acceptable, no further action other than regular control measures. If *Legionella* is found in low numbers in the effluent (range 10 000 – 100 000), reuse of the effluent with nebulization (without additional purification) must be avoided.
Category I/II 10 000 – 100 000 Category III 100 000 – 1 000 000	Presence or growth of *Legionella* The system forms a biotope for the presence or growth of *Legionella* and deserves attention. 1. Initiate the following actions: o Do not (re)use effluent in such a way that nebulization can occur. o Check the WWTP for deviations or changes gene in business operations. o Evaluate existing management measures for implementation in practice and for effectiveness. Formulate and implement improvements. o Deploy as needed simple corrective measures to be carried out. o If work is required in the mist of the infected systems, the preventive wearing of respiratory protection (FFP3) is strongly recommended. After implementation of the measures, have the water analyzed for *Legionella* to check the effectiveness of the measure taken.
Category I/II 100 000 – 1 000 000 Category III 1 000 000 – 10 000 000	High presence or strong growth of *Legionella* The system forms a good biotope for the presence or growth of *Legionella* and deserves special attention. 1. In the follow-up approach (and the severity of the follow-up actions), consider whether it can be concluded on the basis of the available dataset that only *L. pneumophila* is detected in the WWTP. 2. Initiate the following actions: o Do not (re)use effluent in such a way that nebulization can occur. o If possible, reduce aerosol emissions from aerosol-forming components. o Evaluate existing management measures for implementation in practice and for effectiveness. Formulate and implement improvements. o Have an experienced expert carry out research into the growth and spread of *Legionella* fitting to the system. Aim for targeted corrective measures aimed at reducing *Legionella* in aqueous phase and dispersion via aerosols, effluent or sludge. Record the measures in the logbook. o If worked in the mist of the infected systems, the preventive wearing of respiratory protection (FFP3) is necessary. 3. After implementation of the measures, have the water analyzed for *Legionella* to check the effectiveness of the measures taken.
Category I/II > 1 000 000 Category III > 10 000 000	Very high presence or exponential growth of *Legionella* The system is heavily contaminated with *Legionella* and requires immediate attention or there is an unusual occurrence which must be reported. 1. In consultation with an experienced expert, consider having an air sampling carried out as an indication of the degree of spread to the environment. 2. Take immediate action (i.e. not only after confirmation) to spread *Legionella* road "stop" aerosols, effluent or sludge. If this takes too long or is not possible, reducing

measures must be implemented or the source of infection must be covered around, and the surrounding area must be moved away from the source of infection.

3. Immediately initiate the following actions:

- Start an emergency procedure.
- In the follow-up approach (and the severity of the follow-up actions), consider whether it can be concluded on the basis of the available dataset that only *L. pneumophila* is detected in the WWTP.
- Do not (re)use effluent in such a way that nebulization can occur.
- Evaluate existing management measures for implementation in practice and for effectiveness. Formulate and implement improvements.
- Have an experienced expert carry out research into the cause, growth and spread of *Legionella* and into system adaptation. Aim for targeted corrective measures aimed at reducing *Legionella* in water phase and spreading via aerosols, effluent or sludge. Record the measures in the logbook.
- If work is required in the mist of the infected systems, the preventive wearing of respiratory protection (FFP3) is necessary.

After implementation of the measures, have the water analyzed on *Legionella* to check the effectiveness of the measure taken.

3.9.2 Inform and report

The Municipal Health Service (MHS) must be informed if *Legionella* are detected in the WWTP. In addition to offering the possibility of creating a database, this is useful for early source detection by the MHS in the case of solitary cases of legionellosis or small clusters in the environment (preventive effect) [8].
If a sample contains more than 1000000 CFU/L *Legionella*, there is an unusual occurrence in accordance with the Environmental Management Act article 17.2, a report must be made to the Environmental Service as soon as possible. Moreover, the direct users of the effluent or recipients must be informed. To assess the health risks estimating a core expert (occupational hygienist, safety expert) can be called in [8].

3.9.3 Personal protection

For work in WWTPs in which *Legionella* have been found in concentration above 10000 CFU/L, employees must wear respiratory protection. These should be masks of class FFP3 complying with the EN149 standard [8].

3.10 Discussion

The TKI *Legionella* project aims to gather and share information about the control of *Legionella* in WWTPs since knowledge about *Legionella* management and behaviour in WWTPs is very limited. This literature review confirms that available information on *Legionella* control in wastewater treatment plants is very limited. Indeed, there are still knowledge gaps related to the growth of *Legionella* in wastewater, especially in relation to protozoa and the relationship with other microorganisms present in the different biological processes, such as in anaerobic sludge. The spread of *Legionella* also represents a source of questions as the propagation of the bacteria via aerosols depending on the aerators systems and the link between the concentration of *Legionella* in the aqueous phase and in the air are vague. There are still questions remaining concerning air samples and detection methods which make it even hard to monitor *Legionella* and therefore to control it. All the reasons listed above have made the redaction of this report difficult.

Besides, even though there are currently regulations about *Legionella*, they are mostly about drinking water with the purpose of ensuring public health safety. Therefore, measures suggested in governmental documents are mainly oriented to reduce the spread of diseases and not focused on the control of *Legionella* at its source. Furthermore, due to the lack of official regulations concerning *Legionella* in WWTPs, the monitoring of

Legionella concentrations is not controlled as much as it should be and there are only few measures that can be implemented to reduce the growth of *Legionella* and reduce the spread in WWTPs. However, this situation should change in near future as stricter regulations will be adopted in the Netherlands.

Nevertheless, there are still measures that can be implemented in WWTPs and most of them are focused on preventing the spread of *Legionella*. It is challenging to reduce *Legionella* ssp. growth while keeping good balance of the biological processes. Indeed, there are different serogroups of *Legionella* with distinct pathogenicity/resistance. For instance, *L. pneumophila* which is the main target of this report, can survive through extreme conditions thanks to its dormant form called VBNC. They can also be protected by their hosts which make their removal even more difficult.

Since there is no selective (and effective) way to kill *Legionella ssp.*, one of the control actions is to act on the main parameters favouring the growth of *Legionella*. Therefore, reducing the temperature of processes in WWTPs can provide the biggest impact (most effective) on *Legionella* control according to all interviewed experts (partners) and the literature available. The use of a heat exchanger to reduce the temperature of the water stream before entering the aeration process would reduce the growth of *Legionella* and the risk to spread. However, this represents a significant investment for the WWTPs and the effect on both, the presence of *Legionella* (growth control) and the efficiency of the biological process is not fully guaranteed.

Other point of action is to cover the aeration tanks, where the risk of aerosols is particularly high, to limit the spread of the bacteria. A treatment of the air under covers will also be needed but there is little knowledge about this topic. The cover of tanks has already been implemented for odour control strategies. In this specific application, air under the cover is further treated with biofilters. However, in the case of *Legionella* different measures should be taken to ensure the spread like the application of ISO filters. Some partners have expressed their concerns respect to this measure as it reduces their visual control on the process. Furthermore, it might enhance the growth of *Legionella* with the formation of biofilms because of the combination of a greater surface area and the increase of temperature.

Another type of action to undertake is the treatment of the influent or the effluent. Indeed, it is possible to disinfect the influent to avoid the entrance of *Legionella* into the WWTP. However, there are many introduction paths to the system which make the task difficult to accomplish. Moreover, disinfecting the effluent is a potential solution to avoid the spread to the water body. Chlorinated based biocides are already applied in industry to ensure a controlled biological content of effluents before discharge. In addition, monitoring the influent could be an interesting step to control *Legionella* in WWTPs as it could be possible to trace back the source of *Legionella*. Therefore, official measures could be taken by Waterboard to force the company in question to disinfect the water before it is discharged in the sewer system.

Besides the above-mentioned action points, the selection of aerators for oxygen supply in the aeration tank should be taking into account when designing WWTPs. Fine bubbles aerators produce smaller bubbles than coarse bubbles aerators, therefore limiting the aerosols. Using pure oxygen could also be interesting since a smaller quantity of gas would be needed to reach the same amount of oxygen in the aeration basin. Furthermore, since pure oxygen is more expensive, it is typically applied in such a way that it does not escape into the air, thus largely preventing aerosol formation and dispersion.

Nevertheless, to reduce the knowledge gaps remaining, further research should be carried out to test the concrete impact of all the techniques already mentioned. These tests are nonetheless complicated to design. Laboratory tests/pilot tests can be done but it is hard to ensure their representativeness. Experiments on-site will also be needed but as each WWTP is different and detection methods are not completely viable for the wastewater matrix, it remains complicated.

3.11 Recommendations

As mentioned in the previous section the practical knowledge of presence of *Legionella* in WWTP is rather limited and data is typically inconclusive as to the reasons why, in particular cases, *Legionella* thrives. Monitoring of *Legionella* is only performed in specific cases and most cases known are related to industrial wastewater treatment facilities that operate at relative high temperatures. New regulation, possible from next year onwards, will make the monitoring of *Legionella* a compulsory task for WWTP operators. This in turn will give much more insight into the size of the problem. This also opens up possibilities to gain better insight into the risk factors in WWTP operation.

Currently, the potential measures that could be taken to prevent the occurrence and spread of *Legionella* are maximization of the operation temperature, covering of the aeration tank including treatment of the vent air and the use of an aeration system that limits aerosol formation. Even for these measures, their effectiveness is hard to determine accurately. Therefore, we recommend combining results of large-scale monitoring with specific details of the WWTP, in order to be able to determine their effect. Individual WWTP studies may be a starting point, however for generalization they are expected to be insufficient.

Even if such experiments are difficult to design, it is still possible to imagine some tests to measure the efficiency of each measure cited. We can divide these dispositions into three categories: control of *Legionella* growth, entrance prevention of *Legionella* and limitation of the spread.

By using heat exchangers before the aerobic treatment, the temperature of the influent can be reduced. In order to evaluate the impact of this parameter, samples could be taken to detect the presence of *Legionella* and to check that the modifications made still enable the process to achieve its treatment objectives.

Similarly, the effect of pH on the regulation of *Legionella* growth and on the process itself can be evaluated. According to Sabine Kreps, expert on *Legionella* at VITO (Belgium), a low pH would not be efficient to kill *Legionella*. Therefore, the pH of the influent could be increased. However, limitations of pH range for a good biological process performance should be considered.

With the same goal of limiting *Legionella* growth inside WWTPs, the study of iron impact could be investigated, especially the effect of Fe based coagulants on *Legionella* development since a high concentration of iron inhibits the growth of *Legionella*.

Moreover, lab experiments could be done to understand the relationship between *Legionella* concentration in wastewater and the amount in the aerosol. The number of bacteria in the droplets formed from simulated aeration tanks containing different concentrations of *Legionella* could be investigated.

The disinfection of the influent can be achieved with biocides to prevent the entry of *Legionella* in the WWTP. However, the type of biocide and the concentration must be carefully selected to ensure that the chemical has disappeared by the time it enters the process. For instance, chlorine biocide evaporates quickly and therefore does not interfere with biological treatment. Research could be conducted to evaluate the amount required for different types of chemicals such as chlorine- and bromine-based biocides. New biocides with a lower environmental impact could be tested.

The spread of aerosols mainly takes place around the aeration basin. Depending on the type of aeration, the aerosol formation is more or less important. That is why the performance of different aerators from several suppliers could be tested by evaluating the amount of aerosol formed, their size and their dispersal potential. The impact of the use of pure oxygen could also be studied. Moreover, to avoid the aerosol spread, covers can be used. Therefore, the efficiency of different types of covering could be analyzed by measuring the amount of

aerosol around the basin. During these experiments, the temperature and the formation of biofilm should be measured. In addition, water samples could be analyzed to study the impact of covering on *Legionella* growth. To estimate the dispersion of bioaerosols, air samples could be taken around the WWTP, at predefined distances. The air under the cover should be treated, therefore the technologies used for this purpose should be studied. All these tests could be done by monitoring existing plants or by designing specific experiments. However, because the dispersion of bioaerosol is closely linked to weather conditions, it would be interesting to do all these measures under different conditions: ambient temperature, air humidity, wind, light radiation…etc.

Finally, the type of process, such as MBR, related to the life form of *Legionella* and the association to biomass (sludge) that could influence *Legionella* growth and spread could be studied.

Since it is hard to get rid of *Legionella* in WWTPs without affecting the process, the most important is to avoid its entrance and prevent it by regular cleaning and monitoring. Even if no problem concerning *Legionella* has been identified, every WWTP should do a risk assessment and set up a plan of action to react quickly and correctly in case of occurrence.

3.12 Prioritizing further experiments

In order to have a clear insight on further experiments, a ranking list of experiments that could be done by priority can be found below:

1. Impact of temperature on the control of *Legionella*
2. Impact of the different types of aerators
3. Study of efficiency of covers
4. Air treatment under the covers
5. Impact of pure dioxygen in aerosol formation
6. Relationship between *Legionella* concentration in wastewater and the amount of it in aerosols
7. Relationship between the type of process and the life form of *Legionella*
8. Impact of pH on the control of *Legionella*
9. Disinfection of the effluent with biocides, UV systems and oxidation with ozone
10. Impact of the weather on the spread of aerosols
11. Impact of iron concentration on the control of *Legionella*

3.13 Conclusion

Legionella represents a threat to public safety as it can cause diseases, among them Legionnaire's disease being the most dangerous one. WWTPs, especially the biological ones, offer optimal conditions for the growth of this bacteria which endangers both workers and the population spreading through the water or aerosols within the WWTP and even outside.

There are still knowledge gaps about the growth and spread of *Legionella* in WWTPs which make it difficult to design a control strategic plan to act in case of *Legionella* presence.

Monitoring of *Legionella* in WWTPs is not well controlled due to the lack of official regulations. Indeed, in case of *Legionella* occurrence, taking measures as soon as possible should be mandatory.

To limit the growth of *Legionella*, it is possible to act on some parameters of the process, such as temperature. However, it might not be possible to do that without negatively affecting the effectiveness of the processes. In

that case, limiting the spread of *Legionella* should be mandatory. The selection of aerator system in the aeration tank, the use of covers and the effluent disinfection are measured which should be considered.

The concrete impact of these measures on WWTPs remains vague. Further research, especially experimental research, is required to fill these gaps. However, it is important to keep in mind that getting rid of *Legionella* is extremely difficult because of its resistance. Therefore, preventing *Legionella* is the best control strategy. It is advised to regularly monitor the concentration and to maintain and clean the WWTP to avoid the formation of biofilms and sediment accumulation. Every WWTP should also do a risk assessment and set up a plan of action in prevention.

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4 Phase 2: Sample pre-treatment effect on *L. pneumophila* alternative detection methods

4.1 Summary

In the last decade, biological wastewater treatment plants (WWTPs) have been identified as one of the potential sources for *Legionella pneumophila* outbreaks. For proper monitoring and control, a reliable *L. pneumophila* detection method is essential. At this moment, the standard approach for *L. pneumophila* quantification in wastewater samples is culture-based (ISO 11731). However, due to the long analysis time (7-10 days) and a high detection limit ($1x10^5 – 1x10^6$ CFU/L), the method might extend the response time by the plant operators, making *L. pneumpohila* risk management difficult.

This study is part of Phase 2 of the TKI project "Reliable detection and control of *Legionella pneumophila* in wastewater" and its aim was to further test the four alternative L. pneumophila detection methods (Legiolert from IDEXX, MICA Advance from Diamidex, rqmicro.COUNT from rq.micro and QIAcuity dPCR from QIAGEN) that have been previously studied in Phase 1, with a focus on optimisation of the wastewater samples pre-treatment strategies. The effect of sonication, centrifugation, heat treatment and two different diluents (phosphate buffer saline (PBS) and Ringer solution) were tested in different combinations for different *L. pneumophila* detection methods to evaluate whether they improve the methods performance. Legiolert was excluded from the pre-treatment experiments as it already had an optimised pre-treatment protocol dedicated to wastewater samples that was used as a reference. Legiolert and ISO analyses were performed in this study as additional *L. pneumophila* quantification data points.

The wastewater samples in this study came from two industrial WWTPs, a pig slaughterhouse facility (SH) and a paper mill (PM), both sampled at the aeration tanks. They represented varied characteristics, with the SH wastewater having higher content of solids and organic matter. All the methods, except for rqmicro.COUNT (which showed the concentrations to be comparable in both), showed significantly higher concentrations of *L. pneumophila* in the PM wastewater. Moreover, the *L. pneumophila* concentrations detected by Legiolert, MICA Advance and QIAcuity dPCR were lower than those measured with the standard ISO 11731 method. The SH samples were more challenging to analyse by MICA Advance and QIAcuity dPCR than the PM samples, either due to their higher solids and organic matter content, and/or *L. pneumophila* numbers being below their detection limits.

The pre-treatments had a varying effect on different detection methods, with centrifugation being the only one having a significant influence on MICA Advance and QIAcuity dPCR. In MICA Advance, application of centrifugation reduced microbial overgrowth on the filtration membranes, however, the effect on the *L. pneumophila* quantification using this method was unclear. In QIAcuity dPCR it lowered the number of *L. pneumophila* DNA copies, but also decreased the interference potentially caused by compounds from wastewater remaining after DNA extraction.

rqmicro.COUNT results showed no difference between the pre-treatments, however, in these measurements the blanks tested positive, indicating potential issues with off-targeting or autofluorescence signal coming from the compounds present in tap water used for dilutions and/or wastewater.

Overall, detection and quantification of *L. pneumophila* in a complex matrix such as industrial wastewater, remains challenging due to the presence of interfering microorganisms and contaminants. The selected pre-treatment

steps did not show improvement in the detection methods effectiveness, however, their application helped in identifying other points for potential methods optimisation.

4.2 Introduction

The proliferation of *L. pneumophila* in (industrial) wastewater treatment plants (WWTPs), especially in nutrient-rich biological plants, has been recognized as a significant potential source of outbreaks (Pijnacker et al., 2024; van den Berg et al., 2023; Vermeulen et al., 2021). The spread of *L. pneumophila* from WWTPs poses a considerable concern for plant workers and nearby communities due to the formation of aerosols during the treatment process (Pijnacker et al., 2024; Vermeulen et al., 2021, 2019). Aerosols formed above aeration units may contain up to 3300 CFU/m³ of *L. pneumophila* and are capable of traveling several kilometers, depending on the weather conditions, and have been shown to cause infections far from their sources (Blatny et al., 2008).

A significant challenge in addressing *L. pneumophila* growth in WWTPs is the lack of effective and rapid detection methods. Currently, the most widely used and standard method for detecting *Legionella* is the ISO 11731 plate culturing method (ISO, 2017). However, this method has a lengthy incubation period of 7 to 10 days, which delays timely intervention in cases of elevated risk. Additionally, applying the ISO method to highly contaminated water samples, such as sewage water or industrial wastewater, presents challenges due to the overgrowth of the plates by other microorganisms (Heining et al., 2023; van den Berg et al., 2023). As a result, no reliable and fast detection methods currently exist for assessing *L. pneumophila* in wastewater matrices, underscoring the urgent need for improved techniques to facilitate effective monitoring and risk mitigation.

Given the importance of proper risk assessment and management in controlling *L. pneumophila* proliferation and mitigating associated health risks, the TKI program "Reliable detection and control of *Legionella pneumophila* in wastewater" aims to evaluate the reliability of alternative detection methods, optimize them where possible, and explore a potential control strategy for *L. pneumophila* in (industrial) WWTPs. The project consortium comprises knowledge institutions (CEW and KWR Water), consultancy companies, technology providers, and end users. The project consists of three phases with a total timespan of 3,5 years.

The first phase reported separately by KWR focused on testing alternative, faster detection methods for *Legionella* in wastewater samples compared to the "standard" plate culturing method ISO 11731. Four detection methods were selected and provided by the technology providing project partners for further testing and optimization for challenging wastewater matrices. Currently, these detection methods are only validated for *L. pneumophila* detection in "clean" waters, such as potable water samples from taps and non-potable water samples from cooling towers, but not for non-potable (waste)water samples. The four alternative methods represent various technologies, including improved culture-based methods, fluorescence technology, flow cytometry, and digital PCR (dPCR).

In the second phase, potential *L. pneumophila* control strategies and a follow-up optimization of *Legionella* detection methods were studied and evaluated. Industrial and municipal wastewater samples have a complex matrix with often a high microbiological background. Therefore, as a part of this study, multiple wastewater pre-treatment methods were considered and tested to improve the selected alternative detection methods. The results of this phase are reported here.

4.2.1 Goal

The goal of this study was to further test the four selected *L. pneumophila* detection methods and understand whether the additional pre-treatment of the wastewater samples is necessary for better performance of these methods on industrial wastewater.

4.3 Background - detection methods

The four alternative *L. pneumophila* detection methods: Legiolert (IDEXX), MICA Advance (Diamidex), rqmicro.COUNT (rq.micro) and QIAcuity dPCR (QIAGEN) are described briefly in this chapter. A comparison is made to the standard method ISO 11731.

4.3.1 ISO 11731

The ISO 11731 standard is a plate culture-based method for the enumeration of *L. pneumophila* species in both potable and non-potable water samples and is currently approved for use in wastewater matrices (ISO, 2017). This standard contains three methods: a method for samples with a low amount of other microorganisms, e.g., potable water (matrix A), a method for samples with a higher amount of microorganisms, e.g., cooling water (matrix B), and a method for samples with a very high microbiological background, such as sewage water (matrix C). For potable water samples, the method typically begins with membrane filtration to concentrate *L. pneumophila* cells before culturing. For non-potable water samples, if the expected *L. pneumophila* concentration exceeds $1x10^4$ CFU/L, direct plating is permitted. In cases of heavy microbial contamination, the sample can be diluted before plating. To reduce interference from non-target organisms in wastewater samples, pre-treatment steps such as heat or acid treatment may be applied (Toplitsch et al., 2021). These pre-treatments exploit the relative resistance of *L. pneumophila* to brief thermal or acidic conditions. Heat treatment typically involves exposing the sample to 50 °C for 30 minutes, while acid treatment involves exposure to pH 2.2 for 5 minutes.

The detection process involves culturing *L. pneumophila* on buffered charcoal yeast extract (BCYE) agar plates for 7 to 14 days. BCYE agar contains essential growth supplements such as L-cysteine and iron(III) salts, which are critical for the proliferation of *Legionella pneumophila* (Toplitsch et al., 2021). In addition to BCYE, selective media such as GVPC agar, which contains glycine, vancomycin, polymyxin B, and cycloheximide, may be used to suppress background microflora (Scaturro et al., 2020b). The use of GVPC is particularly advantageous when analyzing wastewater, which typically contains a high concentration of interfering organisms. Incubation is conducted at 36 ± 2 °C for 7 to 10 days.

Despite its widespread use, the ISO 11731 method has several limitations when applied to wastewater:

- Viable but non-culturable (VBNC) *L. pneumophila*. Cells that have entered a dormant state due to environmental stress may remain viable and infectious but are not detectable using culture-based methods (Heining et al., 2023; Wickenberg et al., 2022).
- Interference from other microorganisms in heavily contaminated wastewater samples, which can overgrow the plates and conceal the growth of *L. pneumophila*.
- Infections might be missed due to a relatively high detection level of $1x10^4$ CFU/L (van den Berg et al., 2023).
- Time-consuming and laborious analysis. The required 7 to 10 days incubation period (including confirmation of suspected *L. pneumophila* colonies) significantly delays response times, which is critical when monitoring for *L. pneumophila* in wastewater treatment plants to prevent potential outbreaks of Legionnaires' disease.

Due to these challenges, alternative or complementary detection methods are increasingly being considered for *L. pneumophila* monitoring in complex matrices such as wastewater.

4.3.2 Legiolert

The IDEXX Legiolert test is a liquid culture-based, enzymatic detection method specifically developed for the identification and quantification of *L. pneumophila* in both potable and non-potable water samples. Legiolert showed advantages over the conventional culture plate-based method, including a simplified, relatively faster (7 days), and easier-to-interpret detection method. Furthermore, the method showed a higher sensitivity for *L. pneumophila* and lower interference from other micro-organisms (Barrette, 2019; Rech et al., 2018; Scaturro et al., 2020a).

The detection principle of the Legiolert system is based on bacterial enzyme activity. A substrate included in the selective growth medium is metabolized by *L. pneumophila* during active growth, causing a color change in the medium — from colorless to brown. After a 7-day incubation period, positive reactions are visually identified in the wells of the IDEXX Legiolert Quanti-Tray system. These positive wells are then counted to determine the concentration of *L. pneumophila* in the sample, expressed in most probable number per liter (MPN/L). Such cultivation approach makes Legiolert specific and non-destructive, allowing the isolation of strains from positive wells for additional investigations (e.g., outbreak, Sero-grouping).

4.3.3 MICA Advance

The MICA Advance *Legionella* system from Diamidex was originally developed for detecting *L. pneumophila* in drinking water, recreational (potable) water, and non-potable water sources such as cooling towers. It employs a patented labeling technique based on a precursor of legionaminic acid, a specific component of the O-antigen of *L. pneumophila* (Mas Pons et al., 2014; Passot et al., 2023). This precursor, called pLeg-N_3, is modified with a bio-orthogonal azido group that enables highly selective detection. During sample incubation, the pLeg-N_3 molecule is internalized by actively growing *L. pneumophila* microcolonies and incorporated into their O-antigen structures on the cell surface. Following incubation, a tagging solution containing fluorescent molecules is applied. These molecules bind specifically to the azido-labeled O-antigen via click chemistry, resulting in highly selective fluorescent tagging of *L. pneumophila* cells.

The MICA Advance system automatically enumerates the fluorescently tagged *L. pneumophila* microcolonies using the MICA microcolony counter (Passot et al., 2023). Results are reported as colony-forming units per liter (CFU/L). This targeted detection at the microcolony stage enables early identification of *L. pneumophila* and significantly reduces the detection time compared to the ISO 11731 culture method, from 7 to 14 days to 48 hours (Passot et al., 2023; Ya-jie et al., 2024).

4.3.4 QIAcuity dPCR

The QIAGEN QIAcuity dPCR system is a digital polymerase chain reaction (dPCR) platform designed for the reliable quantification of, for instance, *L. pneumophila* DNA in water and wastewater samples (QIAGEN GmbH, 2025, 2024). Digital PCR is an advanced method for nucleic acid quantification that addresses several limitations associated with traditional quantitative real-time PCR (qPCR) (Quan et al., 2018).

The core principle of dPCR lies in sample partitioning: the reaction mixture is divided into thousands of individual micro-reactions (partitions), each containing zero, one, or a few copies of the target DNA. After PCR amplification, partitions containing at least one copy of the *L. pneumophila* target sequence are detected via fluorescence. This is achieved using specially designed primers coupled with target-specific fluorescent hydrolysis probes, which emit a signal when the target DNA is present (QIAGEN GmbH, 2025). The QIAcuity instrument analyses the fluorescent signals and calculates the absolute concentration of *L. pneumophila* DNA, providing a sensitive and accurate tool for monitoring *Legionella* in complex water matrices. For different sample matrices, the method can be customized by selecting an appropriate DNA extraction and purification method, and implementing supporting (d)PCR reaction additives. Moreover, this method is the fastest of all the methods tested in this project, however, it needs to be considered that it does not distinguish between dead and alive cells.

4.3.5 rqmicro.COUNT

The rqmicro.COUNT technology from rq.micro AG offers a rapid (the analysis can be performed within a working day), advanced, and highly specific method to detect *L. pneumophila*, combining immunomagnetic separation with flow cytometry-based quantification (rqmicro AG, 2025). Magnetic particles coated with monoclonal antibodies specific for *L. pneumophila* serogroup 1 cells enable the separation of *L. pneumophila* from other components in the (waste)water matrix (Heining et al., 2023). Quantification is achieved using flow cytometry, which involves labeling the *L. pneumophila* cells with green fluorochromes that bind to the anti-*L. pneumophila* serogroup 1 antibodies. The stained cells are automatically analyzed as they pass through the cartridge channels using the rqmicro.COUNT instrument. A separation between viable and dead cells can be made by staining the cells with the red dye propidium iodide, which enters damaged cells.

An advantage of rqmicro.COUNT over cultivation methods, including ISO 11731:2017, is the ability to detect all intact *L. pneumophila* cells, including viable but non-culturable (VBNC) forms (Heining et al., 2023). These VBNC cells are particularly important since they pose a health risk and cause infections, but are not detectable by conventional culture-based methods (Dietersdorfer et al., 2018; Heining et al., 2023; Nisar et al., 2023; Wickenberg et al., 2022).

4.3.6 Comparison of the detection methods

Table 4-1 provides an overview of the four selected *L. pneumophila* detection methods in comparison to the ISO 11731 norm.

Table 4-1.Overview comparing the different L. pneumophila detection methods studied.

Detection method	Time to result	Detection limit**	Type of detected cells
ISO 11731	7 to 14 days	$1x10^5$ – $1x10^6$ CFU/L	Viable, culturable cells
Legiolert	7 days	$1x10^1$ CFU/L (potable) $1x10^3$ CFU/L (non-potable)	Viable, culturable cells
MICA Advance	48 hours	1 CFU/tested volume of a sample***	Viable, culturable cells
rqmicro.COUNT	< 8 hours*	$1x10^5$ cells/L	All viable cells****
QIAcuity dPCR	< 8 hours*	$<1x10^6$ DNA copy/L	All DNA, from dead or live bacteria****

*Exact time is dependent on the speed at which the experiments are performed.

**Detection limits were provided by the technology manufacturers.

*** MICA Advance provides flexibility for the analyzed sample volume which changes the detection limit.

****Including viable but non-culturable (VBNC) cells.

4.3.7 Purpose of additional pre-treatments

Current *L. pneumophila* detection methods are primarily validated for potable water. Wastewater samples from biological wastewater treatment plants are very rich in microbiology and, therefore, much more challenging. The plates of culture-based methods might overgrow by other microorganisms, concealing *Legionella* colonies (Heining et al., 2023; Wickenberg et al., 2022). Proper sample pre-treatment is therefore essential. *L. pneumophila* is a strong bacterial strain that can resist heat and acid better than some other strains. ISO 11731 contains a heat pre-treatment and an acid pre-treatment step to reduce the number of other microorganisms before plating (Toplitsch et al., 2021). Other possible pre-treatment steps are sonication (to dislodge bacteria from solid particles) and centrifugation (to reduce the amount of suspended solids and to prevent filters from clogging). To prevent the overgrowth of culture-based methods, biological wastewater samples need to be diluted. Different diluents could be used, for instance, isotonic phosphate-buffered saline (PBS) or Ringer solution.

4.4 Methodology

4.4.1 Sampling locations

Wastewater samples were collected at two locations, the paper mill (PM) and the pig slaughterhouse (SH) facility. The facilities were selected due to the increased concentrations of *L. pneumophila* in their wastewater treatment processes. Sampling at these sites was performed on three consecutive Mondays: March 31st, April 7th and April 14th, 2025. The samples were collected and shipped on ice to the commercial laboratory Aqualab Zuid (Werkendam) and the Water Application Centre (WAC; Leeuwarden). Before analysis, the samples were stored at 4 °C for no more than 48 hours, in accordance with the ISO 19458 standard for water sampling and analysis.

4.4.1.1 Location 1: the pig slaughterhouse (SH)

The biological WWTP selected for this study is located at the pig slaughterhouse and meat processing facility. This location was confronted with a *L. pneumophila* outbreak in 2017, and has been studied since (Vermeulen et al., 2021, 2019). The *L. pneumophila* concentrations in this facility have reached levels as high as 1x10^{10} CFU/L. Since the outbreak, multiple measures have been applied, such as, for instance, enclosing the aeration tank and developing alternative preventative strategies, and the *L. pneumophila* concentrations have been effectively reduced, however, they still remain high (1x10^{6} – 1x10^{9} CFU/L). The WWTP consists of the following steps: Buffer tank > Dissolved air flotation > Anaerobic digestion > NAS anammox > Membrane bioreactor > Effluent. The wastewater samples were collected from the NAS aeration tank (Table 4-2A). Samples collected at this location will hereafter be referred to as “SH samples”.

4.4.1.1.1 Location 2: the paper mill (PM)

The second selected location produces cardboard packaging materials from recycled paper. The *L. pneumophila* concentrations in the aeration tank of this facility recorded between 2019 and 2024 were in a range of 10^{6} and 10^{8} CFU/L. The WWTP at this location consists of the following steps: Buffer tank > Anaerobic digestion > Aerobic treatment > Clarifier > Effluent. The wastewater samples were taken from the aeration tank (Table 4-2B). In this report, samples collected at this location will be referred to as “PM samples”.

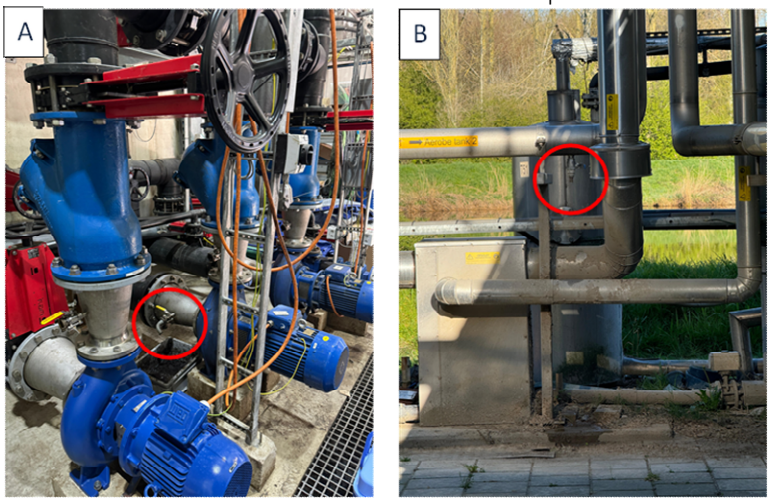


Table 4-2. Sampling points at (A) the slaughterhouse (SH) and (B) the paper mill (PM) facilities.

4.4.2 Wastewater parameters

To determine the differences between the matrices of the PM and SH samples, and their potential effect on the detection methods, some chemical and physical parameters were studied. The analysed parameters included total chemical oxygen demand (tCOD), soluble chemical oxygen demand (sCOD), total suspended solids (TSS), and pH. To determine tCOD and sCOD of the samples, Hach Lange cuvette test LCK 514 was used. Before sCOD analysis, the samples were centrifuged at 1000 x *g* for 5 min and subsequently filtered through a 0.45 µm syringe filter.

To determine the TSS, approximately 50 g of the homogenized wastewater sample was concentrated through a Whatman paper filter (grade 589/2, white ribbon). The filter with the solid residue was placed in a porcelain

crucible and dried overnight at 105 °C. The TSS concentration was calculated based on the difference in weight before and after drying. Lastly, the pH was measured by a HACH HQ40d multi-field meter.

4.4.3 *Legionella* detection methods

4.4.3.1 ISO 11731

The ISO 11731 plate culturing analysis was performed by the commercial laboratory Aqualab Zuid using the protocol for matrix C (wastewater).

4.4.3.1.1 Legiolert

Legiolert from IDEXX is a cultivation-based detection method. Viable and culturable *L. pneumophila* cells will cause a color change of the liquid medium. Sample preparation and analysis were performed according to the "Preliminary wastewater protocol" shared internally by IDEXX and the "IDEXX Legiolert Test protocol for non-potable water". The adapted protocol for wastewater contained the pre-treatment steps that included mixing through inversion, ultrasonic treatment in water bath (35 W, 44 kHz, 9 min), and centrifugation (1000 x*g*, 5 min). Subsequently, the supernatant was placed in a water bath for heat treatment (50 °C, 30 min) and cooled back to ambient temperature before proceeding with the standard Legiolert protocol. After preparation, the samples were sealed and incubated in specially designed 96-well trays at 37 °C for 7 days. After incubation, the brown wells were quantified, and the number of viable and culturable *L. pneumophila* cells was determined.

4.4.3.2 MICA Advance

MICA Advance from Diamidex is a plate-based culture method combined with fluorescence-based detection. In this method, a specific substrate is added to the sample, which is then metabolized only by *L. pneumophila*. This allows further automatic detection and quantification of fluorescently-tagged *L. pneumophila* microcolonies in the MICA plate, providing the presence of viable and culturable cells in a sample (Passot et al., 2023; Ya-jie et al., 2024). The experiments were performed following the manual provided by the manufacturer, including additional adaptations for wastewater samples. The adaptations included decantation of the sample (5-10 min) to remove bigger particles, dilution (100x), and increased volume of pH2 solution for acid treatment (from 5 mL in the original protocol to 20 mL).

4.4.3.3 rqmicro.COUNT

rqmicro.COUNT from rq.micro AG is an immunomagnetic separation and flow-cytometry-based detection method. In the first step, *L. pneumophila* cells are bound to anti-*L. pneumophila* serogroup 1 antibody-coated magnetic particles (Heining et al., 2023). When the samples run through a magnetic field applied in the rqmicro.COUNT unit, *L. pneumophila* cells attached to the magnetic beads separate from the rest of the sample and are detected by flow-cytometry, allowing automatic quantification of *L. pneumophila* cells in a sample. The experiments were performed according to the protocol "*Legionella pneumophila* COUNT kits – User Guide for rqmicro.COUNT wastewater analysis", including additional recommendations for wastewater samples. The samples were diluted 100x.

4.4.3.4 QIAcuity dPCR

The QIAcuity dPCR method from QIAGEN is a DNA-based molecular detection method (QIAGEN GmbH, 2025). The diluted (100x) wastewater samples were collected on membrane filters, from which DNA was extracted using the DNeasy PowerWater kit following the manufacturer's instructions (QIAGEN GmbH, 2022). 10 µL of the extracted DNA was pipetted into an Eppendorf tube, and 30 µL of the prepared reaction mix (Table 4-3) was added. After mixing, the sample was pipetted into the designated wells of a 26K nanoplate. The PCR reaction and subsequent analysis were performed by the QIAcuity dPCR instrument (QIAGEN GmbH, 2024). PCR reaction cycle settings are presented in Table 4-3. After the PCR reaction, the positive partitions were automatically determined, and the number of *L. pneumophila* gene copies per sample was quantified.

Table 4-3. Reaction mix components for sample analysis of QIAGEN QIAcuity dPCR.

Compound	Amount used per sample (µL)
QIAcuity Probe PCR Mastermix	10
PCR water	17
Primer-probe mix for *L. pneumophila*	2
EcoRI restriction enzyme	1

4.4.4 Wastewater sample pre-treatment methods and diluents

For the pre-treatment of the wastewater samples from PM and SH, different approaches were considered for optimal detection of *L. pneumophila*. The selected sample pre-treatment options are based on the methodology from the ISO 11731:2017 method (heat treatment) and on the Preliminary Wastewater Protocol provided by IDEXX for the application on the Legiolert analysis for non-potable water samples. The latter contains three consecutive pre-treatment steps based on ultrasonication, centrifugation, and heat treatment. Since IDEXX has previously developed this pre-treatment protocol for wastewater samples, no further tests were requested for this detection method in this study. For the other three technologies: MICA Advance, rqmicro.COUNT and QIAcuity dPCR, pre-treatments were selected in consultation with the technology providers based on their interests and previous experiences of CEW and KWR. An overview of the selected pre-treatments per technology is given in Table 4-4.

Table 4-4. Overview of the analyzed wastewater pre-treatment methods per L. pneumophila detection method, highlighted in green. The selection of pretreatments per detection method was made in agreement with the technology provider, CEW and KWR.

	Pre-treatments			
	Ultrasonication	Centrifugation	Heat	Alternative diluent
ISO 11731:2017			Included in protocol	
Legiolert	Included in the preliminary wastewater protocol of IDEXX			
MICA Advance	✓	✓	Included in protocol	✓
rqmicro.COUNT	✓	✓	✓	
QIAcuity dPCR	✓	✓		

4.4.4.1 Ultrasonication

Ultrasonication was applied to the samples as it allows dislodging bacteria from the sludge by disrupting the bacterial adhesion. Ultrasonication was performed for 9 minutes with the use of a sonication bath (40 kHz). This protocol was based on the provided preliminary wastewater protocol from IDEXX for Legiolert and was performed for analysis with MICA Advance, rqmicro.COUNT and QIAcuity dPCR.

4.4.4.2 Centrifugation

Centrifugation separates suspended solids in wastewater samples from the liquid phase, reducing clogging during filtration and unexpected interference during analysis. The wastewater samples were centrifuged for 5 minutes at 1000 x*g*. After centrifugation, the supernatant was pipetted into a new sterile sample tube for downstream analysis. This protocol was based on the preliminary wastewater protocol from IDEXX and previous experiences with clogging of equipment used for *L. pneumophila* analysis. The centrifugation of the wastewater samples was performed for the analysis with MICA Advance, rqmicro.COUNT, and QIAcuity dPCR.

4.4.4.3 Heat treatment

The samples were incubated in a water bath at 50°C ± 1°C for 30 ± 2 min. This protocol was based on the ISO 11731:2017 method as a step to suppress the effect of interfering microorganisms in wastewater samples. Heat pre-treatment was performed for wastewater samples before the analysis with rqmicro.COUNT as it was not included in the original protocol.

4.4.4.4 Alternative diluents

Due to the different compositions of wastewater, diluting the samples with tap water could potentially lead to osmotic changes in the sample and result in bacterial cell rupture. Two alternative solutions have been tested, phosphate-buffered saline (PBS) and Ringer solution, as they are both isotonic diluents that assure cell viability during dilution and analysis. The alternative diluents were tested for sample dilution in the MICA Advance detection method.

4.4.4.4.1 PBS

The composition of the PBS is presented in Table 4-5. It was prepared from Sigma-Aldrich reagents which catalogue numbers are also included in the Table 4-5.

Table 4-5. Phosphate buffered saline (PBS) composition.

	Sigma-Aldrich catalogue number	Concentration
Sodium chloride	S9625	0,137 M
Potassium chloride	P4504	0,0027 M
Sodium phosphate dibasic	S0876	0,01 M
Potassium phosphate monobasic	P0662	0,0018 M

4.4.4.4.2 Ringer solution

The Ringer solution was prepared from a commercially available tablet (Ringers solution 1/4 strength tablets, cat. no. 96724, Millipore) following the manufacturer's instructions.

4.4.5 Sample pre-treatment steps and sample information per method

4.4.5.1 Pre-treatment for Legiolert

Since the preliminary pre-treatment for non-potable water samples has already been developed for Legiolert, this method was excluded from the pre-treatment study in agreement with IDEXX. The collected wastewater samples were analysed without any additional adjustments to increase the results database.

4.4.5.1.1 Pre-treatment rqmicro.COUNT

For the rqmicro.COUNT method for *L. pneumophila*, different combinations of pre-treatments were selected for wastewater samples, as presented in Table 4-6. Before filtration, wastewater samples were diluted 100x in sterile tap water. 20 mL of the diluted samples and sterile tap water (blanks) were then filtered and processed following the manufacturer's instructions. The sample pre-treatment was performed in duplicate.

Table 4-6. Sample pre-treatment combinations for rqmicro.COUNT analysis.

Sample	Sonication	Centrifugation	Heat treatment
1	✓	✓	✓
2	✓	✓	X
3	✓	X	✓
4	✓	X	X
5	X	✓	✓
6	X	✓	X
7	X	X	✓
8	X	X	X
9 (blank)	X	X	X

4.4.5.2 Pre-treatment MICA Advance

For the MICA Advance method for *L. pneumophila*, different combinations of pre-treatments were made as presented in Table 4-7. Before filtration, wastewater samples were diluted 100x in sterilized PBS or sterilized Ringer solution. 20 mL of the diluted samples were used for filtration, giving the detection limit of $1x10^4$ CFU/L of the original sample. 20 mL of PBS and Ringer solution were used as blanks. The sample pre-treatment was performed in duplicate.

Due to the overgrowth of undefined microflora present in the wastewater samples obtained from SH, the dilution of the SH wastewater sample was increased to 400x in the 3rd week of analysis, giving the detection limit of $4x10^4$ CFU/L of the original sample. Moreover, images of the membranes after 48 h were taken to visualize general growth on the plates.

Table 4-7. Sample pre-treatment combinations for MICA Advance analysis.

Sample	Sonication	Centrifugation	Diluent
1	✓	✓	Ringer solution
2	✓	✓	PBS
3	✓	X	Ringer solution
4	✓	X	PBS
5	X	✓	Ringer solution
6	X	✓	PBS
7	X	X	Ringer solution
8	X	X	PBS
9 (Ringer)	X	X	Ringer solution
10 (PBS)	X	X	PBS

4.4.5.3 Pre-treatment QIAcuity dPCR

The pre-treatment combinations for QIAcuity dPCR analysis for *L. pneumophila* are presented in the matrix in Table 4-8. Before filtration, wastewater samples were diluted 100x in sterile tap water. 20 mL of the diluted samples and sterile tap water (blank) were used for filtration. The sample pre-treatment was performed in duplicate.

Table 4-8. Sample pre-treatment combinations for QIAcuity dPCR analysis.

Sample	Sonication	Centrifuge
1	✓	✓
2	✓	X
3	X	✓
4	X	X
5 (blank)	X	X

4.4.6 Statistical analysis

A one way analysis of variance (ANOVA) was applied to compare mean *Legionella* concentrations after pre-treatment. This statistical approach evaluates whether observed variations between treatments exceed random variability within pre-treatment groups. When a sample group exceeds a pre-determined *p*-value of 0.05 ($p>0.05$) the sample pre-treatment methods do not differ statistically between pre-treatment groups.

4.5 Results

4.5.1 Wastewater parameters

The pH, TSS, total COD, and soluble COD of the samples from the aeration tanks of the PM and the SH WWTPs were determined and the results are presented in Table 4-9. The main difference between the samples was in their COD values, with SH samples having significantly higher organic matter concentration and also slightly higher solids content.

Table 4-9. Chemical characteristics of the wastewater samples from PM and SH WWTPs. The values are the average of the three separate samplings on March 31st, April 7th, and April 14th, 2025.

	pH	TSS	Total COD	Soluble COD
		g/kg	mg/L	mg/L
PM *aeration tank*	7.6 ± 0.2	7.01 ± 0.10	2 298 ± 302	129 ± 5
SH *aeration tank*	7.0 ± 0.1	10.62 ± 0.77	21 753 ± 3 796	1868 ± 919

4.5.2 ISO 11731 method

For confirmation of the presence of *L. pneumophila* in the collected wastewater samples, the ISO 11731 method was performed externally at the commercial lab (AquaLab Zuid, The Netherlands). Due to application of inappropriate plating volume (0.5 mL) of the samples from March 31st and April 14th that resulted in overgrowth of interfering microorganisms, it was only possible to quantify *L. pneumophila* in the samples from April 7th (plating volumes of 0.001 and 0.01 mL, for PM and SH, respectively). The results are presented in Figure 4-1. On that day, the concentrations of *L. pneumophila* in the aeration tank of the PM plant (4.8x10^{8} CFU/L) were three orders of magnitude higher than in the samples collected at the SH plant (4.0x10^{5} CFU/L).

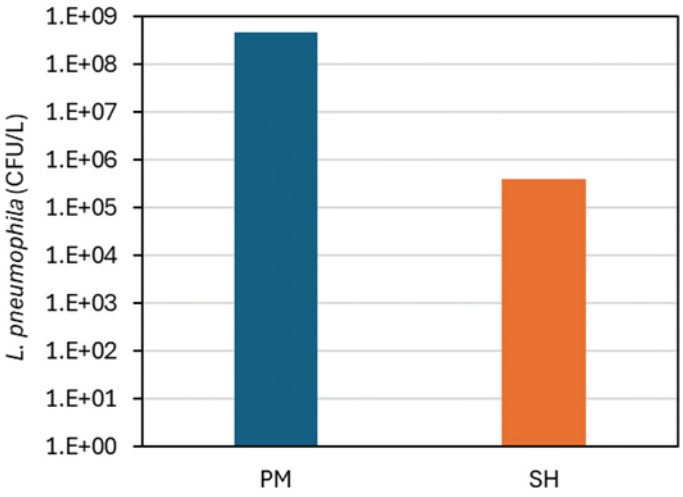


Figure 4-1. L. pneumophila *concentrations determined by ISO 11731 in PM and SH samples from April 7th, 2025. Analysis was performed by Aqualab Zuid. Due to plating mistake only results that day were viable.*

4.5.3 Legiolert

The concentrations of *L. pneumophila* measured with Legiolert are shown in Figure 4-2Figure 4-1. The results were calculated as Most Probable Number (MPN/L), which is equivalent to CFU/L. Compared to the results obtained with ISO 11731 method, the Legiolert values were lower by about four orders of magnitude for PM samples, and

by two orders of magnitude for SH samples on April 7th. PM samples showed *L. pneumophila* concentrations ($5.5 \cdot 10^4$ CFU/L on average) higher by two orders of magnitude than those in SH samples ($5.5 \cdot 10^2$ CFU/L).

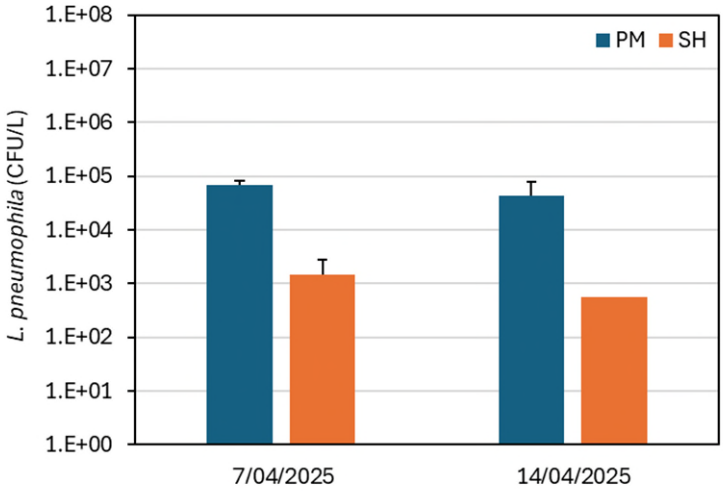


Figure 4-2. L. pneumophila *concentrations determined by Legiolert in PM and SH samples on April 7th and 14th, 2025. The results are averages of triplicate measurements performed on each day.*

4.5.4 Sample pre-treatment: MICA Advance

Figure 4-3 shows the quantitative effect of the sample pre-treatment methods on the MICA Advance performance. Except for the PM samples with "sonication + centrifugation" in combination with different diluents that showed values below the detection limits (except for week 2 with PBS), the results show no significant (p>0.05) differences between the samples without and with pre-treatment. For the SH samples, only two measurements were within the detection range. A comparable result in CFU/L was recorded for both pre-treatment combinations: sonication + centrifugation, with and without Ringer solution. Because of the limited amount of viable results within the detection range (n=1), no statistical calculations could be performed to verify the significance of these results.

It is important to add that the initial analysis showed some weaknesses in the MICA image analysis algorithm on the membranes with a very high load of interfering microbial growth. It has been addressed by Diamidex through an updated algorithm to handle these specific cases without any changes being introduced to the analysis of membranes with a usual microbial load, and the raw images acquired by the MICA Advance unit were re-analysed with the improved algorithm. The results presented in this study are the results of this re-analysis.

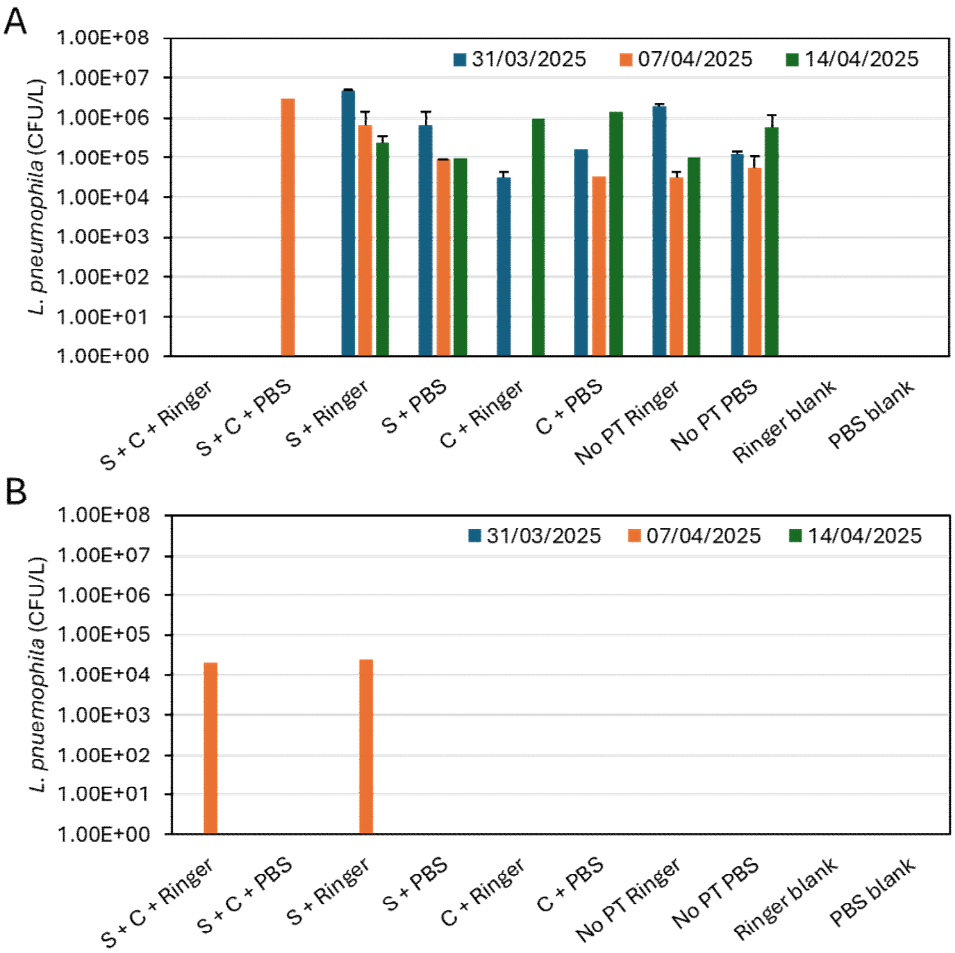


Figure 4-3. L. pneumophila *concentrations determined by MICA Advance in PM (A) and SH (B) samples measured each week after application of various (combinations of) pre-treatments and diluents: sonication (S), centrifugation (C), phosphate-buffered saline (PBS), and Ringer solution (R). No data indicates concentration below the detection range. The weekly results are the averages of duplicate measurements.*

In addition to the scan by the MICA Advance counter, the membranes were photographed for visual inspection as presented in Figure 4-4. In all samples where centrifugation was applied, no microbial cultures could be observed. On the membranes with samples without centrifugation, including the non-treated controls that were only decanted, distinct microbial cultures on PM (white and yellow, with some white fungal growth) and SH (white and black) membranes formed consistently throughout the three weeks of sample collection. Moreover, the microbial cultures on SH membranes were clustered (potentially due to the presence of sludge flocs). In the samples that were sonicated, those cultures appear more dispersed. In week 1, the membranes with SH samples and blanks (not shown) diluted with Ringer solution showed uniform coverage with yellow-coloured cultures in the filtration surface area, indicating diluent contamination used for those samples specifically. Those samples were excluded from the analysis. Otherwise, no effect of the diluent was observed in visual appearance of the microbial cultures. Interestingly, some PM samples that did not show visible microbial growth still tested positive for *L. pneumophila*.

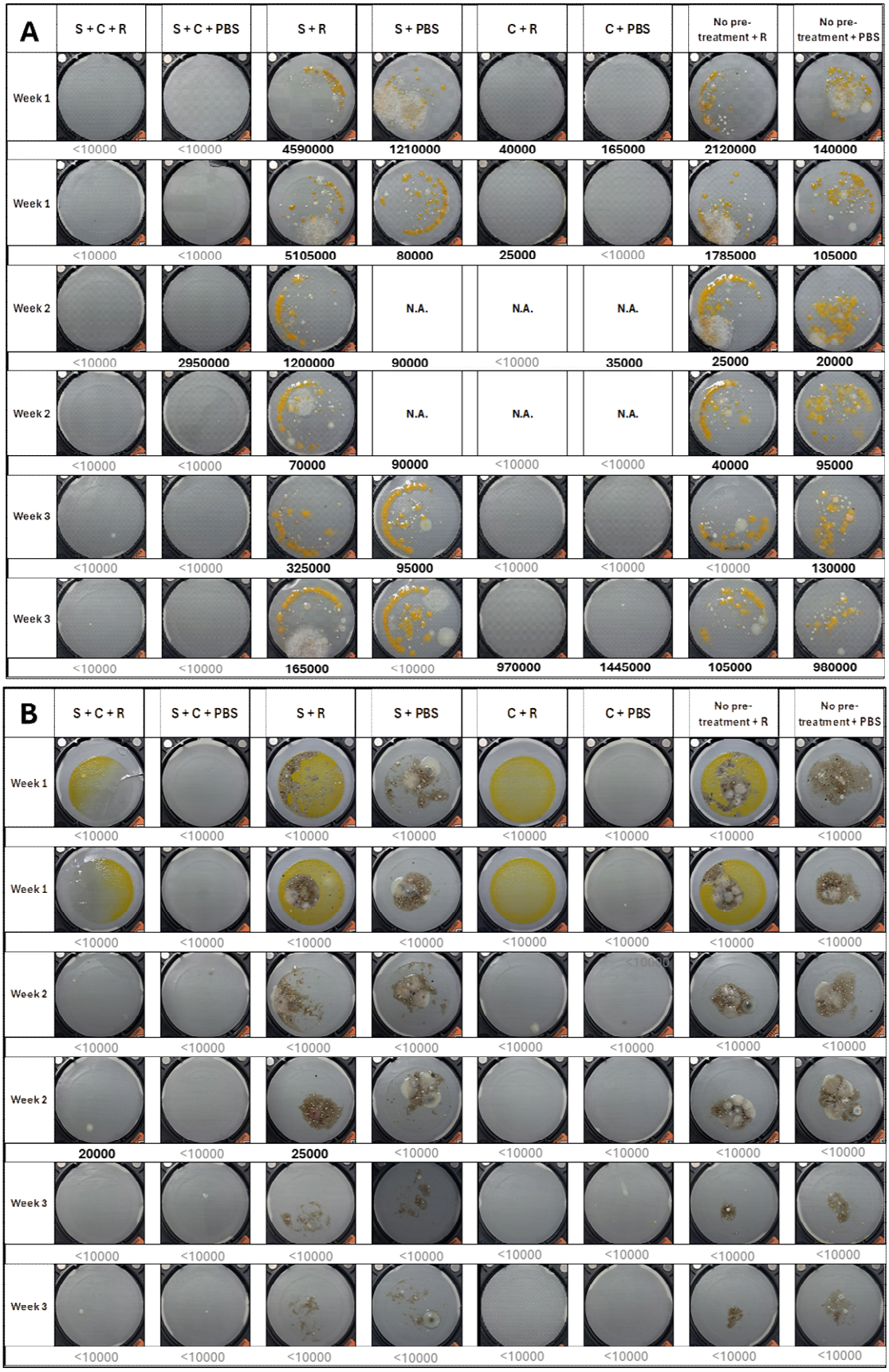


Figure 4-4. MICA membranes after 48 h incubation with PM (A) and SH (B) samples and after applying (combinations of) pre-treatments: sonication (S), centrifugation (C), Ringer solution (R) and phosphate-buffered saline (PBS). Below each membrane the quantitative results in CFU/L are given. Due to a saving error, several pictures of PM samples in week 2 were lost and are shown as "N.A".

4.5.5 Sample pre-treatment: QIAcuity dPCR

For the sample pre-treatment of the QIAcuity dPCR method, the effects of sonication, centrifugation and their combinations were tested and the results are presented in Figure 4-5. For both, the SH samples and the PM samples, the results show a similar trend where the centrifugation step resulted in a decrease of detected *L. pneumophila* DNA copies. In PM samples, centrifugation with and without sonication significantly (p=0.009 and 0.007, respectively) reduced the amount of *L. pneumophila* DNA copies compared to samples without pre-treatment indicating that centrifugation step lowers the *L. pneumophila* concentration. Implementing sonication as a pre-treatment step did not have a significant (p=0.791) effect compared to not pre-treated samples. Due to no DNA copies being detected in the SH samples without pre-treatment (potentially due to a weak signal and dispersed partitions distribution, see Figure 8B), the statistical difference between "no pre-treatment" compared to pre-treated samples could not be evaluated. However, similarly as for PM samples, the sonicated samples resulted in significantly higher concentrations of *L. pneumophila* DNA copies/L, compared to those centrifuged with (p=0.005) and without sonication (p=0.005).

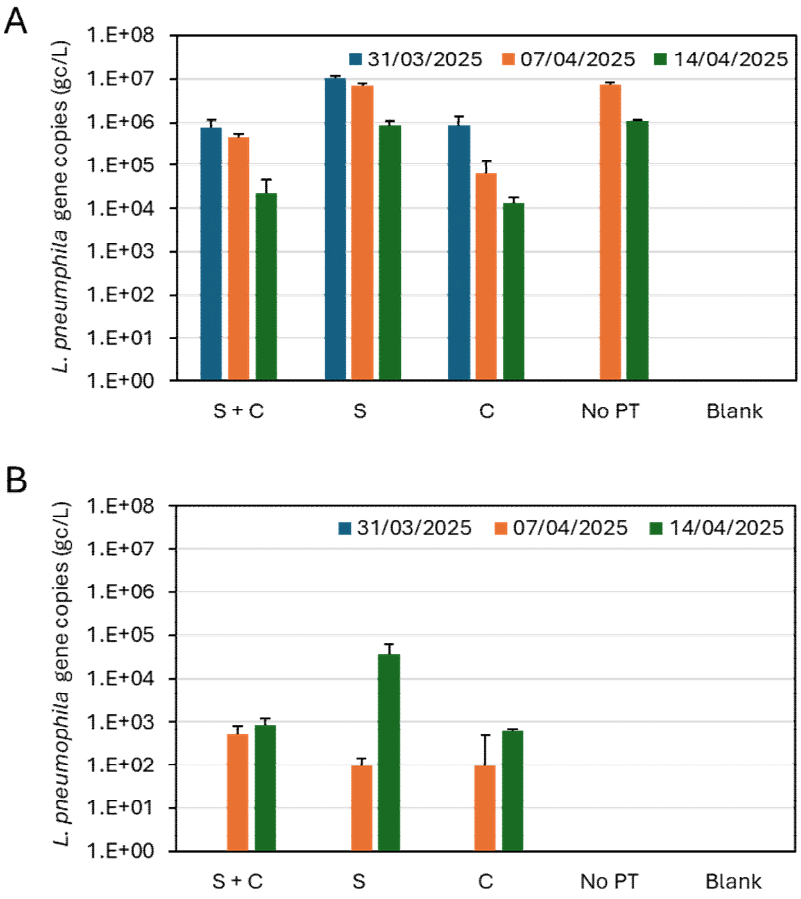


Figure 4-5. L. pneumophila *concentrations determined by QIAcuity dPCR in PM (A) and SH (B) samples measured each week after application of various (combinations of) pre-treatments: sonication (S) and centrifugation (C). No data indicates concentration outside of the detection range. The weekly results are the averages of triplicate measurements.*

4.5.5.1 Distribution of partition signals in QIAcuity dPCR

Next to quantified results of the QIAcuity dPCR analysis, the distribution of signal partition was analysed as it can serve as an indicator for sample purity. The scatterplots of partition signals are shown in Figure 4-6. In both samples the signal was weaker than expected by the technology manufacturer (fluorescence intensity <50 RFU) and relatively high amounts of "rain" (droplets with intermediate fluorescence intensity that fall between the distinct positive and negative clusters in a 1D or 2D plot) were observed. When comparing the positive partition distribution, in the PM samples they gave a stronger signal and were more concentrated (Figure 4-6), while in SH samples, a lot of partitions dispersion occurred with main partition concentration at a low fluorescence intensity

(<15 RFU), making setting a clear threshold difficult and reduces quantification accuracy. Such dispersion seemed to be limited by application of centrifugation (Figure 8B).

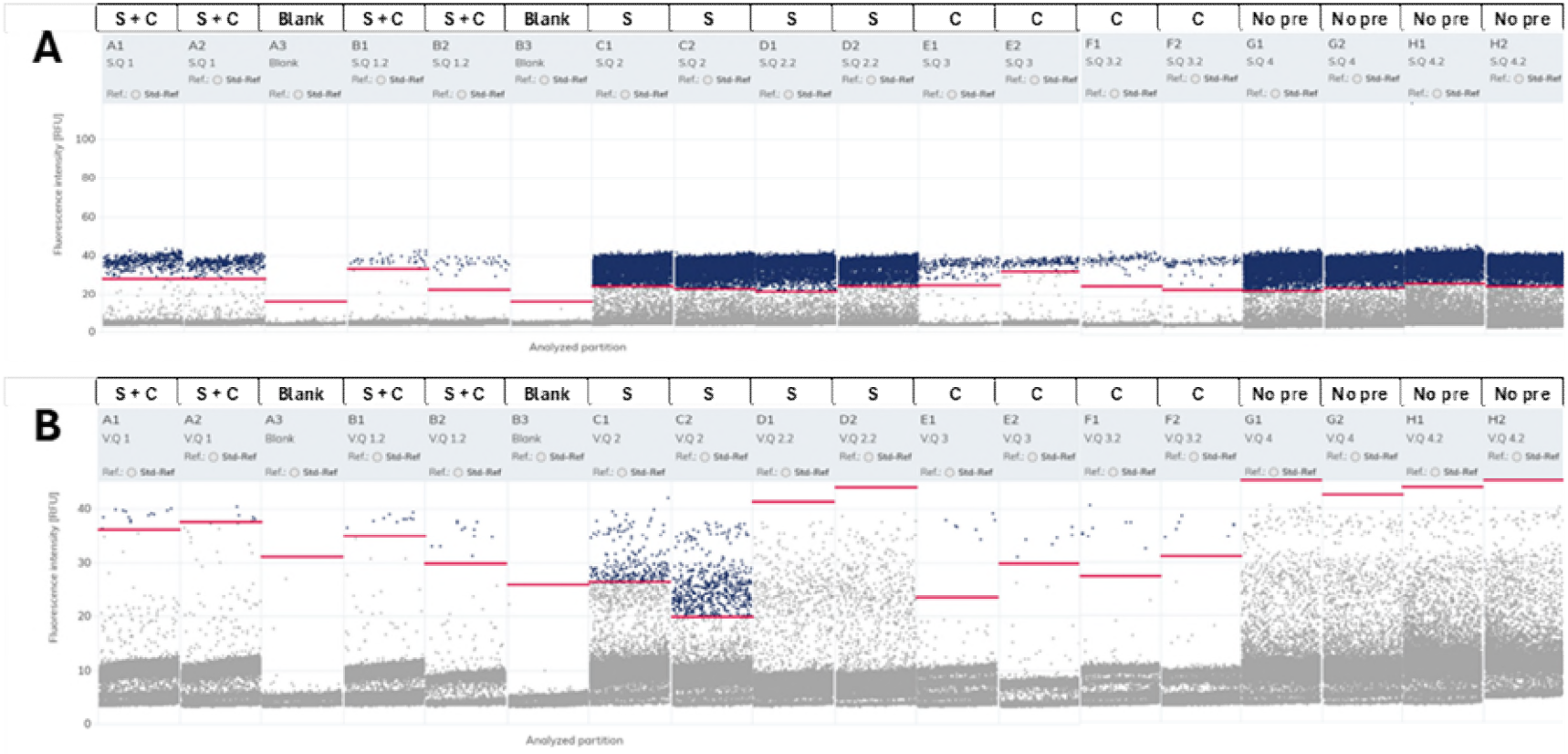


Figure 4-6. Scatterplots of partition signal distribution from PM (A) and SH (B) samples from April 14th, 2025, after applying pre-treatments and combinations: sonication (S), centrifugation (C) and no pre-treatment (No pre). Grey results under the threshold (red line) are negative partitions, blue results above the threshold are positive partitions.

4.5.6 Sample pre-treatment: rqmicro.COUNT

For determination of the effect of various pre-treatment methods on detection of *L. pneumophila* cells with rqmicro.COUNT, eight combinations of pre-treatments were performed on the SH and PM samples. The results are presented in Figure 4-7. The values do not show the pronounced difference between measured *L. pneumophila* concentrations between PM and SH samples as observed with other methods. There was also no significant difference between samples without and without pre-treatment (p>0.05). Also, it is important to note that the negative control samples (blanks) showed positive values reaching up to 10^3 cells/L.

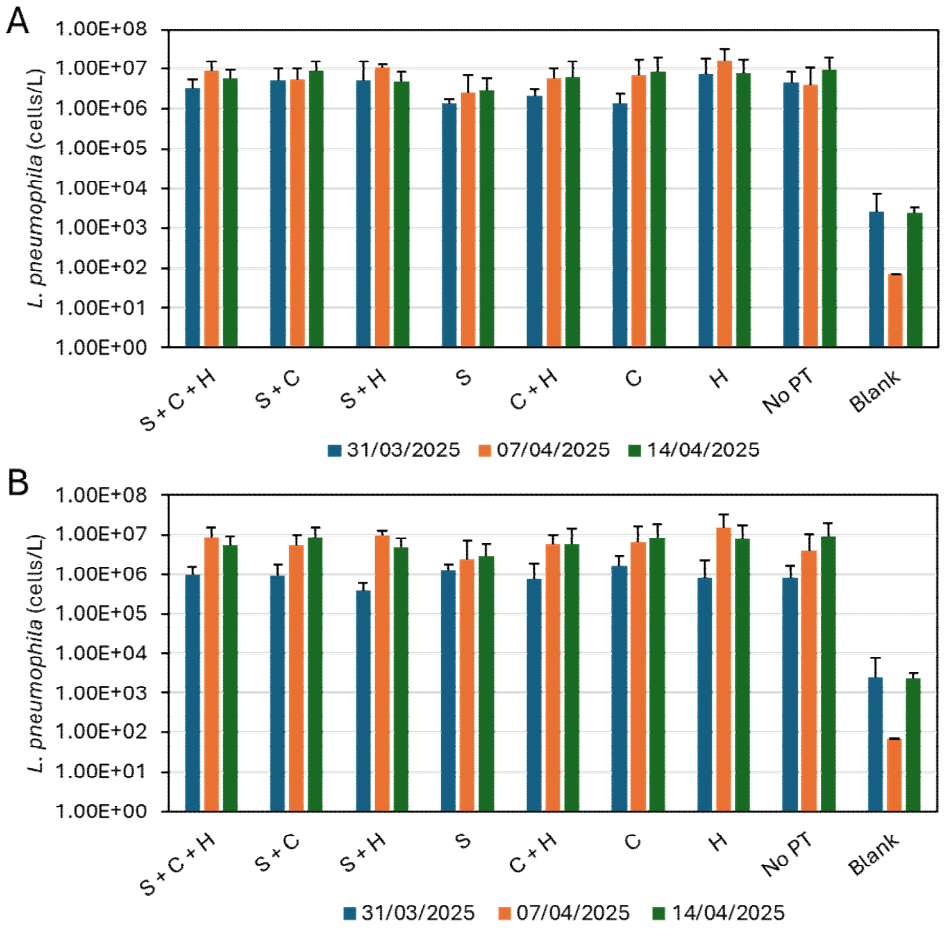


Figure 4-7. L. pneumophila *concentrations determined by rqmicro.COUNT in PM (A) and SH (B) samples each week after application of various (combinations of) pre-treatments: sonication (S), centrifugation (C), heat treatment (H) and no pretreatment (No PT). The weekly results are the averages of duplicate measurements.*

4.6 Discussion

Wastewater creates much more challenges for *Legionella pneumophila* detection than drinking or even cooling tower samples, due to abundance of other microorganisms and compounds that can interfere with the measurements in ways that the existing detection methods are not yet necessarily equipped for. In this study, the selected wastewater samples came from two facilities representing different industries, i.e. paper production and pig slaughterhouse, whose wastewater systems are commonly more affected with elevated *L. pneumophila* concentrations. Samples collected from the aeration basins varied in their composition (Table 4-9), specifically in terms of solids and organic matter (COD) concentrations, bringing to attention another complication for *L. pneumophila* detection, where unlike drinking water, wastewater sample contents can vary considerably between industries and plants. This poses a

challenge in defining one generally applicable detection method for this context. While at the moment the ISO method is a standard technique for *L. pneumophila* in wastewater, the long analysis time and a high detection limit call for an alternative to improve *L. pneumophila* risk management in the WWTP scenario.

In this study, different pre-treatment strategies have been selected and tested to investigate whether they can improve the performance of the four alternative *L. pneumophila* detection methods. Pre-treatment steps included centrifugation, sonication and heat treatment in varying combinations to reduce interference from other microorganisms and compounds present in wastewater and to detach *L. pneumophila* cells from sludge and amoeba for quantification, thereby improving the reliability of the detection methods. However, each of the pre-treatments, if applied inappropriately, can also have negative outcomes. For instance, when sonication or heat treatment are applied, cell lysis or bacteria entering the viable-but-not-culturable (VBNC) state can occur (Nisar et al., 2023).

4.6.1 Sample pre-treatment: Legiolert

The guidance notes offered by IDEXX for wastewater sample preparation for use with the Legiolert protocol were developed prior this project using wastewater samples obtained from KWR and UK Water Utility. A total of 52 samples were analysed with Legiolert using the modified protocol and the standard methodology for non-potable samples. As a reference, samples were also analysed using the ISO 11731 norm (Matrix C). The results showed that using the Legiolert wastewater sample preparation protocol, reactivity was observed in 27 samples, with *L. pneumophila* confirmed in all samples (0% false positivity rate). In comparison, *L. pneumophila* was detected only in 10 samples using the ISO 11731 standard method. Using Legiolert method for non-potable samples without additional pre-treatment, reactivity was observed in 44 samples but the presence of *L. pneumophila* was confirmed in only 6 samples, providing a potential false positivity rate of approx. 84%. Based on these findings, it has been agreed that the proposed Legiolert wastewater sample preparation and protocol for non-potable samples, rather than the standard Legiolert non-potable protocol, should be used throughout the study.

In this work, the lower concentrations of *L. pneumophila* detected with Legiolert method compared to those measured with the ISO 11731 on April 7th could have been due to the implementation of the wastewater pre-treatment steps, which increased the likelihood of confirmed *L. pneumophila* detections at lower concentrations than those found with the ISO method. It is also important to mention that results obtained by Legiolert were consistent between April 7th and 14th and for both wastewater samples proving the method reproducibility.

4.6.2 Sample pre-treatment: MICA Advance

In this study, MICA Advance unit was used for analysis which is an improved version of the MICA that was available for testing in Phase 1 of the project. While the *L. pneumophila* detection principle remained the same, MICA Advance provided improved process workflow through more automated set-up, where up to 18 membranes could be analysed in one run, which allows up to 500 analyses per day, though this limit was not reached in this study.

Quantitative evaluation provided results for the PM samples (Figure 4-4A), while for the SH ones only two individual measurements above the detection limit were recorded (Figure 4-4B) and thereby they were not used to draw conclusions. In case of the PM samples, no significant quantitative effect of pre-treatment nor diluent on the MICA performance was observed, except for “sonication + centrifugation” in combination with both diluents for which the results were below the detection limit in almost all analysed samples (Figure 4-3A). However, when observing the membranes (Figure 4-4), there are clear patterns where in all centrifuged samples (including the SH ones), with and without sonication, no visible microbial growth was observed. Interestingly, the PM samples that were centrifuged without sonication showed positive values, indicating that addition of sonication into the combination of centrifugation and a buffer (both containing NaCl that in some cases can induce bacterial lysis (Logisz and Hovis, 2005) in conditions selected in this study could potentially either damage the cells, make them enter the VBNC state, or increase the lag phase period, leading to difficulties in detection of microcolonies as early as 48 h with MICA Advance.

Visual inspection of the membranes (Figure 4-4), also showed how different microbial colonies from different facilities can be and how differently they distribute on the membranes, both having an effect on the quantification of *L. pneumophila* microcolonies by the MICA unit. Since the wastewater for the analysis was collected from the *L. pneumophila* hotspots, which are the aeration tanks, the samples contained activated sludge flocs and potentially other aggregates coming from wastewater. This can be seen especially on the SH membranes (Figure 6B), where microbial cultures are concentrated as clusters. The MICA method was designed for the analysis of (potable) water samples, and not yet for complex matrices such as wastewater, meaning that such microbial overgrowth can limit the sample volume that can be analysed. While sonication seems to be effective in breaking down those clusters, it did not have a considerable effect on the MICA Advance readings. Such microbial overgrowth could pose the main challenge for MICA Advance application in the wastewater context, therefore, the process optimisation could focus on further limitation of interfering microflora through e.g., improved acid and heat treatments.

4.6.3 Sample pre-treatment: QIAcuity dPCR

From all the three tested methods, the results obtained with QIAcuity dPCR showed the most consistency in terms of the detected numbers of *L. pneumophila* DNA copies between the two wastewater locations and between the different combinations of pre-treatments applied. The sample pre-treatment methods had a significant effect on the *L. pneumophila* detection, which can be especially well observed in the PM samples (Figure 4-5). The use of centrifugation lowered the amount of detected DNA copies compared to non-pretreated sample. This indicates that possibly a part of the *L. pneumophila* cells, its DNA, or dead cells, stay adhered to suspended solids, which are compressed into a pellet during the centrifugation step and removed from the analysed fraction of the sample. The idea behind applying sonication in combination with centrifugation was to first allow the release of *L. pneumophila* cells from the sludge/protozoa so they will remain in the supernatant to be analysed. However, the fact that the *L. pneumophila* concentrations obtained after centrifugation with and without sonication were comparable, and sonication on its own did not have a significant effect on the measurement, contradicts this hypothesis. Similar observation was made for the SH samples, but due to the lack of results for the “no pre-treatment” control and the samples from week 1 it was not possible to provide a conclusion as confidently as it was for the PM samples. This means that neither sonication nor centrifugation improve the *L. pneumophila* detection for QIAcuity dPCR, but there is still potential for improving the method reliability by tackling the issue of DNA samples purity.

Observing dPCR scatterplots can provide additional information about the samples as the partitioning of the fluorescence signals can reflect the presence of contaminant or inhibitory compounds in a sample. The patterns in fluorescence amplitude threshold between positive and negative partitions can be characterized by fluorescence partitions, called “rain”. The presence of rain partitions often indicates a less pure DNA sample with inhibitors (Porco et al., 2022). Rain partitions were observed in the samples from both locations (Figure 4-6), however, their amount was considerably reduced when centrifugation was applied, which seemed to remove (next to *L. pneumophila*) a portion of contaminants. One of the common inhibitors for (d)PCR are humic acids which can be commonly found in wastewater as they form from decomposition of organic matter (including pig manure and slurry) (Jiménez-de-Santiago et al., 2023). They can cause inhibition of fluorescence DNA-binding dye, which results in damping of the fluorescence signals, creating the rain visible in the scatterplots (Jeon and Yan, 2025; Sidstedt et al., 2015) as observed especially in the non-centrifuged SH samples (Figure 8B). While usually (d)PCR inhibitors should be removed during the DNA purification process after the extraction step, the kit used in this study was designed for water samples and its capacity in handling contaminant load from wastewater might not be sufficient. To tackle this, different DNA extraction kits (e.g., for soil) in combination with optimisation of PCR gradient, or implementation of PCR additives, such as QIAGEN Q-Solution could be further explored. Additionally, for more concentrated wastewater samples, less material could be used for DNA extraction to reduce the amount of (d)PCR inhibitors. To understand how much *L. pneumophila* (DNA) remains trapped in the solid fraction, additional analysis of the pellet after centrifugation could be performed.

4.6.4 Sample pre-treatment: rqmicro.COUNT

The analysis of the wastewater samples with rqmicro.COUNT showed no significant difference between the implemented pre-treatments but also between the wastewater samples (Figure 4-7), while all other methods have detected significantly more *L. pneumophila* in the PM samples than those from SH. Those higher concentrations could be justified when compared to culture-based methods, like MICA Advance or Legiolert, as rqmicro.COUNT depends on a different technological principle (flow-cytometry) that allows detection of all the viable cells and is not dependent on their cultivability. However, QIAcuity dPCR also measured significantly higher (up to three orders of magnitude) concentrations of *L. pneumophila* DNA copies in PM wastewater and this method does not depend on bacteria cultivability or viability. Moreover, the detection of *L. pneumophila* in negative controls (blanks in Figure 4-7Figure 4-3) which were sterilized tap water, undermines the accuracy of the results obtained with rqmicro.COUNT in this study. The lack of a significant difference between samples with different pre-treatments also indicates that the issue lays deeper than in the sample preparation. This could potentially be the result of autofluorescence or non-specific binding of minerals to fluorescent dyes in the tap water used for dilution and components present in wastewater itself (Veal et al., 2000). Exploring the off-target detection could potentially be an interesting next step in further evaluation of rqmicro.COUNT suitability for application in wastewater samples.

4.6.5 Comparison of the detection methods and the effect of pre-treatments

Different methods provided very different results for detected *L. pneumophila* concentrations in the tested wastewater samples. Generally, the culture-based methods, Legiolert and MICA Advance, detected significantly lower concentrations than the ISO 11731 standard, but all the results indicated more *L. pneumophila* cells in PM than in SH samples. While QIAcuity dPCR quantifies DNA copies which cannot be directly compared to the *L. pneumophila* cells concentrations measured with other methods, it also confirmed the fact that the PM samples contained more *L. pneumophila*. rqmicro.COUNT as the only method showed no difference in concentrations between the PM and the SH samples. However, the question still remains, whether the results provided by the ISO 11731 norm are representative to the actual *L. pneumophila* concentrations in wastewater, and whether it should be used as a factual qualitative reference point besides the comparison in trends and correlations, taking into account all the challenges with the method reliability in complex matrices. Moreover, considering that two out of three analyses performed by a professional lab in this study failed to provide any results due to microbial overgrowth, indicates that the ISO method as well requires a careful adaptation through selecting a dilution appropriate for different sample sources, and as seen in this study, this is not always done correctly. Considering that the three alternative methods consistently measured lower *L. pneumophila* concentrations could also indicate that ISO 11731 overestimated the real concentrations, either because the results were based on small numbers, because additional microbial growth occurred during samples transportation to the lab and/or storage before the measurement, or due to other unclear cause. Another option is that the pretreatment (centrifugation) causes the loss of L. pneumophila bacteria compared to the ISO method which does not have such a step. This is further discussed in chapter 8.1.

The effect of pre-treatments could be most clearly and consistently observed for QIAcuity dPCR method. The only pre-treatments that showed a significant effect were the combinations that included centrifugation. Adding centrifugation to the sample preparation protocol allowed reduction in noise and reaction inhibition in QIAcuity dPCR, but it also removed a considerable amount of *L. pneumophila* from the analysed fraction (*L. pneumophila* DNA copies removal between 1.2-log and 2-log compared to non-centrifuged samples as measured with QIAcuity dPCR). It might indicate that a big part of *L. pneumophila* cells and DNA is still bound to the solid fraction of the sample, despite application of sonication. Centrifugation has also reduced the amount of interfering microorganisms on MICA Advance membranes. However, it is not clear from the results whether it affects the concentration or condition of *L. pneumophila* cells. Moreover, including centrifugation could be beneficial, not only for increasing the *L. pneumophila* yield, but for optimization of the workflow as well, as high solids content in wastewater samples presents a clogging risk in the filtration step used by the three methods tested (with Legiolert as an exception).

Another important aspect to mention is the difference in the methods performance in analysing samples sourced from two different industrial WWTPs. Even though, QIAcuity dPCR provided significant results for both wastewater streams, the scatterplots still showed a lot of interference indicating a presence of potential contaminants and PCR inhibitors remaining after DNA extraction and purification. When no pre-treatment was applied, the amount of interference made it impossible to obtain viable results the SH samples, indicating that some wastewater streams might require further optimisation of DNA extraction and dPCR protocol. MICA Advance could detect *L. pneumophila* only in two SH samples, while providing measurements for most of the PM samples. However, if we neglect the notion that the *L. pneumophila* concentrations obtained with ISO 11731 method are correct, and they are actually overestimated as discussed above, this could mean that the analysis issues for MICA Advance in this study stem not only from the presence of interfering substances but simply from the fact that *L. pneumophila* concentrations in the SH samples were below its detection limit. This could also explain, why Legiolert analysis showed positive results for the SH samples, since its detection limit for non-potable samples is lower ($1x10^3$ CFU/L) than for MICA Advance.

This study shows that finding one general applicable method for *L. pneumophila* detection in wastewater sample might not be as straightforward as for clear water matrices like (potable) water. In wastewater treatment there are many variables that might be specific to individual WWTP facilities. Therefore, further investigation into various industrial wastewater streams, their characteristics and their effects on *L. pneumophila* detection methods, with extensive database, would be of interest.

4.7 Conclusions and recommendations

4.7.1 Conclusions

- Detection and quantification of *Legionella pneumophila* in a complex matrix such as industrial wastewater, remains challenging due to the presence of interfering microorganisms and contaminants.
- Two out of three wastewater samples could not be analysed using the ISO norm (matrix C) due to insufficient dilution and resulting interfering microflora, showing the limitations of this method.
- Lower *L. pneumophila* concentrations measured with Legiolert, MICA Advance and QIAcuity dPCR might indicate that the ISO method results were overestimated.
- The SH samples were more challenging to analyse by MICA Advance and QIAcuity dPCR than the PM samples, either due to their higher solids and organic matter content or *L. pneumophila* concentrations being below their detection limits.
- All methods, except for rqmicro.COUNT (no difference between PM and SH samples), showed higher concentrations of *L. pneumophila* in the PM samples.
- Legiolert method already contained a sample preparation protocol developed and optimised by IDEXX specifically for wastewater samples that served as a reference point for the tested pre-treatment strategies in this study.
- Despite the need for further optimisation, QIAcuity dPCR was the only method that produced interpretable and trend-consistent results for both wastewater streams in the pre-treatment experiments.
- Addition of pre-treatment steps had a significant quantitative effect in QIAcuity dPCR measurement, where samples treated with centrifugation showed considerably lower concentrations of *L. pneumophila* DNA copies and less interference in the dPCR reaction.

- Centrifugation did reduce the microbial growth on MICA Advance membranes, however, it is not clear how it affects the quantitative analysis. Potentially, if optimised it could improve readability of the membranes by the unit.
- The results obtained with rqmicro.COUNT showed no significant differences between pre-treatments but also between the PM and the SH samples. The latter in combination with the fact that all the negative controls tested positive make it not possible to verify whether the results were valid.

4.7.2 Recommendations

- The effect of wastewater matrices representative to relevant industries on *L. pneumophila* detection should be further studied to understand how different wastewater components affect the analysis.
- Additional sample pre-treatment, as this one developed for Legiolert, should be further studied as a potential optimisation step for the standard ISO 11731 method.
- ISO 11731 method requires further validation to prove that the quantitative data it produces is representative to real concentrations of *L. pneumophila* in wastewater.
- Centrifugation as a pre-treatment for MICA Advance and QIAcuity dPCR could be further studied to see whether it can be optimized to serve as a simple tool for minimizing interference caused by other microorganisms or contaminants present in wastewater samples.
- To improve readings by the MICA Advance unit, additional pre-treatment steps (e.g., improved acid and/or heat treatment) should be investigated to tackle microbial overgrowth on the membranes.
- To reduce the presence of inhibitors and contaminants that interfere with dPCR reactions, alternative DNA extraction kits should be considered (e.g., ones dedicated to soil) in combination with PCR additives, such as QIAGEN Q-Solution, in combination with dPCR reaction optimisation (i.e., temperature, primer and probe concentrations) for different wastewater streams.
- Off-target detection and the effect of autofluorescence should be further evaluated in the rqmicro.COUNT method to validate its suitability for application in wastewater samples.

4.8 References

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5 Phase 2 case study: Predicting *Legionella pneumophila* risk in industrial wastewater treatment plants – From periodic testing to data-drive risk insight

5.1 Overview

Industrial wastewater treatment plants (WWTPs) can create conditions that support the growth of *Legionella pneumophila*, particularly in biological treatment stages with elevated temperatures, nutrient loads, and aeration (Figure 5-1). In the open aerated systems this can lead to spread of aerosols containing *L. pneumophila* leading to increased risk of infections in the area, making industrial WWTPs potential sources for *L. pneumophila* outbreaks (Kusnetsov et al., 2010; Pijnacker et al., 2024).

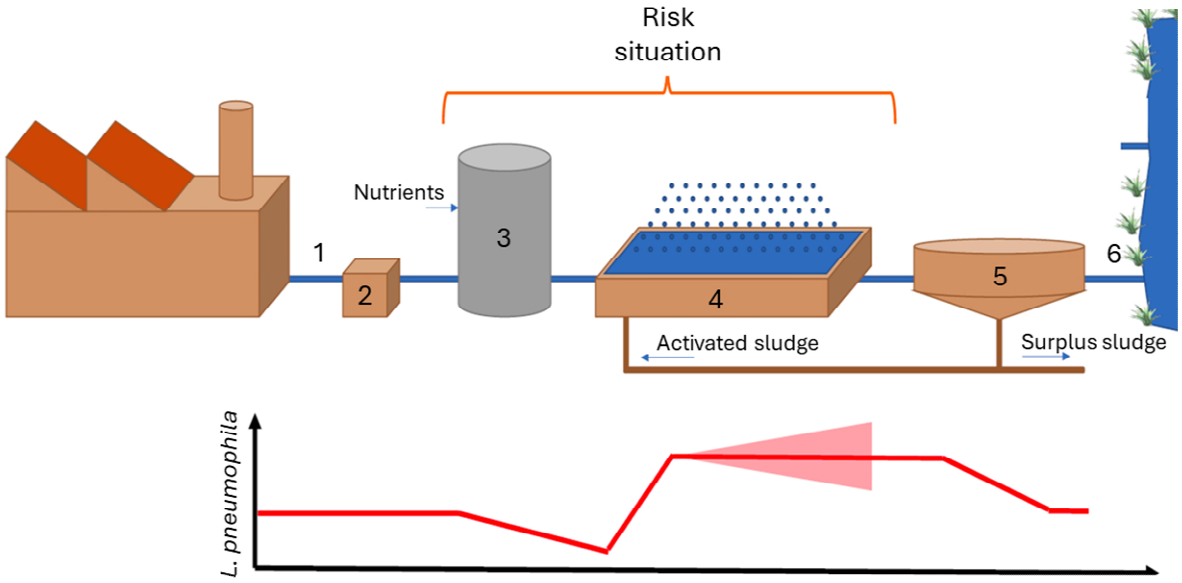


Figure 5-1. Typical hotspots for L. pneumophila *in industrial wastewater treatment systems. The L. pneumophila growth and dissemination risk increases in the aerated activated sludge step where the conditions promote its growth and aeration leads to its spread through aerosols. 1 – Effluent, 2 – Pre-filter, 3 – Anaerobic reactor, 4 – Aeration tank, 5 – Sludge separation, 6 – Effluent.*

Liquisens is a Belgian company specialized in predictive water monitoring using AI where it transforms water quality management through predictive intelligence, linking complex process data time series to laboratory results (Predictive Water Monitoring with AI). The first case study in this project that was carried by Liquisens in the WWTP context was at the facility of a large scale industrial pig slaughterhouse that has been recognized as a *L. pneumophila* outbreak source in 2017. This case study identified the most relevant parameters affecting *L. pneumophila* risk which consisted of a combination of general process parameters and indicators of specific ad-hoc process conditions. The process parameters included, for example, water temperature, which is known to directly affect the growth of *L. pneumophila* (Wallet et al., 2022). In addition, several influential indicators were linked to temporary changes in the incoming wastewater composition or treatment performance, such as proxy parameters for increased incoming protein load, signals indicating reduced performance of the Up-flow Anaerobic Sludge Blanket (UASB) reactor, and

indicators related to the performance of the denitrification step. In practical terms, this means that the measured elevated *L. pneumophila* concentrations were not associated with one single parameter, but rather with a combination of operating conditions and temporary process-related events that may have placed additional stress on the wastewater treatment system. This is where the added value of the model becomes particularly relevant: by analysing multiple parameters simultaneously and over time, the model can identify such combined patterns and interactions more consistently than would be feasible through manual interpretation alone.

The core challenge in developing a predictive model in a WWTP is learning from sparse laboratory data (i.e., *L. pneumophila* concentrations monitored in time) in a context with many operational input variables. Compared to managing drinking water systems, the variety of WWTPs parameters and operational conditions that can differ from facility to facility creates a very complex dataset to navigate. Moreover, *L. pneumophila* analyses are often carried out not more than four times a year which provides a limited historical input needed to identify potential correlations.

The work described in this case study was carried out in cooperation with research and knowledge institutes, consultancy agencies, technology providers and industrial partners as part of Phase 2 of the broader TKI project that focuses on deepening the understanding and control of *L. pneumophila* in industrial wastewater systems. By combining historical laboratory analysis with real-time process data, this work aimed to gather further input in the context of *L. pneumophila* risk in industrial WWTPs to move beyond periodic sampling and establish a foundation for predictive risk monitoring. The ultimate outcome would create a tool that allows WWTP operators to improve and speed up the decision-making process in *L. pneumophila* management field which would prevent hazardous situations before they have a chance to occur.

5.2 Approach

The testing location was a large-scale industrial food processing facility. The facility has been experiencing issues with elevated concentrations of *L. pneumophila* within their WWTP at the MBBR-based biological treatment stage (up to $3x10^6$ CFU/L). Because of these elevated levels, the project started with a comprehensive root cause analysis and risk classification to identify the event or contributing conditions that may have led to the increased *L. pneumophila* values, and to mitigate any potential hazard. The project integrated two primary data sources: (historical) laboratory measurements of *L. pneumophila* concentrations and SCADA data from the treatment process. A total of 37 *L. pneumophila* samples were collected between 02/10/2024 and 21/10/2025. *L. pneumophila* levels were determined in accordance with NEN-EN-ISO 11731. The SCADA data from the wastewater treatment plant – 221 features in total – was collected hourly from 15/09/2024 until 31/10/2025. Operational parameters such as flow rates, pH, oxygen levels, and system status indicators were analyzed in relation to *L. pneumophila* measurements within the MBBR-based biological treatment stage. The limited dataset of 37 samples provided initial direction but required careful interpretation due to its limited size.

A model was developed specifically for the risk classification of *L. pneumophila*, distinguishing between low, moderate, and high-risk conditions. These risk categories are defined by specific thresholds: $<5x10^5$ CFU/L for low risk, $5x10^5$ - $5x10^6$ CFU/L for moderate risk, and $>5x10^6$ CFU/L for high risk. The risk categories were defined to create a balanced distribution of the measurement results across the different classes.

The analysis relied on embeddings as compact representations of the multivariate temporal behavior of the system. These embeddings were used to evaluate the process conditions in the days preceding each *L. pneumophila* measurement and to identify the factors associated with the observed concentrations. In addition, the analysis examined which time series and temporal windows should be included to adequately characterize the trends and evolution of all relevant features over a sufficiently long period. In addition to data analysis, operator input was

incorporated to contextualize production events such as cleaning cycles, load variations, and operational disturbances.

5.3 Results

The analysis showed that *L. pneumophila* risk is not random but linked to specific process conditions within the treatment system, particularly within the biological MBBR (Moving Bed Biofilm Reactor) stage. Figure 5-2 shows two-dimensional visualization of the learned embedding space, where each point represents a *L. pneumophila* measurement sample and colors indicate the corresponding risk category. The circles represent the extent of each cluster and illustrate the absence of overlap between categories, indicating good class separability. In addition, clear relationships were identified between production events (for example incidental discharges) and elevated *L. pneumophila* risk levels, indicating that upstream operational dynamics can disrupt the microbial balance within the system, thereby impacting *L. pneumophila* risk.

Key parameters such as pH, organic load indicators, and sludge-related variables were identified as relevant drivers influencing *L. pneumophila* risk levels, which is in agreement with the previous findings about wastewater parameters effect on *L. pneumophila* growth (Caicedo et al., 2016). The model demonstrated that it is possible to distinguish between different risk categories using process data, providing early indications of conditions that may lead to elevated *L. pneumophila* concentrations.

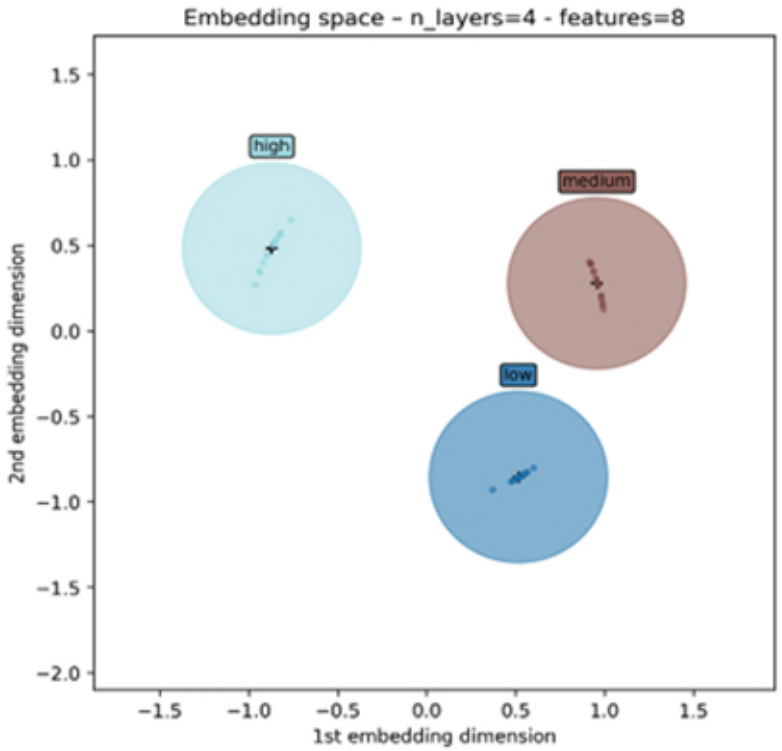


Figure 5-2. Two-dimensional visualization of the embedding space learned by the model, with samples colored according to L. pneumophila *risk category.*

Figure 5-3 illustrates how the model enables the identification of the main drivers of elevated *L. pneumophila* risk for a selected sample. The black point represents the selected sample in the embedding space, whereas the red cross denotes the characteristic pattern of the medium-risk *L. pneumophila* category. By analyzing the direction and

magnitude of change required to move the selected sample toward this target profile, the model identifies which input features have the greatest influence on this transition. Due to confidentiality constraints, the eight most influential parameters cannot be disclosed in detail. However, it can be stated that the four most relevant parameters could each be linked to ad-hoc process events resulting in a substantially increased organic load to the wastewater treatment system. These findings provide insight into which process-related conditions were most strongly associated with the observed situation and may therefore be the most relevant to consider for mitigation.

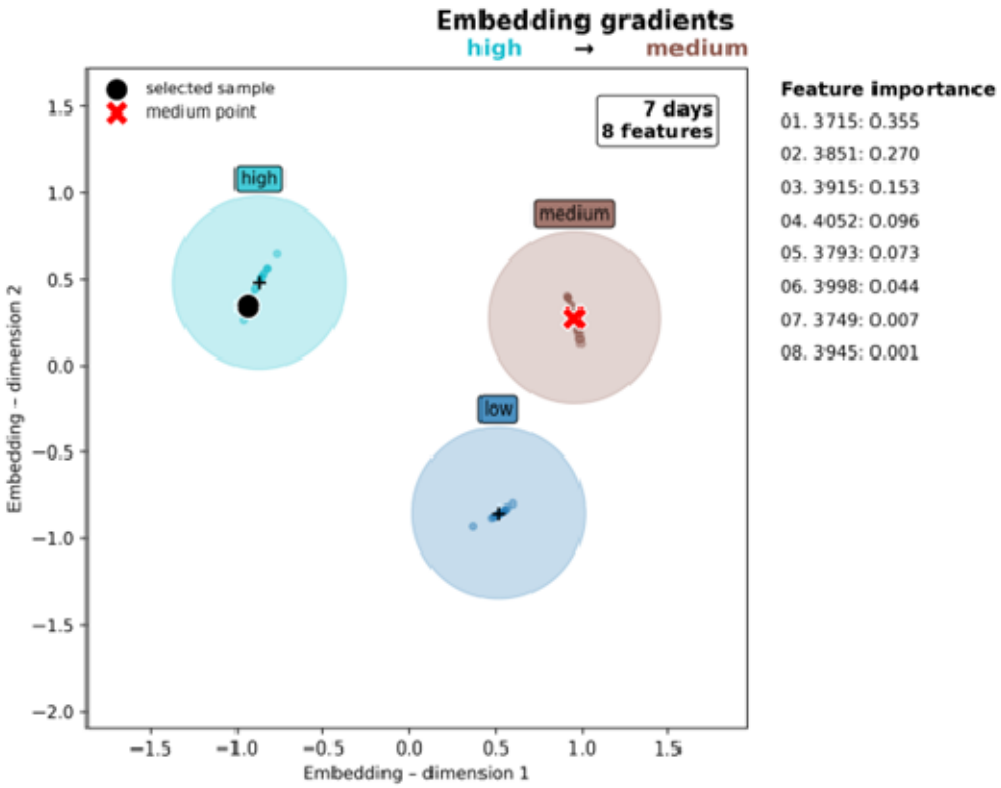


Figure 5-3. Embedding-based gradient analysis for the transition from a selected high risk sample to a medium-risk L. pneumophila pattern.

5.4 Discussion

A comparison with the findings from the case study in this project at the WWTP of the industrial slaughterhouse facility can be done at a conceptual level. In both cases, elevated *L. pneumophila* values could be associated with specific ad-hoc process events or with operational conditions under which the wastewater treatment system was operating close to its process limits. The main similarity is therefore that the elevated values were not interpreted as isolated observations, but in relation to temporary changes in process behavior, organic loading, or operational stress on the treatment system. The main difference is that the specific parameters, process dynamics, and operational context differ between sites. As a result, the relevant triggers and mitigation priorities need to be assessed on a site-specific basis, even if the underlying interpretation is comparable.

The models developed in this use case cannot be transferred one-to-one to another wastewater treatment plant, as they take into account several site- and production-specific factors, including local operating conditions, wastewater characteristics, process configuration and production-related dynamics.

However, the underlying data engine and modelling approach are designed to be reusable. This means that similar models can be developed for other WWTPs by configuring the engine to the specific data streams, process parameters and operational context of that site, without having to rebuild the full methodology from scratch.

The results show that process variability and production-related changes need to be interpreted carefully, particularly because sampling was limited. Further validation is therefore needed. Moreover, these analyses need to be carried out on a site-specific basis, since process behavior, operational conditions, and variability differ from one treatment facility to another. At the same time, the underlying data engine is universally applicable and provides a transferable framework for conducting these analyses across different facilities.

5.5 Conclusions

This case study further demonstrates the potential of combining laboratory analysis, process monitoring, and operational expertise to better understand and control *L. pneumophila* dynamics in industrial wastewater treatment. While the dataset remains limited, the findings confirm that process data can be used to identify conditions associated with increased risk and support the development of early warning strategies. The approach provides a first step toward predictive, risk-based monitoring, enabling operators to move beyond reactive testing and gain deeper insight into the drivers of microbial risk within biological treatment systems. Further steps will require the model validation to verify whether *L. pneumophila*-related events can be correctly predicted using this approach.

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6 Phase 2: Wastewater treatment plant effluent preparation for the UVOX system as a control measure for *Legionella pneumophila* spread

6.1 Introduction

One of the aims of the TKI project "Reliable detection and control of *Legionella pneumophila* in wastewater" was to develop and test new potential mitigation measures for *Legionella pneumophila* in wastewater treatment plants (WWTPs). One of the pathways for *L. pneumophila* spread into the environment is through discharge of the WWTP-effluent to water bodies (Nogueira et al., 2016; Olsen et al., 2010). This is especially risky when the contaminated surface water is used e.g. as cooling water in cooling towers in which *L. pneumophila* can further grow and spread to the environment through aerosolization. Application of ultraviolet (UV) light disinfection of those effluent streams has been shown effective against *L. pneumophila* (Nogueira et al., 2016). The purpose of this study was to test the suitability of UVOX Redox disinfection technology for *L. pneumophila* mitigation in the context of WWTPs. Unfortunately, due to closing of the WWTP selected for this study, this line of research was discontinued. This report provides a summary of preparations that were made before the information about the closing of the facility was received.

6.1.1 UVOX Redox

UVOX Redox combines three disinfection processes within a single reaction chamber. Figure 6-1 provides a schematic representation of the system. Air (green arrows) is drawn into the top of the unit where it is irradiated with UV light at 185 nm. This irradiation converts oxygen (O_2) into ozone (O_3^-; red arrows). The ozone-enriched air is then mixed with the wastewater (blue arrows) which is the first step in the inactivation of microorganisms and oxidation of micropollutants. Subsequently, the wastewater containing ozone is recirculated back into the reaction chamber, where it is directly irradiated with 254 nm UV light. This irradiation provides an additional disinfection effect on its own, but also simultaneously converts ozone into hydroxyl radicals (purple arrows) increasing the oxidation potential, and thereby the disinfection effectiveness of the system (UVOX Redox, 2023). So far, the main application area of the UVOX Redox was in swimming pools (Ekowati et al., 2019), but it has also proven to be effective in pharmaceuticals removal from liquid digestate (Moradi et al., 2024). Disinfection, and specifically deactivation of *L. pneumophila*, in wastewater streams poses additional challenge, mainly due to its high turbidity and consequently, low UV transmittance (UVT) compared to water in swimming pools. Moreover, the high level of organic matter also causes a high ozone demand. While ozone formation and ozonation are independent of the UVT of treated water, the effectiveness of disinfection with UV light and formation of hydroxyl radicals strongly depends on UVT values greater than 90%, a level rarely achieved in untreated WWTP effluent. For that reason, to effectively test the efficiency of UVOX Redox on WWTP effluent, additional pre-treatment is required.

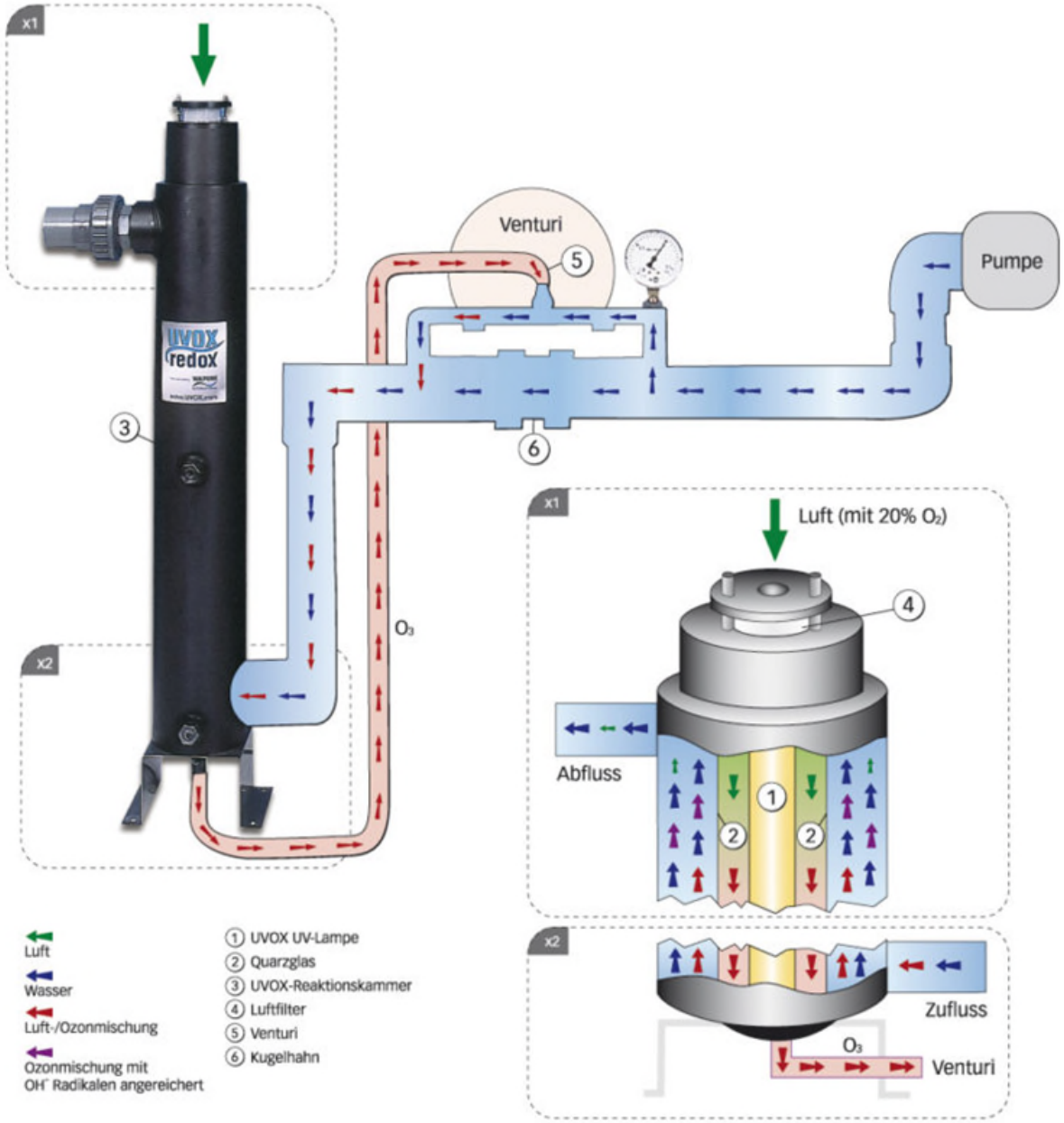


Figure 6-1. Schematic representation of the UVOX Redox system (UVOX Redox, 2023).

6.1.2 Granular activated carbon (GAC)

GAC is a porous medium used for the removal of organic micro- and macro-pollutants from water and wastewater through adsorption onto its extensive internal surface area. It is produced from carbon-rich materials (e.g., wood, coal, or coconut shells) which are pyrolyzed by heating to approximately 500°C in the absence of oxygen. This process carbonizes the material and generates pores. Subsequently, the material is activated through treatment with steam or chemical agents, creating an extensive porous network and increasing the surface area (Altmann et al., 2016). In this study, it was selected as a pre-treatment for the UVOX Redox system as a cheap and effective approach. Due to its adsorption potential, it might also remove a fraction of *L. pneumophila* from the effluent (Rajapaksha et al., 2018).

6.2 Materials and methods

To evaluate the effect of GAC on the effluent UVT, effluents from the WWTPs of a paper mill (PM) and pig slaughterhouse (SH) were collected on March 10, 2025. Two different tests were performed to verify whether the GAC has any effect on the effluent UVT and if so, what is the effective contact time (CT) to achieve an UVT of minimum 90%. The latter was performed using the GAC filtration column. Because it was agreed that the PM plant will be the UVOX Redox testing location, its effluent was selected to be tested in this experimental part. The GAC used in this study came from 8x30 mesh giving particle sizes of 0.59-2.38 mm as described by the manufacturer (Medaad Adsorption Chemicals).

6.2.1 Batch test

Wastewater from the two locations was distributed in volumes of 250 mL into Erlenmeyer flasks with 0 g GAC/L, 1 g GAC/L, and 10 g GAC/L. The flasks were placed on a shaker and agitated at 100 rpm. UVT was measured at 30 min, 1 h, 2 h, 4 h, and 24 h. Before each measurement, GAC particles were removed by brief spinning at 1000 ×*g* for 1 min.

6.2.2 GAC filtration column

The aim of this test was to determine a sufficient CT required to achieve a UVT of 90%. A column filled with GAC was employed for this purpose. The set-up and the column dimensions are illustrated in Figure 6-2. PM WWTP effluent was pumped from the bottom of the column upward through the GAC medium using a peristaltic pump. The CT was controlled by adjusting the pump flow rate. After passing through the GAC, the treated effluent was collected in an Erlenmeyer flask. The effects were evaluated at CTs of 40 s, 50 s, 60 and 120 s. After collection, the treated effluent was centrifuged for 1 minutes at 1000 ×*g* to remove GAC particles. The UVT of the supernatant was subsequently measured using a UV–Vis spectrophotometer.

6.2.3 L. pneumophila removal in the GAC filtration column

Once an appropriate CT was established, the *L. pneumophila* concentration was assessed before and after the GAC filtration using the different detection methods to evaluate the removal efficiency of GAC. All measurements were performed following the manufacturer's instructions (as described in chapter 2.3.1.5). For MICA, rqmicro.COUNT, and QIAcuity, 20 mL of the effluent treated with GAC was used, whereas for Legiolert, 2 mL of that effluent was used. All measurements were conducted in duplicate.

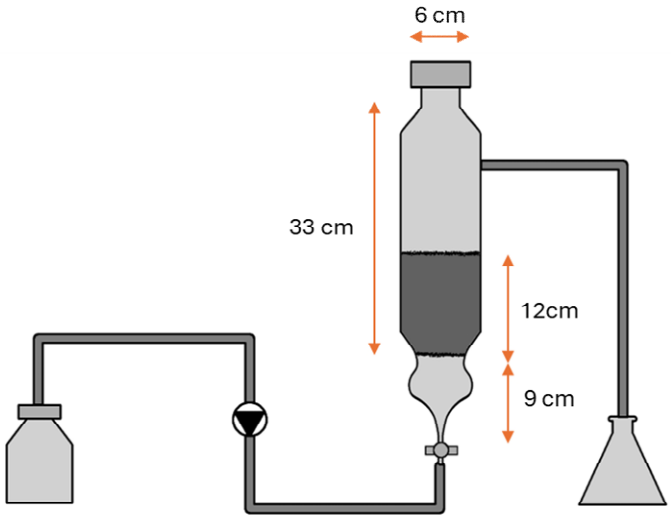


Figure 6-2. Schematic representation of the GAC filtration column. The total volume of added GAC was 340 cm³.

6.3 Results and discussion

To assess whether the GAC influences the UVT of the WWTP effluent, PM and SH effluent samples were mixed with different concentrations of GAC. The results are presented in Figure 6-3. The initial UVT of PM and SH effluents were 13% and 21%, respectively, indicating that none of the streams could be directly treated with the UVOX Redox technology. After contact with 1 g GAC/L, only minimal increase in UVT was observed in the samples. More pronounced changes in UVT were observed in samples containing 10 g GAC/L, where the UVT gradually increased over time reaching 83% for the PM effluent and 78% for the SH effluent after 24 h. These findings proved that GAC treatment has a positive effect on UVT of the selected effluents and therefore, further testing was performed using the GAC column. In the column, GAC is more concentrated and the contact times required to achieve UVT of 90% were expected to be much shorter.

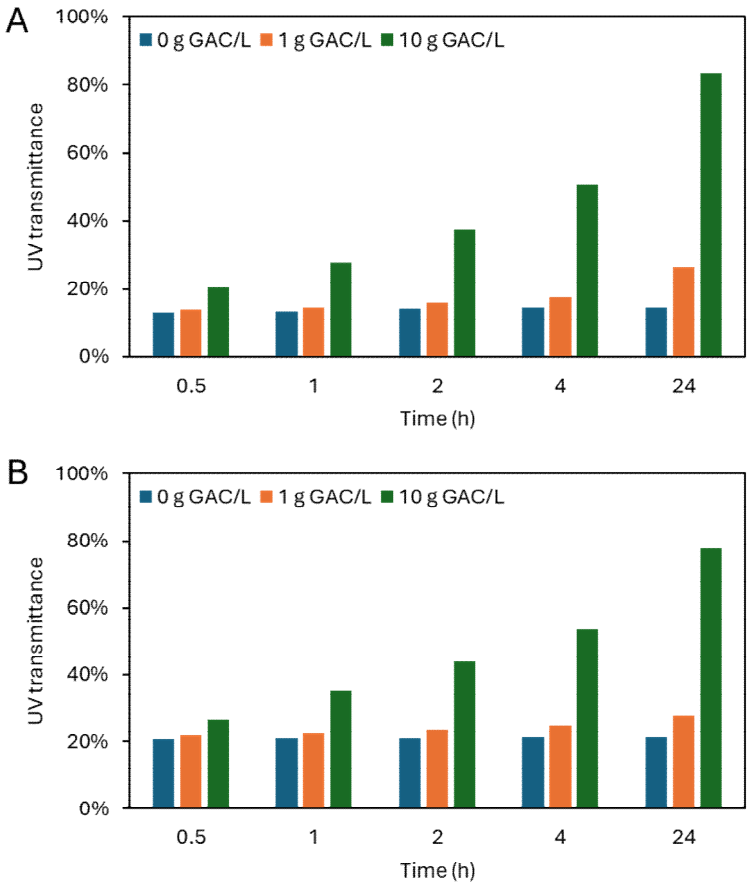


Figure 6-3. UVT in (A) PM and (B) SH effluents after contact with different GAC concentrations.

Figure 6-4 presents the UVT of the PM effluent measured after different CTs in the GAC filtration column. The required minimum UVT of 90% was achieved already after 50 s (90.1%), showing that GAC filtration is an excellent tool to reduce UVT of industrial WWTP effluents. For the UVOX Redox experiments, contact time of 60 s (91.5%) was recommended.

Since, GAC also adsorbs bacteria (Busscher et al., 2008), the concentrations of *L. pneumophila* were tested after the treatment with the CT of 60 s. The removal rates based on the concentrations measured with the four alternative *L. pneumophila* detection methods are presented in Figure 6-5. MICA Advance and rqmicro.COUNT showed comparable removal rates of 0.33-log and 0.28-log, respectively. However, it is important to note that the values detected by MICA Advance ($3x10^4$ CFU/L before filtration) were three orders of magnitude lower than those of rqmicro.COUNT ($2x10^7$ cells/L before filtration). Interestingly, QIAcuity dPCR showed much higher removal of *L. pneumophila* DNA of 1.3-log ($2x10^5$ DNA copies/L before filtration). With the Legiolert no *L. pneumophila* was

detected, potentially due to the expired media used in this experiment. Combination of these findings could indicate that while the PM effluent still carried *L. pneumophila*, some fraction might be present in a VBNC or dead state. However, those results are preliminary and further validation with a wider data set on a bigger scale is needed.

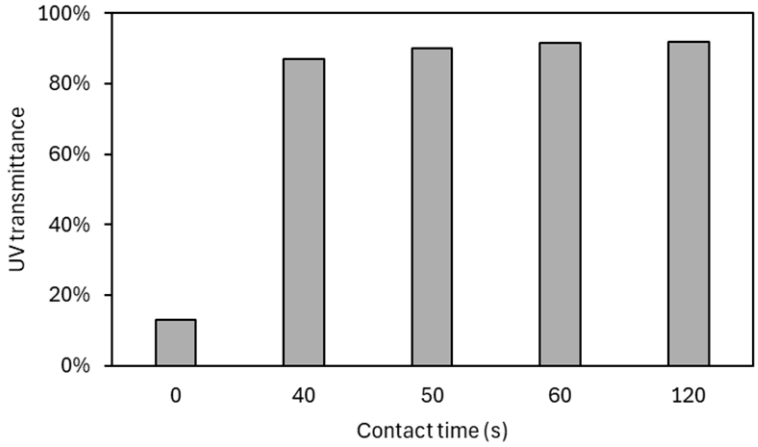


Figure 6-4. UVT of PM effluent after filtration through the GAC column at different contact times. UVT of 90% is a threshold for the effluent quality to be treated with the UVOX Redox unit.

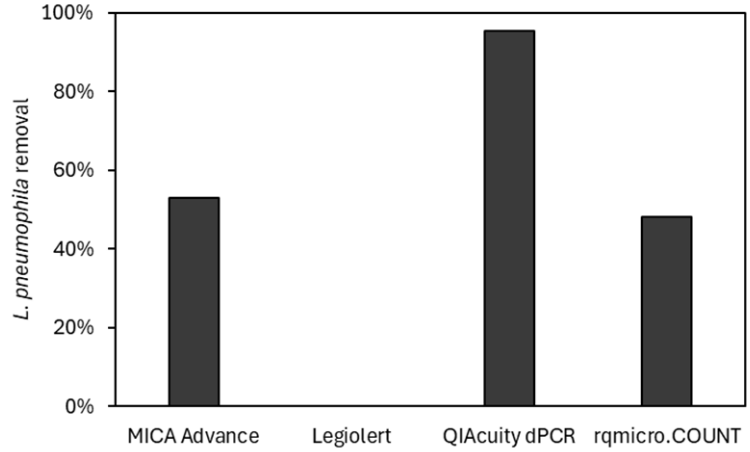


Figure 6-5. L. pneumophila *removal after the GAC column treatment measured with the four alternative detection methods. The Legiolert method was unable to detect L. pneumophila in all the measured samples.*

6.4 Conclusions

- Industrial WWTP effluent cannot be directly treated with the UVOX Redox technology as its UVT values are far below the required UVT of 90%.
- GAC column filtration increased UVT of the PM effluent from 13% to 91.5% in 60 s of contact time. This indicates that GAC filtration could serve as an effective and relatively cheap pre-treatment step for UVOX Redox in (waste)water streams of low UVT.
- GAC filtration could also be implemented as a supporting tool for limiting *L. pneumophila* spread through WWTPs effluent, as it showed to slightly reduce its concentrations. However, the reduction is limited and needs further validation through bigger scale experiments.

6.5 Literature

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7 Phase 3: Online knowledge database

7.1 Introduction

The wastewater treatment plants (WWTPs) being potential hotspots for *L. pneumophila* outbreaks have emerged as an important topic for wastewater treatment facilities, industrial water users, consultants, researchers, and regulatory authorities. Over the past years, increasing attention has been paid to the occurrence of *L. pneumophila* in wastewater systems and the associated occupational and environmental risks. Despite the growing body of knowledge, information remains dispersed across numerous sources, including scientific publications, guidance documents, industry reports, and websites maintained by different organizations. This fragmentation makes it challenging for stakeholders to identify reliable and up-to-date information and determine which sources are most relevant to their specific needs.

To address this challenge, one of the dissemination activities within the TKI project "*Legionella* in wastewater" focused on the development of an online knowledge platform. The objective of the platform is to centralize available knowledge, improve its accessibility, and provide a sustainable resource for all stakeholders involved in assessing, managing, and communicating risks related to *L. pneumophila* in wastewater systems.
This report summarizes the activities carried out during Phase 3 of the project, covering the period from February to August 2026, and highlights the progress made in the development of the website www.legionellainafvalwater.nl.

7.2 Development of the knowledge platform

The development of the online platform was initiated with the aim of creating a user-friendly and future-proof knowledge database dedicated to *L. pneumophila* in WWTPs. The website was designed to function as a central information hub where stakeholders can access both existing knowledge and project-generated insights in a structured and intuitive manner.

The technical foundation of the website was developed by an external web development company (IDS Internet, Leeuwarden) using the WordPress content management system. WordPress was selected because of its flexibility, accessibility, and suitability for future maintenance and content updates. During the initial development phase, multiple graphic design concepts were prepared and presented to the project consortium. Following discussions with project partners, a final design was selected that balances professional appearance, usability, and accessibility (Figure 7-1).



Figure 7.1. Homepage of the Legionella in wastewater platform.

The website can be accessed via www.legionellainafvalwater.nl and is intended to become the primary online resource for information related to *L. pneumophila* in wastewater in the Netherlands.

7.3 Knowledge structure and content development

The content development was coordinated by researchers from CEW. Existing knowledge was collected, reviewed, and transformed into web-based content that is easier to navigate than traditional reports and technical documents.

A key starting point for the knowledge structure was the Hydroscope knowledge document "*Legionella* in Afvalwater" (https://www.hydroscope.nl/nieuws/kennisdocument-legionella-in-afvalwater-v13/), which currently represents one of the most comprehensive publicly available overviews of the topic. Rather than reproducing the document in its original format, the project team aimed to translate the information into a more interactive and user-friendly online environment. This approach allows visitors to quickly locate relevant information without the need to navigate lengthy PDF documents.

To support the conversion of extensive technical content in the Hydroscope document into website-friendly text, Generative AI tools, including Microsoft Copilot, were used during the content development process. These tools assisted in restructuring and adapting the information for online presentation, resulting in content that is more accessible, concise, and suitable for web-based communication. All AI-generated and AI-assisted content was subsequently reviewed, edited, and validated by CEW researchers to ensure technical accuracy, consistency, and alignment with the original source material. In addition, feedback provided by project partners was incorporated into the final content, helping to ensure that the platform reflects both current scientific knowledge and practical experience from the sector.

The platform combines existing knowledge on the topic with information generated within the TKI project itself. In addition to technical and scientific content, the website also presents information about the project objectives, project partners, and project outcomes. In this way, the platform serves both as a knowledge repository and as a dissemination tool for project results.

The current website structure includes or foresees:

- thematic knowledge pages on *L. pneumophila* in wastewater,
- a Frequently Asked Questions (FAQ) section,

- links to external resources and reference materials,
- downloadable documents and reports (including the project report),
- information pages dedicated to project partners,
- contact information of experts for visitors seeking additional support or information,
- link to the Hydroscope document.

7.4 Stakeholder involvement and review process

To ensure that the website meets the needs of its intended target groups, the draft version of the platform was shared with the project consortium for review and feedback.

A review process was conducted involving the project partners, who evaluated the content, structure, and usability of the website prior to its public launch. The comments and suggestions received were used to further optimize the website content, functionality, and overall user experience.

This collaborative review process helped ensure that the platform addresses the information needs of a broad range of stakeholders, including wastewater treatment plant operators, industrial companies, consultants, laboratories, researchers, and policymakers. By incorporating both scientific expertise and practical experience from the sector, the platform was refined into a more relevant and user-oriented knowledge resource.

7.5 Accessibility and dissemination

Although the primary content has been developed in Dutch, the website includes an integrated language-switching functionality that allows visitors to automatically translate the content into English. This feature increases accessibility for international stakeholders and facilitates the dissemination of project results beyond the Dutch water sector.

The website is scheduled to be officially launched with the end of the project in November 2026. Following the launch, the platform will be promoted through communication activities associated with the TKI project. The website will also be referenced in an impact article published through the TKI program, thereby increasing visibility among relevant stakeholders and contributing to broader dissemination of project results.
Long-term sustainability

An important consideration during development was the long-term sustainability of the knowledge platform. While the website is currently hosted and managed by CEW, discussions have taken place regarding its future maintenance after completion of the TKI project.

The intended long-term scenario is that Hydroscope assumes responsibility for the continued management and updating of the platform. The exact timeline and practical arrangements for this transfer will be determined at a later stage. Through continued maintenance and periodic content updates, the platform can remain a relevant and authoritative source of information as new research findings, guidelines, and experiences become available.
This long-term perspective is particularly important given the evolving nature of knowledge related to *L. pneumophila* in wastewater and the ongoing need for accessible and reliable information within the sector.

7.6 Conclusion

During Phase 3 of the TKI project "*Legionella* in wastewater", significant progress was made towards the development of a centralized online knowledge platform. The technical framework was established in WordPress, website content was developed and structured by CEW researchers, and a final graphic design was selected in consultation with project partners. The platform addresses a clear need within the sector by bringing together fragmented information and presenting it through an accessible and user-friendly online environment.

The website not only consolidates existing knowledge but also serves as a dissemination platform for the outcomes of the TKI project and the expertise of the participating partners. With the planned launch in November 2026 and the prospect of long-term maintenance by Hydroscope, the platform represents an important and sustainable project result that will continue to support knowledge sharing and awareness regarding *L. pneumophila* in wastewater beyond the duration of the project.

8 Overall discussion

8.1 Application of *L. pneumophila* detection methods on wastewater

Four different methods, designed for detecting *L. pneumophila* in drinking water, were tested in this study to detect *L. pneumophila* in wastewater: Legiolert from IDEXX, rqmicro.COUNT from rqmicro, MICA from Diamidex, and Qiacuity dPCR from QIAGEN. The methods were tested using i) dilution series of wastewater with high *L. pneumophila* numbers into filtered wastewater, ii) sampling of three warm partial streams of WWTPs for six weeks, and iii) testing of different pretreatment steps.

The four different methods were shortly discussed in the respective chapters of phase 1 (chapter 2) and phase 2 (chapter 4, pre-treatments). In this chapter the results are combined, and the following questions are discussed:

- How do the detection methods behave in phase 1 and 2?
- Which methods are suitable for wastewater and which are not?
- What should be further tested?

Above all it should be noted that the number of experiments is moderate with four dilution series and six monitoring rounds of three WWTPs in phase 1, and several pre-treatment tests in phase 2.

8.1.1 ISO method

The current standard method for *L. pneumophila* detection is the culture-based ISO 11731:2017 method. While widely accepted, this method has several limitations: i) a relatively long incubation period of 7 to 14 days, ii) the method relies on *Legionella* growth on selective agar media, iii) overgrowth of background microorganisms, leading to high detection limits when dilutions are required.

In phase 1 (dilution series and 6-week monitoring) and phase 2 (pre-treatment) the results obtained with the ISO method showed consistently higher *L. pneumophila* concentrations than the Legiolert and MICA. While this difference between the ISO method and Legiolert or MICA was not observed for cleaner water samples (both potable and non-potable water; described in chapter 8.1.3 and 8.1.2). The hypothesis is that this is caused by the difference in the pre-treatment of the three methods. Whereas the Legiolert and MICA remove particles from the water sample by decantation or centrifugation as they interfere with the analysis, this step is not included in the protocol of Matrix C of the ISO method. As the Legiolert and MICA detect lower *L. pneumophila* numbers, this suggests that with decantation or centrifugation not only particles are removed but also *L. pneumophila* attached to the particles, or that the particles take along the *L. pneumophila* bacteria and are removed from the supernatant. This is in agreement with the findings from the pre-treatment experiments where QIAcuity dPCR, showed significant decrease in *L .pneumophila* gene copies in all the samples where centrifugation was applied. The Legiolert and MICA could underestimate the *L. pneumophila* concentration. Another option could be that the ISO method overestimates the number of *L. pneumophila* bacteria, but this seems less likely as colonies are confirmed to be *L. pneumophila* using PCR. These hypotheses have to be confirmed by further testing.

The ISO method was less sensitive than the Legiolert, QIAcuity dPCR and MICA, which both did detect *L. pneumophila* in low numbers when the ISO method did not. In some cases no result with the ISO method was obtained due to overgrowth and analysis of incorrect dilutions. This points to a drawback of the ISO method, that it can be needed to analyze many different dilutions of the water sample to obtain a good result.

8.1.2 Legiolert

In the dilution series and in the experiments of phase 2, Legiolert showed a consistent pattern. The results showed consistently lower *L. pneumophila* concentrations than the ISO method, and this difference increased at higher *L. pneumophila* concentrations. This is in contrast with the validation of the Legiolert which is published as a standard method by ASTM international (International 2021). In another study, in potable and non-potable water samples (containing lower biomass and solids content than wastewater) Legiolert detected slightly higher *L. pneumophila* concentrations than the ISO method (Spies, Pleischl et al. 2018; Checa, Carbonell et al. 2021). This could be caused by the centrifugation step in the pre-treatment of wastewater but not potable water samples. Due to the centrifugation some of the *L. pneumophila* bacteria apparently are collected in the pellet and therefore not enumerated in the Legiolert (for which the supernatant is used).
In wastewater, Legiolert has a lower detection limit as it detected *L. pneumophila* to either the same or lower concentration than the ISO method. The detection limit of Legiolert was lower than the ISO method and was equivalent to 1.2×10^3 to 6.3×10^3 CFU/L (as based on the ISO method).

Before the start of the project, IDEXX developed the pre-treatment protocol for wastewater samples that was used in phase 2 as a reference point for other *L. pneumophila* detection methods. In general, Legiolert performed well, although the pre-treatment suggested by IDEXX does require extra laboratory equipment making the method more elaborate compared to the Legiolert for potable water. Moreover, the use of centrifugation should be looked further into as it might be the cause of lower *L. pneumophila* concentrations than those detected by the ISO method. Further studies comparing Legiolert to the ISO method for wastewater analysis are needed to confirm the difference between these two methods.

8.1.3 MICA

In the dilution series, the results obtained with MICA showed consistently lower *L. pneumophila* concentrations than the ISO method. This is in contrast to earlier measurements in potable water in which the results of MICA and the ISO method are comparable (Passot, Peslier et al. 2022; Hambsch 2022). For cooling water samples, MICA results turned out to be slightly closer to the theoretical *L. pneumophila* concentration introduced by inoculation than the ISO method (Passot, Peslier et al. 2022). In wastewater, the MICA system had a similar or lower detection limit than the ISO method as it detected *L. pneumophila* to either the same or lower concentrations than the ISO method. The detection limit of the MICA was lower than the ISO method and was equivalent to 1.2×10^3 to 6.3×10^3 cfu/L (as based on the ISO method). This is higher than for potable and cooling tower water (100 cfu/liter; (Passot, Peslier et al. 2022)) but still lower than the ISO method.

In the dilution series, the MICA system struggled to reliably quantify the higher *L. pneumophila* concentrations. This could be caused by the high *L. pneumophila* concentration (above the upper detection limit) and/or by a higher concentration of interfering components (as the dilutions were prepared in filtered wastewater, each dilution theoretically contained less of the filterable components). As the MICA system showed comparable results to the ISO method and QIAcuity dPCR in the weekly monitoring of wastewater from full-scale plants, it appears that MICA handles higher concentrations of *L. pneumophila* in practice better than it did during the dilution series. A possible explanation could be that by chance the composition of the wastewater used in the dilution series was less favourable for the MICA system than the wastewater derived from the three WWTPs that were monitored weekly. In phase 2, an improved MICA Advance unit was used in the pre-treatment experiments. With this system the analysis principle remained the same but it provided an advantage of improved work-flow and increased sample load that could be processed within a day. While no significant effect of the selected pre-treatments was observed, the visual inspection of the membranes in this study confirmed the presence of interfering microbial overgrowth and also showed uneven distribution of wastewater components (often in forms of aggregates) on the membrane which could affect the readings by the MICA system. In consultation with Diamidex, it has been agreed that

additional pretreatment (e.g., improved acid treatment) should be considered and tested to reduce that interference.

The MICA system was designed and validated for the analyses of hot (potable) water samples and cooling tower waters (Hambsch 2022, Passot, Peslier et al. 2022), showing to be statistically comparable to the ISO method with a lower sensitivity. In this project, it was applied for the first time to wastewater samples. In general, the MICA system performed well, although improvements are perhaps needed to detect high *L. pneumophila* concentrations in wastewater and to solve possible interference from wastewater components. Diamidex is using the results from this study to improve on this aspect.

8.1.4 QIAcuity dPCR

In the dilution series, the QIAcuity dPCR could detect *L. pneumophila* to either the same concentration or lower concentration than the ISO method, and thus seems more sensitive than the ISO method. The detection limit was quite variable, being equivalent to 6.3×10^{1} to 1.3×10^{5} cfu/L (as based on the ISO method). However, even the upper part of this range would represent a slightly higher sensitivity compared to the ISO method. The QIAcuity dPCR detects both viable and dead cells, whereas the ISO method only detects viable and culturable cells. As the ratio between viable and dead cells may vary for each wastewater and sampling moment, this may explain the larger range in detection limit for the QIAcuity dPCR. For each individual dilution series, for which the viable:dead ratio is similar for each dilution, QIAcuity dPCR did show a consistent dilution pattern, suggesting that this ratio indeed may cause the difference in detection limit. Consequently, it seems not yet possible to calculate the number of *L. pneumophila* bacteria from the number of gene copies.

The different pre-treatments did not improve the analyses, and while centrifugation did remove impurities and reduce interference, it also lowered the *L. pneumophila* concentrations. This indicated that some wastewater components negatively affect the QIAcuity dPCR reaction. Further experiments testing the effect of other DNA extraction kits (e.g., for soil), the PCR gradient, or implementation of PCR reaction additives (e.g., Q Solution by QIAGEN) are recommended for improved QIAcuity dPCR performance in wastewater samples.

In general, the QIAcuity-dPCR performed well in quantifying *L. pneumophila* in wastewater samples. It is important to expand the dataset to investigate whether a constant and reliable ratio exists between gene copy numbers per liter and cfu per liter, that can be used to translate the dPCR results to cfu/L values. Also, it should be noted that if a disinfection procedure is used to kill off *L. pneumophila* (and other microorganisms) the effect might not be measurable with the QIAcuity as, depending on the disinfection procedure, the DNA of *Legionella* might be undamaged and detectable even though the bacterial cell itself is not viable anymore. According to QIAGEN, more work is being done in this direction through removing free DNA before the analysis, or alternatively, using RNA targets as they are expected to degrade quickly after a bacterium dies.

8.1.5 rqmicro.COUNT

The rqmicro.COUNT did not give reliable results with the dilution series. For two (out of four) dilution series several technical errors were encountered resulting in failed measurements. For the two other dilution series continuously high numbers of *L. pneumophila* were detected whereas this was not the case with the other detection methods. Something similar was observed in the pre-treatment tests where the *L. pneumophila* numbers were high in wastewater samples from both industrial locations while all the other methods showed significantly higher numbers in one of the two facilities. Moreover, in phase 2, no significant differences were observed between the samples with different pre-treatments, while it was not the case for MICA Advance, nor QIAcuity dPCR. Considering that also blank samples gave a positive signal, we hypothesize that these high *L. pneumophila* counts are caused by off-targeting, such as autofluorescence or non-specific binding of the fluorescent antibodies to components in the wastewater or tap water used for dilution. Although no experiments were performed to further substantiate this hypothesis, this could potentially be an interesting next step in the further development of rqmicro.COUNT for

application in wastewater samples. Rqmicro suggested using a specific acid pretreatment of the samples, which could not be implemented in phase 1 of this study. Several pretreatment steps during sample preparation (different combinations of centrifugation, heat treatment and sonification) were tested in phase 2, but this did not lead to a clear improvement in the results indicating that the issue might be deeper than only in the sample preparation.

In scientific literature, the rqmicro.COUNT was applied for detecting *L. pneumophila* in aerosols (captured using the Coriolis system) (Heining, Welp et al. 2023), mineral water, artificial process water and cooling water (Redwitz, Streich et al. 2024; www.rqmicro.com). Despite this, the water matrix of wastewater with high biomass load and possible interfering components could not yet be reliably measured with the rqmicro.COUNT. The current method is therefore not yet suitable for application in wastewater.

8.1.6 Conclusions: performance *L. pneumophila* detection methods

8.1.6.1 ISO method

- High detection limit in wastewater samples, sometimes too high for application in wastewater, due to interfering microorganism growth on the culture plates.
- Slightly higher *L. pneumophila* numbers compared to Legiolert and MICA (Advance).
- Correlated to Legiolert, MICA and QIAcuity dPCR.
- Good performance in dilution series and 6-week monitoring of WWTPs (phase 1).
- ISO method requires trained technicians and well-equipped laboratory. Due to the many different pre-treatments and plating on culture medium, much time and resources are needed.
- Matrix C is suitable for wastewater, until now the only official standardized method for wastewater.

8.1.6.2 Legiolert

- Lower detection limit in wastewater than the ISO method.
- Slightly lower *L. pneumophila* numbers compared to ISO method, but comparable to MICA (Advance).
- Correlated to ISO method, MICA and QIAcuity dPCR.
- Good performance in dilution series and 6-week monitoring of WWTPs (phase 1) and pre-treatment optimization (phase 2).
- Legiolert is easy to use, although more laboratory equipment is needed for the necessary pretreatment step which makes the method slightly more technical.
- Suitable for wastewater, but more validation and comparison with ISO method needed.

8.1.6.3 MICA (Advance)

- Lower detection limit in wastewater than the ISO method.
- Slightly lower *L. pneumophila* numbers compared to ISO method, but comparable to Legiolert.
- Correlated to ISO method, Legiolert and QIAcuity dPCR.
- High *L. pneumophila* concentration in dilution series were not quantified correctly, lower concentrations were correctly quantified (phase 1).
- Good performance in 6-week monitoring of WWTPs (including high *L. pneumophila* numbers; phase 1) and pre-treatment optimization (phase 2).
- MICA (Advance) system is easy to use, faster than the ISO method, provides guidance for unexperienced users and gives an automatic enumeration of *L. pneumophila* in CFU/L.
- Suitable for wastewater, but more validation and comparison with ISO method needed.

8.1.6.4 QIAcuity dPCR

- Varying detection limit in wastewater, which might be caused by varying viable : dead ratio of *L. pneumophila* bacteria. The method is still more sensitive than the ISO method.

- Correlated to ISO method, MICA and Legiolert.
- Good performance in dilution series and 6-week monitoring of WWTPs (phase 1) and pre-treatment optimization (phase 2).
- QIAcuity dPCR system requires a trained technician with experience in molecular methods.
- Suitable for wastewater, but more validation and comparison with ISO method needed.

8.1.6.5 rqmicro.COUNT

- No detection limit could be established for wastewater, due to consistently high results even when low *L. pneumophila* numbers were dosed or in control blanks.
- No correlation to ISO method or alternative detection methods.
- Did not perform well in dilution series and 6-week monitoring of WWTPs (phase 1) and pre-treatment optimization (phase 2), probably due to off-targeting.
- rqmicro.COUNT system is easy to use and provides results fastest compared to the other detection methods.
- Not yet suitable for wastewater, more development is needed.

8.2 *L. pneumophila* threshold concentration in wastewater linked to outbreaks

The presence and growth of *L. pneumophila* in WWTPs is especially important in relation to public health. Several outbreaks of Legionellosis in the Netherlands were traced back to WWTPs with high *L. pneumophila* concentrations. In general, it is assumed that outbreaks may occur if the *L. pneumophila* concentration in an aeration basin with fine bubble aeration is above $1x10^6$ cfu/L at which concentrations *L. pneumophila* is also present in high enough concentrations in aerosols to potentially infect people surrounding the WWTP (Oesterholt & Hollebekkers, 2022). The $1x10^6$ cfu/L threshold was set with the ISO method, but setting the threshold with the alternative detection method is important. Only if this threshold is known, the WWTP can be operated in such a way to try to stay below this threshold value while monitoring with the alternative detection methods.

8.3 Modelling growth of *L. pneumophila* in WWTP

The tools available so far for *L. pneumophila* control in industrial WWTPs mostly focus on its management, but not on prevention. When a wastewater facility experiences increased concentrations of *L. pneumophila*, aeration tank containment through its coverage or enclosure are suggested to prevent the aerosols from spreading. This is accompanied by providing clear safety gear and instruction for the facility employees. The Liquisens predictive model approach could provide a solution that is more proactive and allows plant operators to manage *L. pneumophila* in their plant before it reaches concerning concentrations.

In this project, using the Liquisens algorithm at an industrial WWTP (chapter 5), the increase of the *L. pneumophila* concentration in wastewater was linked to specific process related conditions, such as pH, organic load indicators, and sludge-related variables. Although the dataset remains limited, the findings confirm that operational process information can be used to identify conditions associated with increased *Legionella*-risks and support the development of early warning strategies. The collected input is the first step toward predictive, risk-based monitoring, enabling operators to move beyond reactive testing and gain deeper insight into the drivers of microbial risk within biological treatment systems.

The dataset of 37 *L. pneumophila* samples over a period of one year was limited and ideally a larger dataset, including events, should be used to feed the Liquisens algorithm. Combining the algorithm with more monitoring data and thus *L. pneumophila* concentrations as measured with the ISO method and the three successful

alternative detection methods (QIAcuity, Legiolert, MICA (Advance)) would allow to provide extra information and input for the algorithm. As the alternative methods are sometimes more sensitive and easier to perform, this could yield additional and more relevant information for the algorithm as more data (with low *L. pneumophila* concentrations) becomes available and initial growth at lower concentrations can be included in the model. It would therefore be worthwhile to continue with the Liquisens approach and combine it with sampling and *L. pneumophila* analysis with the three alternative detection methods and the ISO method. This will yield more insight into factors influencing the *L. pneumophila* concentration in wastewater, such as temperature, pH and operational parameters. By applying this method at different WWTPs it is possible to study factors that determine the proliferation of *L. pneumophila* in different situations and to see if more general conditions can be extracted that trigger *L. pneumophila* growth. This would help in defining more general measures. By using *L. pneumophila* data from four different detection methods, possibly also lower *L. pneumophila* concentrations can be included in the algorithm allowing for an even earlier warning signal that *L. pneumophila* levels are rising. Assuming that the costs will be lower than the ISO method more measurements might be performed thereby improving the algorithm.

I Measurement results phase 1 (chapter 2)

Table 9-1: Table with all measurements in the project, including data received from technology suppliers with samples they received through KWR during the project. ND: No detection. NA: Missing result (either not performed or error). Note 1: Legiolert done with simple pre-treatment instead of modified pretreatment for wastewater. Note 2: Legiolert results noted as off target by IDEXX. Note 3: Measurements done after 12th of March 2024 use MICA pretreatment protocol version 2, those before use pretreatment protocol version 1.

Location/partner	Sample date	ISO method	Legiolert	MICA	rqmicro. COUNT	QIAcuity dPCR	Note
Boxtel (Hydroscope)	19-2-2024	5.71E+04	4.75E+04[1]	7.20E+02	6.25E+05	2.82E+06	Aeration basin
Boxtel (Hydroscope)	26-2-2024	1.48E+05	7.25E+04	1.93E+03	1.88E+05	9.00E+06	Aeration basin
Boxtel (Hydroscope)	11-3-2024	1.17E+06	8.07E+03	2.28E+04	1.75E+07	1.87E+06	Aeration basin
Boxtel (Hydroscope)	25-3-2024	8.45E+07	4.87E+03	6.11E+05	1.31E+06	1.58E+08	Aeration basin
Boxtel (Hydroscope)	8-4-2024	2.95E+07	1.02E+05	1.54E+06	2.55E+07	2.92E+07	Aeration basin
Boxtel (Hydroscope)	15-4-2024	7.97E+07	2.71E+05	1.66E+06	NA	2.00E+08	Aeration basin
Boxtel (Hydroscope)	3-10-2023	5.00E+04	1.00E+03	NA	NA	NA	Effluent
Boxtel (Hydroscope)	3-10-2023	1.50E+05	3.60E+04	NA	NA	NA	Aeration basin
GETEC	8-4-2024	ND	ND	ND	ND	ND	
GETEC	25-9-2023	ND	ND	NA	NA	NA	Effluent
GETEC	25-9-2023	ND	ND	NA	NA	NA	Aeration basin
GETEC	2-10-2023	ND	1.00E+03	NA	NA	NA	Effluent
GETEC	2-10-2023	ND	1.00E+03	NA	NA	NA	Aeration basin
Leuven	19-2-2024	ND	9.61E+05[1]	1.05E+04	5.00E+05	1.59E+05	
Leuven	26-2-2024	ND	4.40E+04	1.18E+04	6.52E+06	3.04E+05	
Leuven	11-3-2024	ND	8.23E+03	2.73E+03	4.89E+07	1.61E+05	
Leuven	25-3-2024	6.67E+04	2.39E+06	1.65E+04	1.81E+06	1.42E+05	
Leuven	8-4-2024	1.43E+06	4.22E+04	1.92E+04	1.19E+09	8.55E+05	
Leuven	15-4-2024	8.91E+05	2.26E+04	ND	3.81E+08	7.78E+05	
RWZI Ede	8-7-2024	8.00E+05	NA	NA	NA	NA	Location not in project, known high *L. pneumophila*, used for dilution series
RWZI Ede	16-7-2024	6.00E+05	6.80E+04	1.68E+03	NA	2.96E+07	Location not in project, known high *L. pneumophila*, used for dilution series
RWZI Ede	17-9-2024	2.16E+07	6.34E+05	1.68E+03	NA	6.20E+07	Location not in project, known high *L. pneumophila*, used for dilution series
Circle Wastewater Services	19-2-2024	NA	NA	NA	NA	NA	
Circle Wastewater Services	26-2-2024	ND	ND	ND	1.97E+06	1.80E+04	
Circle Wastewater Services	11-3-2024	ND	9.00E+02	ND	9.19E+06	1.80E+04	
Circle Wastewater Services	25-3-2024	ND	7.00E+02	ND	2.40E+06	ND	

Circle Wastewater Services	8-4-2024	ND	1.00E+03	ND	NA	ND	
Circle Wastewater Services	15-4-2024	ND	1.00E+03	2.35E+02	2.42E+06	1.77E+04	
Circle Wastewater Services	25-9-2023	ND	2.00E+03	NA	NA	NA	Effluent
Circle Wastewater Services	25-9-2023	ND	ND	NA	NA	NA	Aeration basin
Circle Wastewater Services	25-9-2023	ND	6.00E+03	NA	NA	NA	Aeration basin
Circle Wastewater Services	25-9-2023	ND	2.00E+03	NA	NA	NA	Aeration basin
Tyson	20-11-2023	2.50E+04	6.31E+02	<100	3.26E+06	ND	No longer in project, aeration basin
Tyson	25-9-2023	ND	ND	NA	NA	NA	No longer in project, effluent
Tyson	25-9-2023	ND	6.00E+03	NA	NA	NA	No longer in project, aeration basin
Boxtel (Hydroscope)	3-10-2023	ND	4.00E+03[2]	NA	NA	NA	Effluent, Legiolert result noted as off target by IDEXX
Waterstromen	2-10-2023	ND	ND	NA	NA	NA	Reactor effluent
Waterstromen	2-10-2023	ND	ND	NA	NA	NA	Reactor effluent
Waterstromen	2-10-2023	ND	ND	NA	NA	NA	Effluent
Waterstromen	25-9-2023	ND	2.00E+03[2]	NA	NA	NA	P removal; reactor effluent, Legiolert result noted as off target by IDEXX
Waterstromen	25-9-2023	ND	ND	NA	NA	NA	N removal; reactor effluent
Waterstromen	25-9-2023	ND	ND	NA	NA	NA	Effluent

Table 9-2. Table of NA measurements

Location/partner	Sample date	ISO method	Legiolert	MICA	rqmicro. COUNT	QIAcuity dPCR	Note
Boxtel (Hydroscope)	15-4-2024	7.97E+07	2.71E+05	1.66E+06	NA	2.00E+08	NA was an error that occurred during measurement
Boxtel (Hydroscope)	3-10-2023	5.00E+04	1.00E+03	NA	NA	NA	All NAs: not performed or no data on hand
Boxtel (Hydroscope)	3-10-2023	1.50E+05	3.60E+04	NA	NA	NA	All NAs: not performed or no data on hand
GETEC	25-9-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand
GETEC	25-9-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand
GETEC	2-10-2023	ND	1.00E+03	NA	NA	NA	All NAs: not performed or no data on hand
GETEC	2-10-2023	ND	1.00E+03	NA	NA	NA	All NAs: not performed or no data on hand
RWZI Ede	8-7-2024	8.00E+05	NA	NA	NA	NA	All NAs: not performed
RWZI Ede	16-7-2024	6.00E+05	6.80E+04	1.68E+03	NA	2.96E+07	NA: error that occurred during measurement
RWZI Ede	17-9-2024	2.16E+07	6.34E+05	1.68E+03	NA	6.20E+07	NA: error that occurred during measurement
Circle Wastewater Services	19-2-2024	NA	NA	NA	NA	NA	All NAs not performed
Circle Wastewater Services	8-4-2024	ND	1.00E+03	ND	NA	ND	NA: error that occurred during measurement
Circle Wastewater Services	25-9-2023	ND	2.00E+03	NA	NA	NA	All NAs: not performed or no data on hand
Circle Wastewater Services	25-9-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand
Circle Wastewater Services	25-9-2023	ND	6.00E+03	NA	NA	NA	All NAs: not performed or no data on hand
Circle Wastewater Services	25-9-2023	ND	2.00E+03	NA	NA	NA	All NAs: not performed or no data on hand
Tyson	25-9-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand
Tyson	25-9-2023	ND	6.00E+03	NA	NA	NA	All NAs: not performed or no data on hand
Boxtel (Hydroscope)	3-10-2023	ND	4.00E+03[2]	NA	NA	NA	All NAs: not performed or no data on hand
Waterstromen	2-10-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand
Waterstromen	2-10-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand
Waterstromen	2-10-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand
Waterstromen	25-9-2023	ND	2.00E+03[2]	NA	NA	NA	All NAs: not performed or no data on hand
Waterstromen	25-9-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand
Waterstromen	25-9-2023	ND	ND	NA	NA	NA	All NAs: not performed or no data on hand

II Appendices Phase 2 (chapter 3)

II.I Risk assessment: Monitoring

3 different situations can be identified:

1. Unknown WWTP: nothing else has been regulated and there are no regulations.
2. Well-known WWTP: something has been arranged and/or there are regulations.
3. Unusual occurrence: there is an unusual event because of which there are adverse consequences for the environment due to possible exposure to *Legionella* from WWTP.

- *Situation 1*

The aim is to identify whether there is a risk according to *Table 6* and therefore the measures that need to be taken.

When visiting a company with a WWTP, a checking should be done to see if there is a risky installation and if people are familiar with the problem. *Tables 6 and 7* are important for this step. The following questions can be relevant to ask [8]:

- Is there a WWTP within the company?
- What is the type of WWTP (biological (aerobic/anaerobic)/physical-chemical/combined)?
- What is the wastewater temperature?
- What is the operating temperature in the aeration tank of the WWTP?
- Does the company know the origin of the influent?
- Does the company know where their effluent and sludge go?
- How can the purification be classified according to *Table 6* and *Table 7* and which approach is involved? If there are risks of *Legionella*, is the current situation sufficient or do ex officio regulations still need to be added?
- If there are no regulations yet, has a (plan for a) management plan, risk analysis and monitoring plan already been drawn up and assessed by the competent authority?
- In case of presence of *Legionella* when sampling, possibly coordinate with MHS (Municipal Health Service)?

- *Situation 2*

Regulations can be monitored, and the documents or implementation can be assessed.

When visiting a company with a WWTP where regulations have been imposed regarding *Legionella* prior or during the visit, management plan, risk-analysis and monitoring plan are assessed.

The following questions can be relevant to ask during a visit [8]:

- Has the company made a risk assessment? If so, is it of sufficient quality (see section Risk assessment, management plan and monitoring plan)?
- Is there already a management plan containing possible measures?
- Are these measures realistic and available?
- Are there measures being implemented? Is there anything registered about it?
- Have there been any changes since the preparation of the risk assessment or management plan ?
- Is there a monitoring plan in place to check/manage the WWTP for the presence of *Legionella*?
- Is there a *Legionella* sampling plan in relation to the risk profile and risk category (see section Risk assessment, management plan and monitoring plan)?
- Is there an accredited person or an accredited laboratory that can sample wastewater?
- Is the recorded trend in *Legionella* results reliable? If so, are there sufficient measurements that give an indication of an expected range of *Legionella* content in the wastewater?
- Does the company know where their effluent and sludge go?

- Are *Legionella* analyses carried out correctly? The aim should be to achieve the lowest possible detection limit.
- Are these results stored correctly to determine a trend? Is the management of the data analysis entrusted to the official or in the company's broader environmental management system?
- Is the plant management familiar with the analysis results?
- What action levels does the company use? At what level are positive values reported to the competent authority?
- Is there a plan of action and is there research into possible measures?
- Are there any measures in the picture (see section Risk assessment, management plan and monitoring plan)?

- *Situation 3*

When an unusual event is reported (e.g: following a very high level of *Legionella* in the WWTP), it is important to determine the possible spread to the environment. If the MHS reports that there is at least one patient in area, they might carry out a source investigation. Then, the company must take urgent measures to reduce the contamination of *Legionella* in WWTP. In case of illness caused by the WWTP, MHS can advise to close the WWTP [8].

II.II Risk assessment: Management plan and monitoring plan

Below is a checklist with the most relevant topics to be monitored. This checklist corresponds to the methodology presented in the risk assessment part [8].

Table 9-3. General methodology for risk assessment

Part: General	
General data risk assessment process	*Definition*
• Is the author sufficiently knowledgeable? • Does the company have a reliable partner for the research? • Is the laboratory in question sufficiently qualified to detect *Legionella*?	Carrying out a *Legionella* risk assessment for wastewater requires a lot of expertise and is tailor-made. The person responsible for the risk assessment must therefore be an expert in that field, must have demonstrable experience with *Legionella* prevention in general, and have at least basic knowledge of the water purification processes at WWTPs. The person must also be aware of the latest legislation, technical guidelines at the time of preparation of the risk assessment. It is highly recommended that a risk assessment be carried out by a specialized agency that controls the combination between *Legionella* and wastewater.

Table 9-4. Methodology for the inventory of existing situation

Component: inventory of existing situation	
Corresponds to step 2b in chapter 6: Inventory of existing situation. The inventory of the existing situation means visualizing all process components of the WWTP (components), relevant operating conditions and the environment.	
Subject	*Definition*
Has sufficient distinction been made between different components of the WWTP?	Check for the presence of a process diagram and a logical classification by components as part of the risk assessment.
Is the risk assessment still up to date?	Check if changes to the installation, operations or environment have been made since the last risk assessment requiring a reassessment.
Have the specific operating conditions been inventoried in sufficient sizes?	Check whether the following aspects have been described as part of the inventory of the existing situation. • The nature and quality of the wastewater and graft sludge used/treated. • The temperature and acidity of the water in the various process steps (components). • The type of aeration in components. • The management of the biological (waste) water treatment (where aerosol formation/misting can take place). • Reuse of effluent directly/indirectly. • Is there a link with occupational health and safety policy?
Have the environmental factors been sufficiently inventoried?	Check that the following aspects are described as part of the inventory • Has the location of the installation within the company and in relation to the environment (risk category classification, distance from residential areas and distance from areas where vulnerable people are located) been taken into account? • Presence of other aerosol-forming water systems in the immediate vicinity • Presence of intake points downstream of the discharge point for the WWTP effluent
Is there an inventory of existing quality data regarding the content of *Legionella* bacteria?	Check if the company has started monitoring *Legionella* in the effluent and at relevant positions in the WWTP.

Table 9-5. Methodology for risk assessment

Component: risk assessment	
Corresponds to steps 3 and 4 in the risk assessment section: Risk assessment by component and risk assessment Sludge/surplus sludge and reuse effluent. The risk assessment shall be carried out based on the risk qualification in Table 1 focusing on risk criteria.	
Subject	*Definition*
Does the risk assessment cover the entire company?	This can be assessed based on a description in the risk assessment and the actual installation. • Is there perhaps already a regular *Legionella* management plan available within the establishment due to cooling towers or fountains and the like? • Would this trigger a procedure with the occupational health and safety part?
Are the risks of presence, growth and dispersion of *Legionella* sufficiently examined per component?	Check whether each component has been tested against the growth conditions throughout the year based on the temperature trend. Furthermore, it must be indicated per component whether there is Misting of the wastewater takes place, for example, by aeration or by other causes.
Are the possible risks to the use and origin of Inoculum sludge and the disposal and processing of surplus sludge included in the assessment?	Check whether the origin of the sludge is known and whether the quality of the sludge has been inquired about. For its own surplus sludge, it must be known how it will be disposed of and processed.
Are the possible risks of reusing effluent included in the risk assessment?	This concerns direct reuse at the WWTP or outside the WWTP for, e.g, cleaning activities or to prevent foaming with nozzles. It may also involve indirect reuse of the effluent downstream of the discharge point.
Is the risk assessment confirmed by the available readings for *Legionella*?	Check that the *Legionella* levels found in the wastewater and in the effluent correspond to the result of the risk assessment. (In other words, *Legionella* is found when it was not or was expected based on the risk assessment). Is the lab where the samples are analyzed sufficiently expert?
Is the risk assessment still up to date?	Have there been any changes to the installation, operations or environment since the last risk assessment that require a new assessment?

Table 9-6. Methodology for an action plan

Component: action plan	
Corresponds to step 5 in the risk assessment section: Drawing up an action plan. Based on the picture of relevant characteristics of the WWTP (step 2b), the risk assessment per component (step 3) and the risk assessment of sludge/effluent (step 4), it is clear whether there is a (potentially) risky installation. The operator of a high-risk installation shall then draw up an action plan on the corrective and control measures to be taken. The action plan is aimed at mitigating the risks identified during the risk analysis. It identifies the steps to be taken and sets deadlines for these steps to be taken.	
Subject	*Definition*
Is there a plan of action with a period within which measures will be investigated? And are the costs transparent?	Check on the following aspects: • Has a trade-off been made for the application of corrective (structural) measures over control measures? • Have the operator made any proposals in the action plan for further research? • Does the action plan contain a timetable for the implementation of measures? • Is that timeframe realistic? Consult the MHS to check whether there is an increased number of patients in the area.
Does the action plan contain an overview of corrective (structural) measures?	Check on the following aspects: • Has research been done into available techniques? • Have the possibilities for covering the aeration basin been investigated (as one of the possible solutions)? • Has a trade-off been made in terms of effectiveness and efficiency of measures? • Has the possible undesirable effects of measures been watched? • Is the expected implementation period stated and is it realistic and acceptable (partly in view of the available analysis results in relation to information from the MHS about patients in the environment)? • Has the effectiveness been monitored?
Does the action plan contain an overview of the control measures?	Check on the following aspects: • Has a trade-off been made in terms of effectiveness and efficiency of measures? • Have the possible undesirable effects of measures been considered? • Is the expected implementation period stated? • If a measure has been implemented: is it included in the management plan?

Is the need for further research sufficiently proved?	Check on the following aspects: • Are there any measures that require further research? • Is there a time limit for the duration of the investigation? • Are there options available when the research does not give the desired result?

Table 9-7. Methodology for the management plan

Component: management plan	
Corresponds to step 6 in the risk assessment section: Drawing up a management plan. After the risk assessment and the action plan have been drawn up, the risk is identified, and the possible measures are known. After this, attention can be paid to the *Legionella* management of the WWTP in the long term. To this end, a management plan should be drawn up. This management plan can be a separate document or part of a work instruction or business action of the WWTP. The management plan shall include how the risk is handled responsibly. It is a document that must be kept updated regularly, especially about adjustments in installation, environment or results of analyses.	
Subject	**Definition**
Does the management plan contain drawings and/or diagrams of the current layout of the WWTP?	Check the following aspects: Is the installation well visible? • In addition to a general overview, are the various connected installations (parts) in the picture? • How detailed are the drawings? Are the drawings up to date? Does the level of detail match the risk?
Does the management plan contain a description of the operation of the WWTP?	• Is the operation and functioning of the installation clear? Are possible malfunctions and the options to fix them in the picture?
Does the management plan contain an inventory of the environment?	• Are the relevant factors analyzed in the risk assessment listed?
Is there a description of all checks to be carried out at the WWTP with regard to the presence of *Legionella*?	Is there a clear overview with the relevant parameters? • Temperature, pH, *Legionella* content in aqueous phase at the relevant installation components? Where, when and how do the checks go? Is anyone responsible for the implementation and the registrations?
Does the management plan contain action levels and a description of the approach taken if those levels are exceeded?	Are the sampling sites clearly indicated? • Does sampling always take place at the same place and time? • Which analytical method(s) are used?
Is there a logbook?	• Is the logbook sufficiently clear and uncluttered? Does the logbook contain a summary of the results of checks performed (analysis and inspection results), the control measures implemented, the changes made to the WWTP, the changes made

	to the maintenance of the WWTP and the consequences of these changes for the existing risk assessment?
Is there a matrix overview that captures the duties and responsibilities of involved officers within the company?	Is it sufficiently clear who is ultimately responsible, who bears what responsibility within the process, who is consulted and who should be informed?
Certified sampling and analysis	• Are the samples taken by an approved undertaking? • Are analyses carried out according to the prescribed method? • Is it an accredited laboratory? What is the average value? Is there a trend line?

II.III Circle Wastewater Services meeting report

Date: 30/05/2023

Participants: Ger NOTERMANS (Circle Wastewater Services), Bego OSUNA GONZALEZ (CEW), Jade KAN (CEW intern), Camille GOLLION (CEW intern) and Coline TROPHARDY (CEW intern)

Circle Wastewater Services is specialized in WWTPs treating wastewater for polymers industries. They are currently treating the wastewaters of 55-60 companies. Circle Wastewater Services joined the project because they are interested in getting information about the management of *Legionella* and more precisely about how to deal with it, what biocides can be used (compatibility with the organisms in the sludge). Indeed, they currently do not have any knowledge about how to manage *Legionella* if it occurred in their WWTP.

At the moment, *Legionella* concentration is maintained below 20 CFU/L. Wastewater usually has a flow that can go up to 3500 m^3/h. The wastewaters have different compositions, but they all have high concentrations of hydrocarbons because of the polymers and nitrogen, which are all mixed.

The general process of the WWTP is the following:

1) Nutrients are divided. Phosphoric acid is dosed, it must be in little excess. For the dosage of nutrients, extra COD is added if the nitrogen concentration is too high in the influent. Ammonia should be prevented.
2) Denitrification
3) Nitrification: Nitrogen is oxidized into nitrate whereas COD is oxidized into CO_2, ammonium is eliminated.
4) Denitrification
5) Wastewater goes through a tank to get rid of the sludge.
6) Wastewater is discharged into the mass.
7) Some sludges can go back to the denitrification step.

All the steps involved are aerobic as air is always present to keep the sludge moving and prevent its sedimentation, the aim is that the wastewater is saturated in dioxygen. A part of the wastewater is sprayed at high pressure in the basin to aerate it. The basin is not covered. If needed pure oxygen can be added.

The influent has a COD of 2 g/L.

The characteristics of the WWTPs are summarized in the following table:

	Temperature	pH	O_2	Nutrients	Iron	Protozoa
Value	20-33°C	6 – 9.5, average is 8	Around 2 mg/L	C/N = 5 P: above detection limit	Presence of iron but the precise concentration is unknown	No information

The iron level in the process is not known, however they use iron chloride as an electrolyte. Protozoa in the sludge are not quantified either. The ratio carbon/nitrogen is about 5 and phosphorus is added if its concentration is below the limit detection to be sure that there is phosphorus.

The sludge contains a lot of calcium carbonate. A part of the used sludge is reused to make the balance in the nitrification basin to keep a concentration between 11 and 15 g/L. The rest is treated and burnt externally in Belgium.

Circle Wastewater Services does not encounter *Legionella* problem, however, 20 to 25 industrial companies using cooling towers detect *Legionella* in their wastewater. These are killed using a biocide, chlorine bleach, before going to the WWTP.

Circle Wastewater Services has one cooling tower to cool water under 30°C during the summer. This is the stage of the process with the higher risks with the aerated denitrification basin.

The infrastructures are made of concrete.

A turnaround is done every 15 years for the maintenance of the WWTP. Then one of the two basins is stopped. The capacity of the plant is reduced at the minimum and extra dioxygen is used to be more performant.

Circle Wastewater Services tests *Legionella* every month and every two weeks during the summer period by taking a sample from the denitrification basin. They do not know how to measure the concentration of *Legionella* in the air.

They do not know how to act in case of *Legionella* problem, they would certainly warn the neighbourhood and the employees who should wear a PF2 mask around the basins.

II.IV Normec meeting report

Date: 01/06/2023

Participates: Naomi SCHILDERINK (Normec Kalsbeek), Bego OSUNA GONZALEZ (CEW), Jade KAN (CEW intern), Camille GOLLION (CEW intern) and Coline TROPHARDY (CEW intern)

Normec is consultancy company that gives advice on *Legionella* management for drinking water systems, cooling towers and industrial WWTPs. These industrial WWTPs can treat wastewater from brewery, food industry, paper company and waterboards. Normec decided to join the project to gain knowledge about the control of *Legionella* in WWTP by cooperating with other partners. Indeed, knowledge about controlling *Legionella* is minimal.

To advise companies, Normec follows the "Handreiking *Legionella* preventie in AWZI" (Guidance on *Legionella* prevention in WWTP) written by KWR. Their most reliable source for the parameters of the *Legionella* growth is the Hydroscope document written by Kevin Kanters. It is currently the most complete document. Normec will provide us with a database gathering values of operating conditions. Stages the most at risk are aeration tanks and when processes change. The most common problems faced by their clients are the following:

- The company took 2 samples at the same place, one of them contains *Legionella* and the other one does not.
- They have very high concentrations of *Legionella*, if it is the case, there are very few means of action.

Normec also takes and analyzes samples to detect the presence of *Legionella* by following a standard. Samples are collected around aeration tanks and in the effluent, therefore most of them are water samples. However, they can do air samples if the concentration of *Legionella* is high or if the process at risk is not covered. If needed, they make additional samples of the influent for example, to identify the source of contamination. The ideal situation is to treat the air in advance.

According to Normec, to limit the risk of *Legionella*, WWTPs should have the knowledge and the level of awareness concerning *Legionella*. Then, a risk assessment should be made. Finally, controlling steps and an effective monitoring system should be implemented. There are currently no effective techniques to eliminate *Legionella* in WWTPs.

It is hard to design WWTP to prevent *Legionella* when the type of influent is not known. They advise to follow the "Handreiking *Legionella* preventie in AWZI" (Guidance on *Legionella* prevention in WWTP) written by KWR. Currently, Normec has no technology against *Legionella* in WWTP. They advise to lower the temperature, to lower or raise the pH to make it less comfortable for *Legionella* growth. During the biological treatment, the pH can be moved around 0,5 to 1 unit without affecting the process. These adaptations are done in dialogue with the client according to what is possible for the process.

The use of biocide is not possible for a WWTP. In fact, the BOD level would also decrease. However, they can be used before to treat the influent to make sure that *Legionella* do not enter in the process via the effluent. Biocides are also used in cooling towers in addition to corrosion inhibitors. This is the most effective and cheapest technology.

Technologies that Normec provides, available on its website, targets essentially drinking water. For instance, the *Macronox* technology can be used upstream WWTP to treat the influent. *Tapper* is an app to monitor and detect *Legionella* in buildings drinking water systems. It could be used as a data base in WWTPs but otherwise it would not be interesting.

Retention time and flow rate are important parameters for drinking water installations but in WWTPs, because there are already nutrients and favorable temperature, these parameters have not a big influence, it would not be so useful to control them.

Covering the tank is a possibility but the temperature would therefore increase. They do not know if a UV treatment could be used.

When *Legionella* is present, workers must wear a PF3 mask when they are in a place where the risk of spreading is high.

II.V GETEC meeting report

Date: 06/06/2023

Participants: Ger Joosten (GETEC), Dries Buitenwerf (GETEC), Bego Osuna Gonzalez (CEW), Jade Kan (CEW intern) and Coline Trophardy (CEW intern)

GETEC Platform Netherlands supports companies offering a wide range of climate-friendly and smart energy solutions for the entire Benelux. GETEC PARK.EMMEN is located in the northeastern part of the Netherlands nearby Emmen and provides wastewater treatment solutions to local chemical companies and industries like plastic factories, milk powder factories and gelatin factories. The wastewater is collected and is treated in a centralized wastewater treatment plant. The wastewater treatment is a biological treatment. Wastewater is treated in several steps and the final effluents are discharged to the Bargermeer canal.

GETEC is interested in joining this project because they care about everyone's safety and they are worried about the current lack of knowledge on control of *Legionella*, especially from the government. The current control solutions provided are highly costly and they are concerned about efficiency.

Their WWTP consists of a desulfurization of a fraction of the water stream entering the plant before entering the anaerobic treatment. The total stream is then treated by an aerobic process in which both nitrification process (aerobic conditions) and later the denitrification process (under anoxic conditions) are taking place in the same tank by recirculating the water stream through different compartments.

The WWTP is mainly made of concrete, except the anaerobic digestor which contains a plastic coating layer and the water pipes are also made of plastic (HDPE).

The main operating conditions of the processes are gathered in the following table:

	Anaerobic	Aerobic	Sulfates removal
Temperature (°C)	30 - 35	20-30 (sometimes 30)	35 - 37
pH	7 – 7.5	7.5 - 8	8 - 9
Redox potential (mV)	450	200	/
O2 (mg/l)	-	2.5	-

The total volume of water treated is 7500 m^3, which corresponds to a flow rate of water around 300 m^3/h. Suspended solids concentration in the aerobic tank is kept around 4 g/L with a SRT corresponding to 2 to 3 weeks.

For the sludge treatment, the remaining excess of sludge goes through a concentrating process (centrifuge) to enter into a thermal dryer at 130°C. The resulting dry sludge is finally externally burnt (incinerating).

To dose oxygen required during the process, they use a fine bubble aeration operating at 5,5 m from the surface. In the aerated tank, oxygen is kept at 2,5 mg/L. During the denitrification process, DO level is about 1,5 mg/L dropping at the end of this process, they try to have an oxygen concentration of 0 mg/L by the end of the denitrification step. Liquid oxygen is used in case of need (emergency) and in a reserve space, oxygen is in excess to around 6-7 mg/L.

They do not dose nutrients as wastewater is rich in phosphates and sulfates.

Their clients have encountered *Legionella* problems in their cooling towers. To eliminate it, they used biocides containing chloride or bromide.

GETEC does tests of the effluent for *Legionella* control 4 times a year. The concentration of *Legionella* in their wastewater effluent is kept below 100 CFU/L. An accredited laboratory takes the samples, and they are analyzed by accredited laboratory (WLN).

No samples are taken in the aerated tank or during the process because it is hard to detect *Legionella* in such a matrix.

To maintain their WWTP, GETEC tries to keep it clean by avoiding the formation of biofilms. Maintenance is done only when a problem comes, for example a blocked pipe. In this case, they empty the WWTP, clean it and fill it again. Also, they keep the water flow high to avoid sedimentation in pipes and tanks. They do regular visual inspections.

There is currently no safety measure for employees, except when there is a problem, they advise their employees to wear a mask.

If a high concentration of *Legionella* ever occurs in their WWTP, they would alert the government, but they currently have no plan of action or measures to take. They will try to avoid the spreading of the bacteria in the air.

In case of *Legionella* problem, reducing the temperature will be a difficult measure to implement because a temperature ranging from 20 – 30 °C (which is also the optimal temperature for the growth of *Legionella*) is required for their processes.

II.VI VITO meeting report

Date: 22/06/2023

Participants: Sabine KREPS (VITO), Bego Osuna Gonzalez (CEW), Jade Kan (CEW intern) and Coline Trophardy (CEW intern)

Sabine KREPS is the responsible of VITO microbiology laboratory. Vito is controlling and analyzing water by following accreditations such as the ISO procedure. Furthermore, 3 times a year, they verify that samplers have accurate results and that their analysis methods are correct. They also write down procedures to sample *Legionella*.

Last year, they did a study to compare the different methods to detect *Legionella* such as the ISO method, PCR... Vito is also currently doing a study in which they will sample wastewater from 65 places from all types of industries to do a screening, among them, WWTPs with high *Legionella* risks. They have currently sampled 20 places. To select the WWTPs to test, they select the ones which have risky operational conditions (temperature for example) and aeration systems. Wastewater is a very complex matrix, therefore it is complicated to analyze it. It is necessary to treat the sample: direct plating, followed by an acid treatment and finally antibiotics are added. This gives great results.

Then, they noticed that samples were divided in 2 phases. They did 2 experiments: the first one to mix the phases and the other one to separate the layers. Then, the concentration of *Legionella* was measured in the single phase for the first experiment, and then measurements were taken in the two separate phases. The result is the following: in the organic material phase, the concentration of *Legionella* was 10 times higher. Therefore, *Legionella* bacteria fix to the sludge.

According to Sabine, it is difficult to eliminate *Legionella*. Indeed, it is a very resistant bacteria that can survive the acid solution used for the detection. In fact, *Legionella* is so persistent that, according to Sabine, it is impossible to get rid of it once it has infected a WWTP. The cheapest measures would be to lower the temperature and/or the organic material concentration. Lowering the pH would not be efficient, however, increasing the pH could be tested. Biocides made of chlorine, used in cooling tower or in the influent before the WWTP, evaporates into the atmosphere before going into the process, so the microorganisms involved in the treatment are not killed. Other biocides could be more persistent and could therefore damage the process.

The detection method used by Vito, the ISO method, does not allow us to distinguish the dormant form, the dead and the living cells. It would maybe work with the PCR method, but some tests confirm this. In the lab, some researchers add *Legionella* in pure water to develop some detection methods. However, the results obtained are not reliable for the wastewater because *Legionella* does not have the same behavior in all the matrix.

Concentrations over 10^5-10^6 CFU/L resulted in a possibility of high risk of *Legionella* in aerosols. It may not be possible to have zero *Legionella* in WWTP but to reduce the amount under these concentrations would be a potential solution.

II.VII RQmicro meeting report

Date: 27/06/2023

Participants: Silvana Gloor (Rqmicro), Christophe Gutknecht (Rqmicro), Bego Osuna Gonzalez (CEW), Jade Kan (CEW intern) and Coline Trophardy (CEW intern)

Rqmicro is a technology supplier that provides kits to detect *Legionella* in water. Its technology will be tested during the first phase of the TKI *Legionella* project, in collaboration with KWR. The method used is not a culture method. It labels the bacteria with antibody and get results in 2 hours. It allows us to detect not only the cultivable cells but all the alive bacteria, even the VBNC form. It is not possible to difference both alive forms, however the difference between alive and dead cells can be made. The method involves a filtration step that breaks the protozoa, so *Legionella* which were inside the host are released and can be detected as well. This detection of *Legionella* in host works about at 95%. The kit includes a positive control which allows to be sure that the device works by testing a sample with a known level of *Legionella*.

This method has not been tested in large scale in wastewater, but they have a lot of experience in cooling towers. Their interest in the project is to get more knowledge about WWTP to target a new market application and extend the application of their kit to other matrices.

Their customers are big companies which possess cooling towers, for example steel factories. Their goal is to determine the amount of *Legionella* in their cooling towers by using Rqmicro's *Legionella* detection kits. Since their method is fast, companies can quickly act and add a treatment to kill *Legionella* if needed. Moreover, it is currently not possible to use Rqmicro's *Legionella* detection kits in wastewater samples as wastewater is a complex matrix. However, research is being conducted in order to optimize the protocol to adapt it to wastewater.

Rqmicro does not have knowledge concerning *Legionella* in WWTPs however, they have some about *Legionella* in cooling towers. Indeed, every 6 months, companies with cooling towers are controlled and companies aim to not have any *Legionella* in it. There are many different ways to remove *Legionella* in cooling towers, the most common one being the use of biocides, many different compositions are available. However, there are also other less common techniques such as the use of electricity by EDF in France used in a small scale. It is also possible to use other bacteria to kill *Legionella*.

Moreover, it is not possible to automate their detection method as some of the steps required human supervision such as the incubation, the filtration, etc. Detection of *Legionella* in aerosols research is conducted in Munich University. However, it is currently impossible to detect *Legionella* in the air with their method because it needs to be concentrated first.

II.VIII Tyson Foods meeting report

Date: 29/06/2023

Participants: Bannie OLDENBANNING (Tyson Foods), Bego Osuna Gonzalez (CEW), Jade Kan (CEW intern) and Coline Trophardy (CEW intern)

Absent: Bregie Nabben

Bannie OLDENBANNING is an operator for Tyson Foods WWTP and he is an expert on wastewater treatment.

Tyson Foods is interested in joining this project to learn more about the possibilities of measures to take in WWTPs in the presence of *Legionella*. They constructed a new WWTP that should start functioning at the end of summer.

They encounter *Legionella* problems in their previous WWTP. Indeed, they took air samples near the calcium carbonate stage and noticed the presence of *Legionella*. It infected people and some of them were sick because of it. It was impossible to take water samples because the wastewater matrix is too complicated to analyze. According to Bannie, *Legionella* had the opportunity to develop because of the water temperature which was around 40°C. To protect their workers from *Legionella*, they provided white suits and masks. Tyson Foods tried to limit the spread of *Legionella* by aerosols by the stages at risk for spreading of the bacteria.

There was no biological treatment in their previous WWTP, but they added one in their new one. The wastewater has a neutral pH around 7. They have a turbulent streaming with a velocity of 3.2 m/s. Their new WWTP is mainly made of metal on the outside and isolation material in this inside. The reaction tank is made of steel and the membrane bioreactor is in stainless steel. The tank containing the sludge is made of plastic and there is not any cooling tower in the process. However, no specific installation was implemented in the new WWTP to prevent *Legionella*. There is a disinfection stage using NaCl, but for cleaning purposes. The WWTP will be cleaned once a year, but the frequency can be adjusted depending on the problems encountered.

II.IX QIAGEN meeting report

Date: ***29/06/2023***

Participants: Saskia Soestbergen (QIAGEN) Max Van der Ploeg (QIAGEN), Cathleen Vander Lee (QIAGEN),), Bego Osuna Gonzalez (CEW), Jade Kan (CEW intern) and Coline Trophardy (CEW intern)

QIAGEN provides isolation kits and detection kits for several microorganisms. KWR already used the isolation kits and asked them to join the TKI project to improve the dPCR detection method in addition to the isolation method.

The dPCR detection technique is a hydrolysis probe-based assay used for accurate and sensitive identification of a wide range of microorganisms. Up to 680 microbial targets can be detected.

DPCR stands for “limiting dilution PCR”. It is based on end point analysis and Poisson statistics to quantify the number of nucleic acid molecules in a sample. Indeed, each sample is partioned into a large number of individual and parallel reactions. This is done thanks to a microplate containing different capillaries or channels. There is a PCR amplification in each partition. Then, they are counted and those containing amplified products show a fluorescent signal. The kit contains a primer pair and a hydrolysis probe with a dye. They are lyophilized in a tube. The method can detect the presence of a very low concentration of DNA. It has other advantages such as a high reproducibility and high tolerance to inhibitors.

However, because it is based on DNA detection, it is not possible to differentiate the dormant form, the alive or the dead cells as they are genomically the same. A solution might be to look at the RNA, but because of its instability, it would be hard to process it.

These kits have already been used for wastewater, it should not be a problem to use it to detect *Legionella* in WWTPs but some tests have still to be made. If the *Legionella* concentration is enough, it should not be a problem to isolate and detect it even if the bacteria live in a host such as protozoa.

This technology can be automated, which is very promising to facilitate the monitoring of WWTP. It will be tested for phase 1 of the TKI *Legionella* project which focuses on the detection of *Legionella* in wastewater.

II.X Idexx meeting report

Date: 29/06/2023

Participants: Kristin MAJESKA (Idexx), Philippe ROUSSELIN (Idexx), Jeff BATES (Idexx), Brett BREWIN (Idexx), Bego Osuna Gonzalez (CEW), Jade Kan (CEW intern) and Coline Trophardy (CEW intern)

IDEXX is a technology supplier specialized in animal care and analysis of dairy products and water.

IDEXX decided to join the project because they are interested in developing excellent technologies to decrease health risks. Therefore, they created a test that detects *L. pneumophila* (all serogroups) based on a liquid culture method which means that the isolates can be stored and serotyped. Their technology is called Legiolert. It is simpler than ISO methods as it requires less steps. For instance, no confirmation step is required. IDEXX has specific protocols depending on the matrix, for example cooling towers. This technology is currently suitable for potable and non-potable water (cooling towers, pools, spas, decorative fountains and more). The pH has no impact on the method since it uses buffer and dilution. It is a sensitive and specific method for *L. pneumophila*.

Legiolert is based on bacterial enzyme detection using a substrate in the Legiolert reagent. *L. pneumophila* cells need nutrients to grow, and they reproduce by using the ones present in Legiolert reagent. When *L. pneumophila* use the added substrate to grow, they produce a brown color which is an indicator. It can detect *L. pneumophila* with a sensitivity of 1 organism in 100 mL within 7 days.

Even though they have not tested their *Legionella* detection tool in wastewater, they are confident on its effectiveness in this matrix as they have already worked with this type of media before. Their technology will be tested for phase 1 of the TKI *Legionella* project which focuses on the detection of *Legionella* in wastewater. Furthermore, they do not know if their tool can detect *Legionella* in amoebae or the VBNC form, but it might be the case as they detect more *Legionella* CFU in a sample compared to the ISO method.

II.XI Hydroscope meeting report

Date: 30/06/2023

Participants: Kevin KANTERS (Hydroscope), Sjoerd KREMERS (Hydroscope), Bego Osuna Gonzalez (CEW), Jade Kan (CEW intern) and Coline Trophardy (CEW intern)

Kevin Kanters is the CEO of Hydroscope. Hydroscope is a consultancy company giving advice on water management. They are interested in joining this project to know more about the behavior of *Legionella* in WWTPs. Indeed, there is currently limited knowledge about the latter.

They are also interested in getting more information about the dose-effect response as people might be able to get infected by a single *Legionella* bacterium in the air. They would like to study to model the spread of *Legionella* from the source (the WWTP) and its surroundings depending on the distance.

Future regulations concerning *Legionella* in WWTPs based on the RIVM document and the Handreiking *Legionella* preventie will be published in 2027 in The Netherlands. Among them, a WWTP that has a biological treatment operating at a temperature above 25°c will have to redact a risk assessment. According to Kevin Kanters, even though *L. pneumophila* is the main responsible for infections, monitoring other types of *Legionella* is also important. It is also easier to monitor other parameters than *Legionella* because it can grow very quickly.

The most important parameter to control the growth of *Legionella* is the temperature. Furthermore, he noticed that the concentration of *Legionella* is more important in processes when there is an anaerobic step followed by an aerobic step. Indeed, after the anaerobic phase, there is not very much competition and *Legionella* multiplies faster than the other bacteria, the first moments in the aerobic phase are crucial. Removing the anaerobic step could help, however since it might be difficult to do so in WWTPs, monitoring the anaerobic process is essential. High concentration of fatty acids and proteins provide optimal growth condition for *Legionella*. They also need specific amino acids (such as cysteine), oxygen and the right temperature to grow. However, controlling *Legionella* with nutrients concentration is most likely impossible as other organisms in aerobic processes require nutrients as well. Moreover, putting a great quantity of buffer at the beginning of the treatment in WWTPs might help as they will increase the effectiveness of the anaerobic treatment, thus *Legionella* would grow less. The sludge retention time can also have an impact on the growth of *Legionella*.

Controlling the iron level could be a lead in the investigation of growth control, but currently no more information is known. The addition of coagulant containing iron could have an impact on *Legionella* concentration, this could be studied further. Such coagulant becomes more and more expensive, therefore companies choose aluminum coagulant whose impact could also be studied.

Protozoa are a key factor for the first step of *Legionella* growth, this affects the type of protozoa present by competition. Indeed, some protozoa cannot be infected by *Legionella*. In France, a biocide has been developed based on amoebae that kill *Legionella*, however these amoebae could not survive long enough in wastewater to be applied in WWTP, therefore the research has been stopped. Furthermore, it will most likely be impossible to add bacteria that is in competition with *Legionella* to use amoebae as a host since most bacteria are also pathogenic to humans.

It is important to know the size of *Legionella* cell and if it lives inside an amoeba to know if it can go into droplets and thus spread via aerosols. The type of aeration is a key factor in the spread of *Legionella*. Research about how big the droplets are depending on the type of aeration is needed. Small bubble aeration provides less aeration but avoids the formation of aerosol unlike aeration using larger bubbles. The ideal aeration is to use fine or nano bubbles with pure oxygen. Cover can be a problem because it increases the temperature of the water and so *Legionella* can grow very fast. The effect of covering is a log 1 reduction in aerosol in the air. The Hydroscope WWTP is covered and there is plenty of air circulation under the cover. According to Kevin, it is more effective to have a great aeration system than to use covers. Moreover, operators dislike the covering because they cannot see, there is therefore a loss of control in the process.

In the mesophilic process, the contact time is longer than in thermophilic process, it allows to kill more *Legionella*. In a membrane bioreactor, there is a low risk of *Legionella* spread, however, during the cleaning process of the membrane, using air, aerosols are generated which increases the risk. If *Legionella* is already present in the influent, the risk is also increased.

The disinfection of the entire WWTP would not have any effect because the *Legionella* would come again.

The way and the place where samples are taken to detect *Legionella* are more important than detection itself.

II.XII Waterstromen meeting report

Date: ***20/07/2023***

Participants: Bernadet Otten (Waterstromen), Bego Osuna Gonzalez (CEW), Jade Kan (CEW intern) and Coline Trophardy (CEW intern)

Waterstromen owns five WWTPs in the Netherlands, which treat wastewater from different types of companie from different fields such as food industry (Aviko: potatos industry and Noblesse Proteins), leather field, paper industry.

All the plants have biological processes, mainly with a combination of aerobic and anaerobic processes. The plant where Bernadet works, which treats Aviko's wastewater, has four steps: anaerobic, aerobic, another biological treatment with less aeration and finally the collection of biomass to sell it. The temperature remains stable, ranging from 30°C to 35°C. Indeed, the wastewater is always cooled down at 34°C to make sure that the temperature is in the good range. During the first step, the anaerobic one, the pH is around 7 and the retention time is 15h. Biogas is formed at that stage. The next step for the wastewater is to go through a reaction with aeration. In this step, the pH is between 8 and 8.5, the temperature ranges from 30°C to 35°C and the dissolved oxygen is 3 mg/L.

Then, the wastewater undergoes through a small aeration in which the dissolved oxygen level is 0.5 mg/L. For the three first steps, all the tanks are covered. Mechanical aerations and membranes are used for the aeration in which small bubbles are diffused.

Finally, the biomass is kept alive in an open tank and sealed. This is a risky stage for the development of *Legionella*.

At first, *Legionella* was tested in their WWTPs with the qPCR method twice a year. However, this technique detects both living and dead *Legionella*. They will start to collect data with a new method next month and will keep on sampling and testing their WWTPs monthly for a whole year. The WWTP in which Bernadet is working was built in 2006, nevertheless they decided to test for *Legionella* in 2020. Only samplings in the effluent are being taken because they have decided to do research on the effluent and not on the influent. Using qPcR, the results show that from one measure to another, the concentration rises from under 10 000 CFU/L to 500 000 CFU/L without any changes in the process. However, the *Legionella* bacteria detected was not *Legionella pneumophilia* but *non-pneumophilia.*

Furthermore, their aeration processes are covered. Indeed, the reactor is made of concrete containing openings. Therefore, metallic covers can be put on top. It is possible to open them and close them. These covers are opened and inspected every day by operators, to make sure that everything is working correctly. During these checks, the risk of *Legionella* spread is present. However, it is not possible to go inside. The air under the cover is not treated and discharged directly into the ambient air via a pipe at the top of the cover. No effect on the temperature was observed.

Waterstromen has currently only taken preventive *Legionella* measures and they have identified four stages at risk in their WWTPs following the Handreiking. They do not really know how to act in case of *Legionella* occurrence. At certain locations in the WWTP, such as when the tanks are opened, operated should wear a mask.

On the one hand, the steel pipes allowing the transport of wastewater are cleaning weekly with either an acidic solution (pH < 2) or a basic solution (pH > 11) for 2h. They must be careful handling the resulting solution, therefore it is stocked aside to not affect the biomass. On the other hand, reactors and pipes related to the reactors are not cleaned as often since it is necessary to take out the biomass to do so. For this reason, they are cleaned once a year.

In the two WWTPs that treat water from Aviko, differences were observed concerning *Legionella* concentration. Both have the same operating conditions, only two parameters defer. In the first one where no *Legionella* has been found, the influent pH is about 4.5 – 5 and the plant is in the same place as the factory. The plants where *Legionella* have been detected have an influent pH of 5.5 and the water must travel 5 km in the pipe to reach the WWTP.

Their expectations of this project are to have guarantees about the reliability of the detection and control methods. Indeed, they are willing to invest to prevent and control *Legionella* but they have to be sure that the implemented measures will work. They also care about the safety of their workers and of the environment.