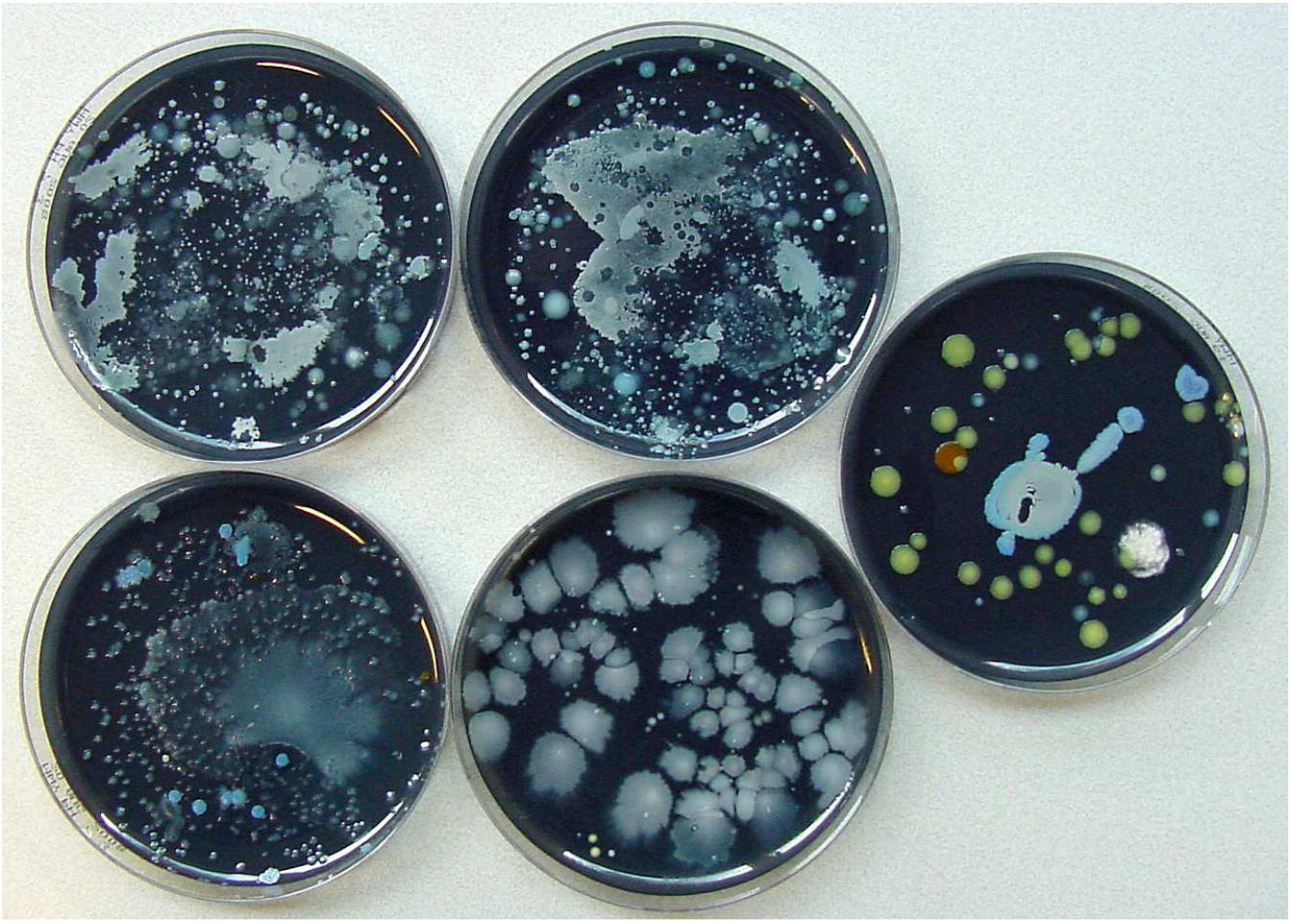

A new method of screening cooling water and process water for *Legionella pneumophila*

KWR 2009.004 (Eng;P)
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KWR Watercycle Research Institute

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Title
A new method of screening cooling water and process water for *Legionella pneumophila*.

Project number
A307531

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This report is public; all names of companies and laboratories involved are made anonymous.

Summary

The industry needs a fast and reliable analytical method for *Legionella* detection or monitoring, to enable optimal management and control of *Legionella* concentrations in cooling water and process water systems.
Taking the latest developments in the field of analysis as the starting point, over a 6-week period this study used 10 different analytical techniques to examine a total of 52 samples from 15 cooling water systems and 4 process water systems for the presence of *Legionella* and/or *Legionella pneumophila*. The main aim of the study was to test and evaluate the usefulness of an analytical scheme for cooling water and process water samples, which involved using Q-PCR analysis for *Legionella pneumophila* (in accordance with draft NEN 6254) and following this in the case of a positive result with a specific cultivation method for *Legionella pneumophila* (based on NEN 6253 with MWY medium). KWR developed both methods.

The other project objectives were:
- Development of a standard monitoring protocol that enables a representative sample to be taken of a complex cooling water or process water system.
- Comparison of the analysis results obtained using the aforementioned analytical techniques with the results of similar Q-PCR techniques (Applied Biosystems, according to protocol v.2.0; Pall Genesystems according to AFNOR XPT90-471) and other cultivation methods for *Legionella* (according to NEN 6265, NEN 6265:2007 with MWY medium and ISO 11731) and *Legionella pneumophila* (according to draft NEN 6253).
- Testing and evaluating the accuracy of a qualitative field test on the basis of an immunochromatographic assay for *Legionella pneumophila* sero-group 1 (Nalco, Fastpath™).

The sampled process water systems were systems in the paper industry. This process water has a high concentration of undissolved substances, organic matter and micro-organisms. This makes performing a specific microbiological assessment in the field particularly difficult, for example one involving a *Legionella* sp. cultivation method. As the conditions in a paper factory appear to be favourable for the growth of legionella bacteria, the paper industry also needs a robust analytical technique to provide greater clarity about the presence of these micro-organisms in process water.

The following method of working was adopted in this study:
- Each participating company selected 3 cooling water or process water systems with a demonstrable 'legionella history' (select sample).
- Each participating company assigned responsibility to one or more people for taking samples in accordance with the protocol, which KWR provided (appendix I), and for registering the current operating data for the selected systems (appendix II).
- All the participating companies were given 27 sterilised 1-litre bottles with a standard content comprising a thiosulphate solution for neutralising oxidising biocides and an NTA solution for complexing heavy metals.
- The company then sampled each cooling water or process water system on three different days, after which it sent the samples to KWR by courier. Three sample bottles were filled for each system. A note of the current operating data for each system was made on the information sheet.
- The contents of the three bottles with samples from the same system were homogenised by KWR on the same day and divided into new sample bottles. These sample bottles were sent to the participating laboratories by courier on the same day (see table 2).
- Analysis started the day after sampling.
- The participating laboratories sent the analysis results to KWR for further processing.

The analysis results have been processed and partially tested statistically. This has led to the following conclusions and recommendations:

- This study has demonstrated the usefulness and added value for analysing cooling water and process water samples in accordance with the analysis scheme based on using Q-PCR analysis for *Legionella pneumophila,* followed in the case of a positive result by a specific cultivation method for *Legionella pneumophila*. Besides providing managers of cooling water and process water systems with information faster, this new screening methodology also provides them with more specific information on the presence of *Legionella pneumophila* in the system. The choice of the specific Q-PCR for *Legionella pneumophila* in the scheme does not affect the result. The choice of the specific cultivation method should be based on the recommendations of this study and possibly the results of a further study.
- Variance analysis showed for the cooling water and process water samples used in this study that there were no significant statistical differences between the results of three Q-PCR techniques for detecting *Legionella pneumophila* performed by three different laboratories in accordance with three different standards.
- In most cases, the results of the Q-PCR techniques for the cooling water samples studied meet the requirement for a detection limit of at least 1,000 units/litre, based on table 12 of Health & Safety Information Sheet AI-32. If using Q-PCR technology systematically leads to more units per litre being detected than the number detected using the cultivation method, table 12 of the Health & Safety Information Sheet will have to be expanded for the interpretation of Q-PCR results. With regard to the specific situation at a company, when switching to Q-PCR, the method should preferably be used in parallel for a while with the cultivation method normally used up to that point, so that the results of the old and new method can be correlated. This can then be used as a basis for adjusting the action levels in the legionella control plan.
- The practical usefulness of the analysis scheme based on Q-PCR, followed by a cultivation method specifically for *Legionella pneumophila*, may be limited by the observation that using Q-PCR for the specific detection of *Legionella pneumophila* in the cooling water samples analysed does not lead to less positive samples than detection based on a cultivation method for *Legionella* in total. In comparison with drinking water systems, *Legionella pneumophila* is apparently fairly generally present in cooling water systems.
- This study has shown that applying NEN 6265 with BCYE medium for cooling water and process water samples leads to unsatisfactory results, owing to disruptive additional growth on the culture medium. NEN 6265 was revised for this in 2007 by also describing the MWY medium for samples with excessive additional growth.
- This study has shown that pretreating cooling water and process water samples with acid in accordance with ISO 11731:1998(E) and counting more frequently on day 3 and day 5 after incubation, leads to fewer outcomes for which no result can be stated on account of additional growth on the culture medium.
- Variance analysis has shown for the cooling water and process water samples used in this study that there are no significant statistical differences between the results of the cultivation method according to ISO 11731 and the cultivation method according to NEN 6265:2007 using an MWY culture medium, provided that the agar plates are also assessed on day 3 and day 5.
- Taking into account the method's limitations, the Fastpath™ method is a useful addition to the set of instruments available to the process operator responsible for the daily management of cooling water systems. The method's main benefits are speed, simplicity and the possibility of on-site implementation. However, it is important to realise that specificity in respect of *Legionella pneumophila* sero-group 1 means that the other sero-groups are not taken into account. (Nevertheless *Legionella pneumophila* sero-group 1 is the main cause of legionella pneumonia; up to 90% of registered cases worldwide). Moreover, the information is of such a qualitative nature that it cannot replace the measurements that are normally made.
- This study has not provided any indications of the presence of high concentrations of legionella bacteria in process water samples from the paper and cardboard industry. Nevertheless, the results indicate that *Legionella* or *Legionella pneumophila* can be detected, at least in a number of samples.
- It was notable for practically every cooling water system examined in this study that disinfection hardly ever consistently resulted in a legionella bacteria concentration of less than 1,000 cfu/l.
- The laboratories participating in this study have demonstrated that it is possible to achieve reliable results using a cultivation method for *Legionella* and/or *Legionella pneumophila.* A contributory factor

to this was that these laboratories were experienced and did everything possible to achieve good analytical results, without attempting to cut costs or avoid difficulties. The interlaboratory comparisons organised by KWR also confirmed that a certain degree of reliability can be achieved using a cultivation method.

Recommendations:

- It is recommended on the basis of the experiences from this study that the specific cultivation method for *Legionella pneumophila,* with an MWY cultivation medium based on NEN 6265:2007 and the draft version of NEN 6253, should be further optimised by means of pretreatment with acid and more frequent counting.
- As good results in this study were obtained using the cultivation method according to ISO 11731, it would be advisable to examine the extent to which the method could also be made suitable for the specific cultivation of *Legionella pneumophila.*
- A further recommendation – assuming a sufficiently large non-select sample of industrial cooling water systems – is that the two specific cultivation methods for detecting *Legionella pneumophila* should be checked both separately and in combination with Q-PCR assessment to determine their performance in terms of yield and cost.
- It would be advisable to set up interlaboratory comparisons for the Q-PCR techniques, as is done for the cultivation method.
- The cultivation method in accordance with ISO 11731 is recommended for detecting *Legionella* in process water from the paper and cardboard industry, subject to pretreating the sample with acid and reading the culture medium counts more frequently after incubation.
- A further examination of the role that 'full stream' or 'side stream' filtration plays in the effectiveness of disinfection in cooling water systems is recommended. Relevant variables in this are the type of disinfectant and the dose and method of dosing (continuous/discontinuous).

Contents

1 Introduction

1.1 Background

Employer's obligations under the Dutch Working Conditions Act include the requirement to take appropriate measures to prevent employees from being exposed to legionella bacteria. The introduction of health and safety policy rule 4.87-1 obliged employers to conduct a hazard identification and risk assessment (HIRA) for possible growth of *Legionella* in their cooling water and process water systems. In practice, the HIRA often results in management and control measures in the systems concerned. Experiences at Corus and Dow in recent years showed that the complex matrix of cooling water makes it particularly difficult to monitor *Legionella* in cooling water using the existing cultivation method in accordance with NEN 6265 (as described in the policy rule, as well as the alternative cultivation method according to ISO 11731). Moreover, there are major differences between the analysis results of different laboratories and the result can only be determined after at least 7 days.

The industry clearly needs a fast and reliable analytical method for *Legionella* detection or monitoring, to enable optimal management and control of *Legionella* concentrations in cooling water and process water systems. On the grounds of the limit values stipulated in Health & Safety Information Sheet (AI-32)[5] for *Legionella* in cooling water, an analytical method of this kind should have a detection limit of at least 1,000 legionella bacteria per litre.

Three recent developments at KWR concerning the analysis of legionella bacteria were recently extensively reported in the journal H_2O[1][2][3]:

- The newly developed quantitative real-time PCR method (Q-PCR) enables the concentration of *Legionella pneumophila* in a water sample to be measured within a few hours[1]. The Q-PCR method's reproducibility is better than that of the standard cultivation method. Chemical disinfection reduced the concentration of *L. pneumophila*, by more than 90 percent but thermally disinfecting mains water had hardly any effect on the results of the Q-PCR method. Correctly interpreting the results obtained therefore requires information on the water sample's origin/history.
- A cultivation method was developed at KWR that specifically demonstrates the presence of *Legionella pneumophila* on the basis of an increase in pH in the culture medium and a higher incubation temperature[2].
- Existing information has shown that *Legionella pneumophila* is by far the most important cause of legionella pneumonia. The researchers therefore concluded in a third article that the policy in the Netherlands should primarily focus on combating *Legionella pneumophila* in mains water/water systems[3].

A fourth development concerns an amendment of the existing standard NEN 6265 for the analysis of *Legionella*. A revised version of NEN 6265 was published in October 2007, and included a modified method of working for "isolating *Legionella* in a sample expected to contain disruptive flora" (such as cooling water and process water). In addition to making use of the usual BCYE medium, this method of working uses plates with an MWY medium [1].

The selective detection methods for *Legionella pneumophila* (Q-PCR and cultivation) make it possible to specifically tackle this organism in water systems. The most obvious use of these detection methods in practice is for rapidly screening a series of samples (e.g. from cooling tower systems) using Q-PCR, possibly followed by the selective cultivation method for *Legionella*

[1] MWY = Modified Wadowsky Yee Agar; BCYE = Buffered Charcoal Yeast Extract Agar

pneumophila, if *Legionella pneumophila* is detected in excess of a certain concentration (see scheme below).

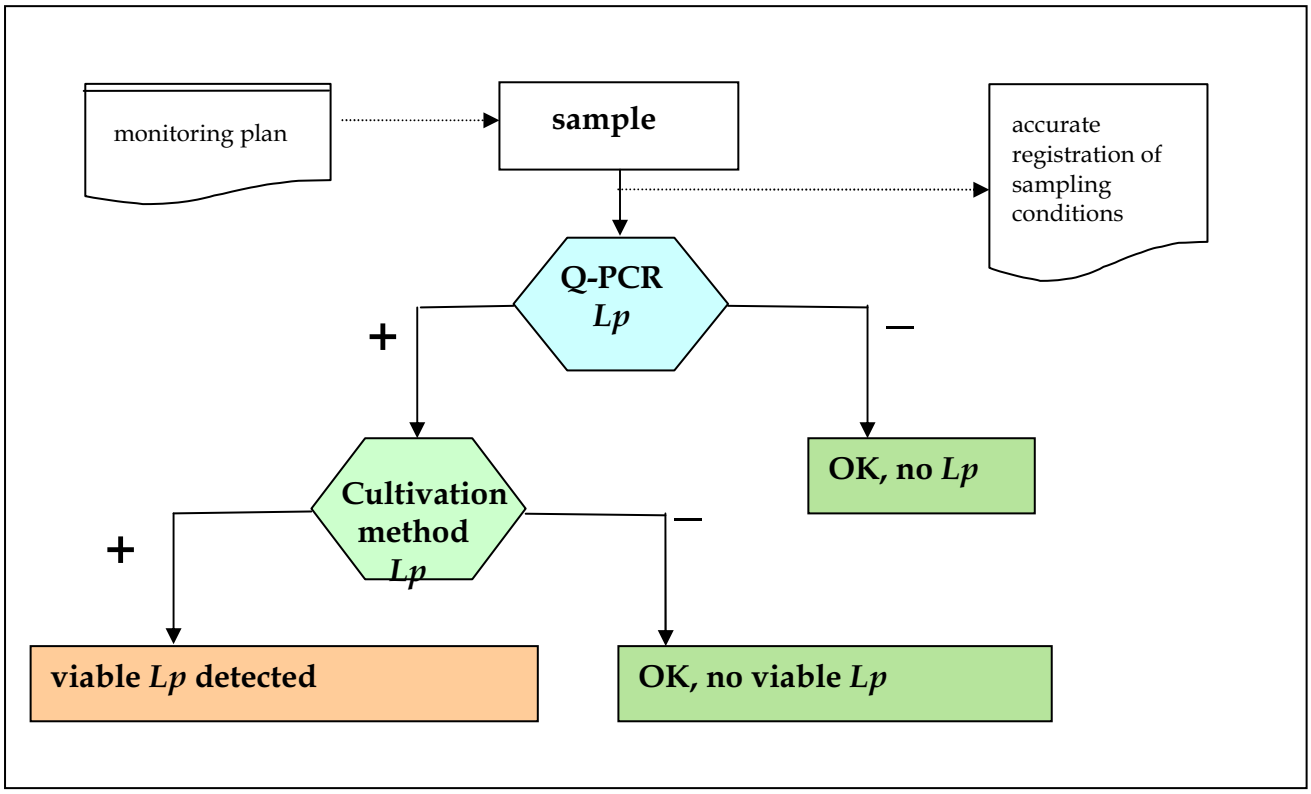


Figure 1. Proposed scheme for Legionella pneumophila screening in cooling and process water.

If the scheme shown in figure 1 proves to be economically and technically successful, it could provide an alternative to present cultivation methods referred to in documents such as the Health & Safety Information Sheet[5] (also referred to as AI-32) as standard methods for sampling and analysing *Legionella* in cooling water systems. It should be noted in connection with this that the government has not yet taken the step from detection of all viable varieties of legionella to specific detection of *Legionella pneumophila*.

The results of an analysis to detect *Legionella* or *Legionella pneumophila* may have major consequences that affect a factory's business operations and the environment. It is essential in connection with this to have a representative water sample from the water system being investigated. In practice, the analysis and analysis results receive a great deal of attention but not enough attention is paid to the quality of sampling. It is difficult to take a representative sample in a large cooling water or process water system. A standard sampling protocol is therefore required, in which the best options for the time, place and method of sampling are laid down. Moreover, to enable interpretation of the analysis results, it is important to keep a meticulous record of the conditions under which samples were taken.

Besides a reliable analytical technique that can be used for testing against statutory frameworks and company-specific guidelines, operators in the industry also need a fast, simple and cheap method for daily system monitoring. In practice, ATP measurements and colony number measurements (dip slides for example) are often used for this, both of which provide an idea of the system's general microbiological state. The immunochromatographic assay is a new development in this field and can be used for qualitative detection (present/absent) of *Legionella pneumophila* sero-group 1 (*Lp* SG1). This method is fast, simple and can be used on site. A major advantage of using this method is that it provides specific information on the presence of the most relevant pathogen of the *Legionella* genus. The immunochromatographic assay thereby offers a potentially useful addition to the operator's set of instruments.

1.2 Project objectives

The project objectives are:

- Development of a standard monitoring protocol that enables a representative sample to be taken of a complex cooling water or process water system.
- Testing and evaluating the usefulness of the scheme in figure 1 for cooling water and process water samples.
- Comparison (at KWR) of the analysis results obtained using the analytical techniques shown in figure 1 with the results of similar Q-PCR techniques and cultivation methods at other laboratories.
- Testing and evaluating the accuracy of the qualitative immunochromatographic assay for *Legionella pneumophila* SG 1 in cooling water and process water.

1.3 Companies and laboratories involved

This project was initiated as a multi-client project in which the aim was for all the project's industrial participants to supply cooling water and process water samples from their own systems. Table 1 provides a list of companies that provided samples for this project.

Table 1 List of companies involved in this study

Company	Sample types
A	cooling water from 2 systems and process water from 1 system at the same site
B	cooling water from 3 systems at the same site
C	cooling water from 3 systems at the same site
D	cooling water from 4 systems at various sites
E	cooling water from 3 systems at the same site
F	process water from 3 different systems at 3 different sites

The Royal Association of Dutch Paper and Cardboard Manufacturers (Koninklijke VNP) represents 21 paper and cardboard manufacturers in the Netherlands. These factories make repeated use of their process water. This results in water with a high concentration of undissolved substances, organic matter and micro-organisms. It is therefore particularly difficult to perform a specific microbiological assessment, for *Legionella*, for instance. Besides it being difficult to filter sufficient water for detection purposes, there is also often excessive additional growth on the culture medium. Conditions in a paper factory (high temperatures, much aerosol formation, high humidity) constitute all the reasons for monitoring the process water for *Legionella*. VNP is consequently extremely interested in evaluating new methods, such as the ones that are the subject of this project.

Table 2 provides a list of laboratories participating in this study. Chapter 2 provides a more detailed description of the methods used.

Table 2 List of laboratories involved in this project

Laboratory	Method(s) used
Lab I	cultivation methods related to NEN 6265 and ISO 11731
Lab II	Q-PCR Pall Genesystems
Lab III	Q-PCR
Lab IV	Q-PCR cultivation methods related to NEN 6265 Nalco Fastpath™ (immunochromatographic assay)

2 Methods

2.1 Sampling protocol

A small committee of representatives from the industry and cooling water technology experts drew up a sampling protocol for sampling cooling water and process water systems in aid of legionella analysis. The complete protocol is included in appendix I. The protocol includes an information sheet (list for completion) for recording current data on the sampled system. This information sheet is included in appendix II.
The protocol was primarily intended for sampling within the scope of this project and was distributed to the participating companies. It can also be seen as a general sampling protocol for cooling water and process water systems.

In summary, the following recommendations apply to sampling cooling water systems:

- Discontinuously disinfected cooling water systems should be sampled at the end of the disinfection cycle, which means just before introducing a new dose of disinfectant. The time of sampling is irrelevant in systems that are continuously disinfected. However, the position where samples are taken is important. Samples should preferably be taken just before the location of the dosing point.
- The following preferred order for the position where samples are taken should be used when sampling cooling water systems:
    - Sampling of the falling water in the free space above the cooling tower basin (*at least 1 metre from the side and using special tools*).
    - Sampling of the water in the cooling tower basin from a position as close as possible to the recirculation pump's suction pipe (*at least 1 metre from the side and within 10 to 20 cm below the water's surface*).
    - Sampling from a sampling tap in a main pipe on the outlet side of the recirculation pump, as close as possible to the cooling tower (*tap-out pipe should preferably not be made of rubber or plastic, should be shorter than 3 metres and should be flushed for 3 minutes before samples are taken*).

  The starting point for the preferred order is that the sample should represent the quality of the cooling water as far as possible at the point where it is sprayed and could be spread into the surrounding area as an aerosol. Spraying of cooling water takes place above the cooling section, which is generally an unsuitable place for sampling. The positions suggested here are intended to provide samples that are as close as possible to the aforementioned representative quality. Wall effects, namely factors which affect the quality of the sample owing to a biofilm or sediment (in the basin or in a pipe), are a key factor here and should be prevented as far as possible. This is the reason for the chosen distances to the wall of the cooling tower and the flushing time for the sampling point.
- It is particularly important to ensure homogeneous water distribution across the cooling tower when directly sampling falling water. This is also a standard precondition for a cooling tower to operate properly and is therefore in practice an important point for the operator's attention. Poor distribution is an indicator of local clogging in the cooling section, which reduces the system's cooling capacity and increases the likelihood of legionella bacteria growing.
- It is important to keep a record of the conditions under which samples were taken and especially of the disinfection regime.

In the case of process water systems, large variations in the types of systems make it impossible to provide standard recommendations for the best place to take samples. A few recommendations are provided in appendix II for the paper and cardboard industry and for the steel industry. The following general recommendations apply for process water flows:

- Discontinuously disinfected process water systems should be sampled at the end of the disinfection cycle. The time of sampling is irrelevant in systems that are continuously disinfected. However, the position where samples are taken is important. Samples should preferably be taken just before the location of the dosing point.

- It is important to keep a record of the conditions under which samples were taken and especially of the disinfection regime.

2.2 Analytical methods

2.2.1 Summary of analytical methods used in this study

Table 3 provides a list of the analytical methods and associated standards used in this study.

Table 3 List of the analytical methods used in this project

Analytical technique	Standard	Laboratory that performed the work in this study
cultivation method for *Legionella* with BCYE medium	in accordance with NEN 6265	Lab IV
cultivation method for *Legionella*	in accordance with ISO 11731	Lab I
cultivation method for *Legionella* with MWY medium	in accordance with NEN 6265:2007 based on NEN 6265:2007 (with more frequent reading)	Lab IV Lab I
cultivation method for *Legionella pneumophila* with BCYE medium	in accordance with draft version of NEN 6253	Lab IV
cultivation method for *Legionella pneumophila* with MWY medium	based on NEN 6265:2007 and draft version of NEN 6253	Lab IV
Q-PCR for *Legionella pneumophila*	in accordance with draft version of NEN 6254; growth of a fragment of the *mip* gene (DNA) with PCR and specific primers.	Lab IV
Q-PCR for *Legionella pneumophila*	Applied Biosystems. Detection and Quantification of *Legionella spp*. and *Legionella pneumophila*. In accordance with protocol v.2.0	Lab III
Q-PCR for *Legionella pneumophila*	Pall Genesystems. Quantitative PCR for *Legionella pneumophila*. Validated by AFNOR in accordance with XPT90-471 standard. Additional interpretation of the PCR result in accordance with draft version of NEN 6254.	Lab II
Fastpath™	Nalco	Lab IV

Fastpath™ is a qualitative field test especially for detecting *Legionella pneumophila* sero-group 1. The method is based on a chromatographic test that uses an immunoassay in which a reaction occurs between antibodies on the detection equipment and antigens of *Lp* SG1. The method uses a test strip on which the preconcentrated sample has to be placed. The sample has to be pretreated by filtration to obtain a sufficiently low detection limit. The test strip has a test band and a control band. The control band always indicates a red line; the test band shows a second red line for the presence of *Legionella pneumophila* sero-group 1 in excess of the detection limit. The detection limit is 100 cfu/l following pretreatment of 250 ml of the sample by filtration and 100,000 cfu/l without filtration. All pretreatment and detection requirements were provided by Nalco. Laboratory technicians have been trained to use the method.

2.2.2 Differences in performing the various cultivation methods

The analytical method described in NEN 6265 is generally used for *Legionella* detection in water/drinking water samples in the Netherlands. The method requires use of a specific culture medium (BCYE = Buffered Charcoal Yeast Extract). This culture medium is known to lead to problems when used for water samples with a high concentration of disruptive flora.
The internationally used ISO 11731 method is based on the same type of medium but with the addition of various other substances, the names of which are indicated in the name of the medium (GVPC = Glycine Vancomycine Polymixine Cycloheximide agar).
The KWR study of 2005[4] showed that for samples with a high concentration of disruptive flora – such as samples of cooling water and process water – additional growth is best inhibited by using MWY medium (MWY = Modified Wadowsky Yee agar) as the culture medium. This medium was consequently introduced in 2007, in the revised version of NEN 6265.

Besides the culture medium's composition, the pH value and incubation temperature are also important parameters, in connection with the *Legionella* yield in the analysis. Research has established that the variation in pH and incubation temperature can be used to make the cultivation method specific for detecting *Legionella pneumophila*[2]. With respect to NEN 6265:2007, the culture medium's pH is increased from 6.9 ± 0.1 to 7.3 ± 0.5 and the incubation temperature from 36 ± 2 ˚C to 40 ± 0.5 ˚C. The method for the specific analysis of *Legionella pneumophila* has been set out in the draft version of NEN 6253.

When performing the method set out in NEN 6265:2007, using MWY medium, lab I adopted a method that differed in the following way from the usual standard:

- The culture media were assessed after 3, 5, 7 and 10 days. Standard practice is to count the colonies on a culture medium after an incubation period of at least 7 days. Reading the culture media more frequently makes the method more labour intensive and more expensive but may offer the advantage of better assessment, as additional growth is still limited.

2.2.3 Differences in performing the three Q-PCR techniques

The quantitative PCR method, referred to simply as Q-PCR, is based on the Polymer Chain Reaction, in which a specific DNA fragment can be amplified to produce large numbers of copies under the influence of thermal cycles and with the aid of enzymatic reactions. Specific amplification is made possible by using short synthetic DNA molecules, known as primers. The base sequence or DNA sequence in the primers is chosen so that it binds selectively to the DNA, in this case of *Legionella pneumophila*. An enzymatic chain reaction occurs in the PCR once the primers bind to the DNA obtained from the water sample and the DNA fragment is amplified between the primers during each temperature cycle. Amplification can only occur if the sample contains this specific *L. pneumophila* DNA. In Real-time PCR, the formation of the amplified DNA is measured during each cycle (real-time). A synthetic DNA molecule known as the probe is used for this and is labelled with a fluorescent dye. The fluorescence occurs after the DNA has formed. Quantification is possible because there is a clear link between the time in the reaction at which it becomes possible to detect the DNA fragment that has formed (C_T value) and the *L. pneumophila* DNA concentration in the sample.

2.2.3.1 Q-PCR according to the draft version of NEN 6254

The Q-PCR method according to the draft version of NEN 6254 was developed and validated at KWR. Detection of *L. pneumophila* is carried out by specific primers that amplify a small fragment of the *mip* gene. The possibility of inhibiting the PCR and yield of DNA isolation is quantified by adding control DNA to every sample. The control is quantified at the same time in a multiplex PCR. The quantitative result of *L. pneumophila* detection is corrected using the yield produced by the control. Around 20 samples can be analysed per analysis.

2.2.3.2 Q-PCR according to Pall Genesystems Technology

The Q-PCR method is based on a method that uses a completely standardised PCR system comprising a DNA extraction, a specific PCR machine and a gene disc that includes everything necessary for the specific DNA amplification. Besides detection of *L. pneumophila* and/or *Legionella* spp., the detection

method also includes various quality controls, such as an external quantitative positive control. No information is available on the DNA fragment that is amplified, the DNA sequence of primers or the probe. Six or 12 samples can be analysed simultaneously per analysis run. The method was validated by AFNOR. The interpretation was also performed (limit of detection (LOD) and lowest quantity determinable (LQD)) in accordance with the draft version of NEN 6254 when the results in this study were evaluated.

2.2.3.3 Q-PCR according to the Applied Biosystems detection kit

Applied Biosystems developed and marketed the Q-PCR method. The Q-PCR detection and quantification kit for *L. pneumophila* includes an internal positive control and Amperase UNG. Amperase UNG treatment prevents the reamplification of PCR DNA fragments that were formed in earlier PCR experiments. This method also lacks information on the DNA sequence of primers, probes and controls.

2.3 Method used in the study

As described in section 1.3, the project's participants supplied samples form 15 cooling water systems and 4 process water systems. KWR collected and homogenised the water samples and then distributed them to the participating laboratories for further analysis.

The following method of working was used for this:

- Each participating company selected 3 cooling water or process water systems with a demonstrable 'legionella history' (select sample).
- Each participating company assigned responsibility to one or more people for taking samples in accordance with the protocol, which KWR provided (appendix I), and for registering the current operating data for the selected systems (appendix II).
- All the participating companies were given 27 sterilised 1-litre bottles with a standard content comprising a thiosulphate solution for neutralising oxidising biocides and an NTA solution for complexing heavy metals.
- The company then sampled each cooling water or process water system on three different days, after which the samples were sent to KWR by courier. Three sample bottles were filled for each system. A note of the current operating data for each system was made on the information sheet.
- The contents of the three bottles with samples from the same system were homogenised by KWR on the same day and divided into new sample bottles. These sample bottles were sent to the participating laboratories by courier on the same day (see table 2).
- Analysis started the day after sampling.
- The participating laboratories sent the analysis results to KWR for further processing.

The samples were consecutively coded from OPIW1 to OPIW54. The contents of sample bottles OPIW 1, OPIW 2 and OPIW 3 from company D were mistakenly mixed with each other while the work was underway. The resulting sample was further analysed as OPIW 1 and codes OPIW 2 and OPIW 3 were removed from the overviews.

The study led to a results matrix for 52 samples analysed using 10 analytical techniques, which produced 520 results. The matrix is shown in appendix III.

2.4 Comparison of the analysis results of the various analytical methods

A more in-depth statistical examination of some parts of the study was conducted by a statistician with the aid of variance analysis (ANOVA method, see appendix VI).

International standard ISO 16140 was also used for a qualitative comparison of the analysis results. The aforementioned ISO standard includes a protocol which describes how alternative microbiological

analytical methods can be compared with a reference method. The following parameters and definitions are important for this:

- Relative accuracy (AC). Degree of accordance between the result of the reference method and the result obtained with the alternative model using the same samples.
- Relative sensitivity (SE). Ability of the alternative method to analyse for the parameter concerned when it is detected by the reference method.
- Relative specificity (SP). Ability of the alternative method not to analyse for the parameter concerned when it is not detected by the reference method.

These parameters can be calculated using a results matrix, as shown in table 4, and using the following formulas:

- AC = (PA + NA)/N * 100%
- SE = PA/N_+ * 100% = PA/(PA + ND) *100%
- SP = NA/N_- * 100% = NA/(NA + PD) * 100%

Table 4. Results matrix for the qualitative comparison of various analytical methods

Results (total N samples)	Reference method positive (N_+ samples)	Reference method negative (N_- samples)
Alternative method Positive	+/+ number of samples with a positive accordance (PA)	-/+ number of samples with a positive deviation (PD)
Alternative method Negative	+/- number of samples with a negative deviation (ND)	-/- number of samples with a negative accordance (NA)

3 Results and discussion

3.1 General

The main aim of this study was to test and evaluate the usefulness of the scheme in figure 1 for cooling water and process water samples. It essentially involves using Q-PCR for *Legionella pneumophila*, possibly followed by a cultivation method specifically for *Legionella pneumophila* to confirm the viability of the bacteria.
Appendix IV includes a summary of the results achieved after applying the analytical methods referred to in figure 1, on the understanding that the specific cultivation method according to the draft version of NEN 6253 was used in every case and not only when Q-PCR produced a positive result.
The results can be summarised as follows:

- 33 of the 52 samples (63.5%) showed a positive result after using Q-PCR in accordance with the draft version of NEN 6254.
    - A positive result was also obtained for 16 of the 33 corresponding samples when the specific cultivation method was used.
    - Excessive additional growth on the agar plate meant that no result could be stated for 9 of the 33 corresponding samples when the specific cultivation method was used.
    - However, DNA copies were found for 8 of the 33 corresponding samples when Q-PCR was used but no viable bacteria were detected using the specific cultivation method (< detection limit).
- The result for both Q-PCR and the cultivation method was below the detection limit for 11 of the 52 samples (21%).
- No Q-PCR could be stated for 5 of the 52 samples (9.6%) because the yield of the internal control was below the limit value for reliable analysis of the sample.
- For 3 of the 52 samples (5.8%), the result using Q-PCR was below the detection limit and too much additional growth on the culture medium meant that no result could be stated for the cultivation method.
- No false negative results were obtained using the Q-PCR method, which means that no DNA copies were found using Q-PCR but colonies were found on the culture medium when using the cultivation method.

The case in which 8 of the 33 results were positive using Q-PCR and negative using the cultivation method indicates that, although there may not have been any viable *Legionella pneumophila* bacteria in the sample at the time of sampling, it nevertheless contained insufficiently lysed legionella bacteria which would still be detected. (The DNA of dead legionella bacteria that has completely disintegrated or lysed is fairly present in the solution, so it passes through the filter during filtration and no longer affects the PCR result.) This is a typical result after using the scheme in figure 1 when thermal or chemical disinfection has been used in the sampled system. In such situations it indicates effective disinfection but also the presence of legionella bacteria during certain periods (such as between disinfection operations) or in certain parts of the system. The latter may apply in the case of continuous disinfection, for example. The overview in appendix IV, which also includes a summary of the disinfection method used per system, shows that this is indeed the case in systems that undergo discontinuous disinfection by means of shock dosing and in systems that are continuously disinfected. Table 5 shows a simplified scheme with a proposal for a general qualitative interpretation of the screening according to figure 1.

Table 5 Scheme for interpreting results of the screening method according to figure 1

Q-PCR	specific cultivation method	interpretation
< detection limit	< detection limit	The sampled water contains no detectable *Legionella pneumophila* or its remains. Depending on the specific circumstances (size of the system; representativeness of the sample) this is an indication of a microbiologically stable system in which no *Legionella* growth occurs.
positive result	< detection limit	The sampled water contains no viable *Legionella pneumophila* in excess of the detection limit but there is an indication that growth can nevertheless occur in the system at certain times (periods between two shock doses, for example) or at certain positions (outside the range of the sampling point and outside the range of continuous disinfection). Situation requires further study.
positive result	positive result	The system contains viable *Legionella pneumophila* bacteria. The situation has to be assessed on the basis of established concentration levels according to applicable protocols. If disinfection is carried out, its effectiveness should be further ascertained.

3.2 Comparison of Fastpath™ with other techniques

The Fastpath™ method was executed at lab IV using test kits provided by the supplier. During execution, it emerged that reading the test strip can actually be a point for discussion, in the sense that interpretation of some results involves a certain degree of subjectivity.
The results of Fastpath™ - as an alternative method - were compared with those of the Q-PCR carried out by lab IV (see appendix V). As Q-PCR focuses on *Legionella pneumophila* and the immunoassay only on *Legionella pneumophila* sero-group 1, the interpretation also took into account the results of serotyping carried out by lab III, in the case of the ISO 11731 and NEN 6265 analysis. This makes it possible to determine whether, for example, a negative result of the Fastpath™ method is correct.

The results can be summarised as follows:

- A total of 47 samples were assessed (there was no result for 5 samples when Q-PCR was used as the reference method).
- The Fastpath method gave a false positive result 4 times, a false negative result 5 times and a correct negative result 6 times. It should be noted in connection with this that two false positive results were obtained for samples from the paper industry, whereby the detection limit for Q-PCR was much higher.
- The relative accuracy (AC) of Fastpath™ was 81% of that of Q-PCR.
- The method's relative sensitivity (SE) was 85%.
- The relative specificity (SP) was 71%.
- When determining these parameters, a correct negative result of Fastpath™ was interpreted as a positive accordance (PA). A correct negative result arises when *Legionella pneumophila* is detected using Q-PCR, for example, but the serotyping indicates that sero-group 1 has not been found in the sample concerned.

In view of the good results obtained using the ISO method (see section 3.4), this cultivation method's results were also compared with those of Fastpath™ (see appendix V).

The results can be summarised as follows:

- A total of 49 samples were assessed (no results for 3 samples with ISO method as reference method).
- The Fastpath method gave a false positive result 5 times, a false negative result 5 times and a correct negative result 10 times. The sample codes for the 5 false negative results correspond exactly with those obtained from the comparison with Q-PCR. In two of the 5 false negative results, relatively low concentrations were measured using the ISO method (200 and 530 cfu/l) but in the three other cases the concentrations were around 60,000 cfu/l.
- The relative accuracy (AC) of Fastpath™ was 80% of that of ISO.
- The method's relative sensitivity (SE) was 86%.
- The relative specificity (SP) was 58%.
- When determining these parameters, a correct negative result of Fastpath™ was interpreted as a positive accordance (PA).

On the basis of these results, the degree of accordance of Fastpath™ with the two reference methods is approximately 80%. It is ultimately up to the users to determine according to their own criteria whether this level of accuracy is sufficient for using this fast screening method in practice. It is also important to realise that the method is limited to *Legionella pneumophila* sero-group 1; however, this is the main cause of legionella pneumonia (up to 90% of registered cases worldwide).

3.3 Comparison of Q-PCR techniques

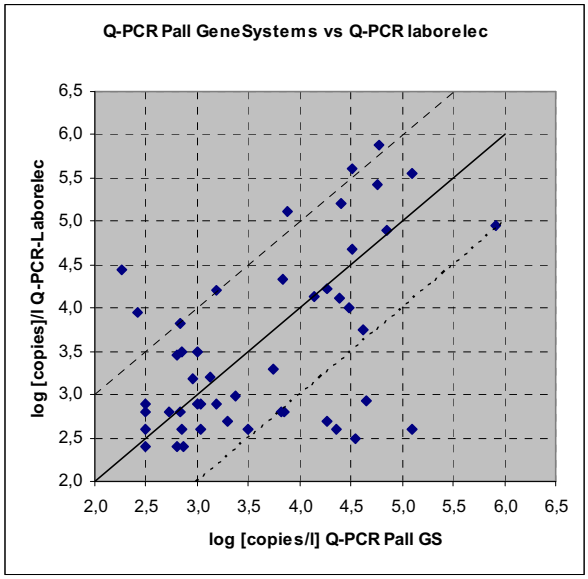
Appendix VI provides an overview of the Q-PCR analysis results obtained by three different laboratories using three different methods. The main characteristics are summarised in the table below.

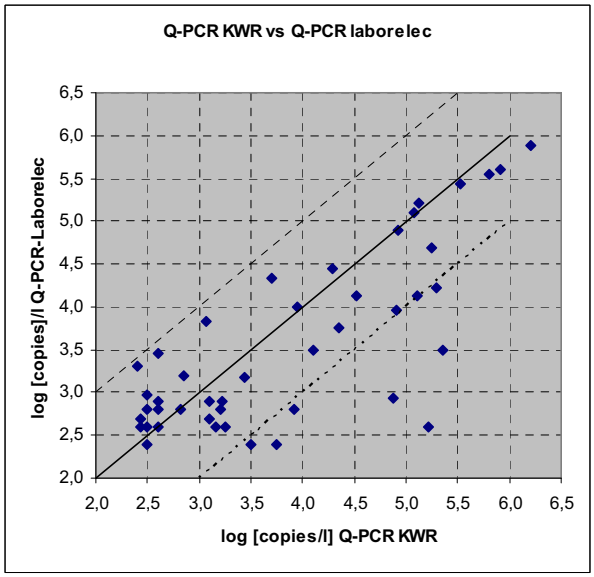
Table 6. Main characteristics of the results of the three Q-PCR techniques

	Q-PCR lab IV	Q-PCR lab III	Q-PCR lab II
number of positive samples	33	26	47
• which were > 100,000	• 11	• 5	• 3
• which were > 1,000	• 22	• 19	• 30
• which were ≤ 1,000	• 3	• 2	• 14
number of negative samples (< detection limit)	14	22	5
number of samples with no result	5	4	0

No value could be determined for 5 of the Q-PCRs performed by lab IV, as the yield of the internal control was below the limit value for reliable quantitative analysis. However, in 4 of the 5 cases *Legionella pneumophila* was detected. In 4 samples in the Q-PCR performed by lab III the level of inhibition produced an unreliable result. No value is stated for these cases.

Figure 2 sets out the Q-PCR results of each of the three laboratories against each other in a double logarithmic diagram. An analysis result below the detection limit is shown here as the logarithm of half of the detection limit concerned (log{detection limit/2}). The good accordance between the three methods is striking. It is also notable that the Q-PCR at lab IV detected a higher number of copies more often than the other two methods. No immediate explanation is available for this but it may be an effect of the yield correction that was made.





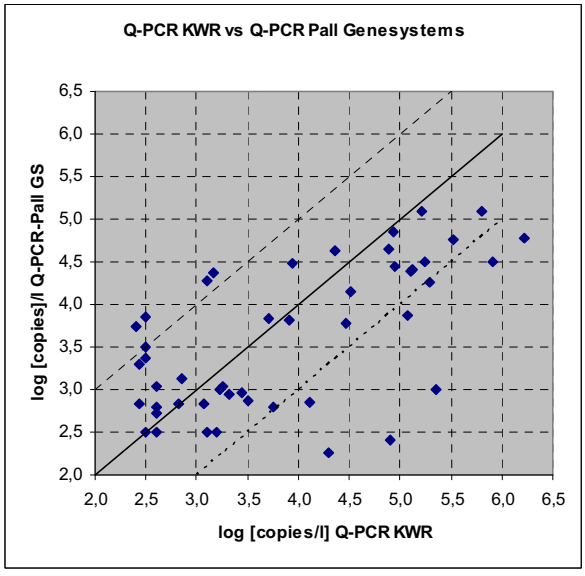


Figure 2. Comparison of numbers of Legionella pneumophila detected using the three different Q-PCR techniques in the water samples that were studied. The dotted lines show the relationship between the numbers detected using the two methods that have been compared (respectively a factor of 10 higher and a factor of 10 lower).

Table 7 Results of the qualitative assessment of the three Q-PCR techniques vis-à-vis ISO 11731 as the reference method.

	Q-PCR lab IV	Q-PCR lab III	Q-PCR lab II
relative accuracy (AC)	91%	78%	82%
relative specificity (SP)	89%	82%	33%
relative sensitivity (SE)	92%	76%	97%

A comparison was made in accordance with ISO 16140 (see section 2.4) to enable an initial qualitative assessment of the results of the three Q-PCR methods and the results of the cultivation method according to ISO 11731 as a reference. When making comparisons it must be remembered that the Q-PCR is concerned with *Legionella pneumophila* and the ISO method with *Legionella as a whole*. The ISO method's serotyping was therefore also taken into account, to ascertain whether a negative Q-PCR result was correct. A correct negative result was then deemed to be a positive accordance (PA) in the qualitative comparison. Table 7 shows a summary of this qualitative comparison's results. The calculations shown in

tabular form in appendix VI only included those samples for which both analytical methods produced a result. Table 7 generally shows a high level of accordance between the Q-PCR results and the results obtained in accordance with the method of ISO 11731. Only the specificity of the Pall Genesystems Q-PCR was relatively low. This was because this method detected a quantifiable number of legionella cells in 92% of the samples examined, which is a relatively high figure (8% of the samples were below the detection limit).

A variance analysis was used to compare the results of the three different Q-PCR techniques with each other (see appendix VII). The zero hypothesis was tested in this analysis that the mean of the analysis results is the same for the three different Q-PCR methods. It is 95% certain that this hypothesis cannot be rejected. Statistically, this means that there are no significant differences between the three methods.

The detection limit of the various Q-PCR techniques was generally lower than 1,000 copies/litre for cooling water samples. It was only necessary to state a higher detection limit than that on a number of occasions when using the Q-PCR of lab III. The 1,000 cfu/l requirement stated in the Health & Safety Information Sheet could not be met in those cases. However, the question is whether the table in question, which was drawn up on the basis of the interpretation of results of the cultivation method, can still be used for interpreting Q-PCR results. The main point is whether using Q-PCR is a reason for significantly higher numbers of copies per litre with respect to the number of colony-forming units per litre. This is examined in figure 3 by setting out the average results of the Q-PCR methods against those of the specific cultivation method for *Legionella pneumophila* using MWY medium (in accordance with the draft version of NEN 6253). In both cases, values below the detection limit have been included in the graph with a value corresponding with half the value of that detection limit.

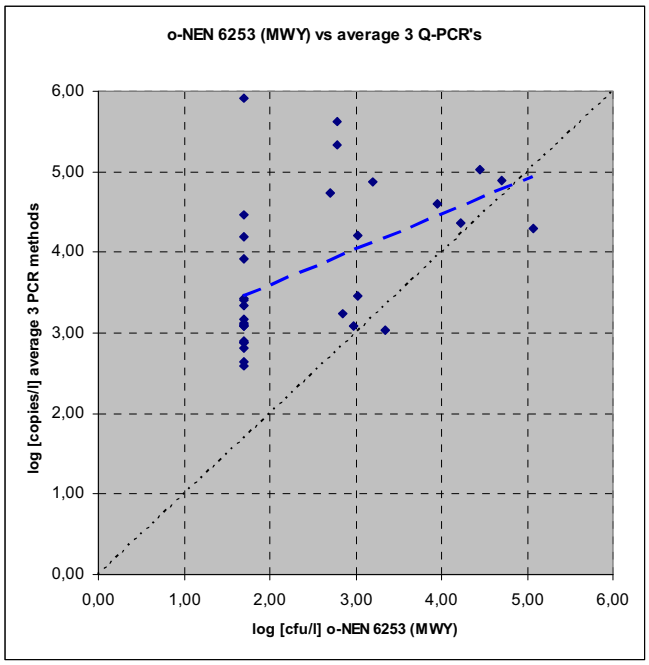


Figure 3. Comparison of the specific cultivation results for Legionella pneumophila, according to the draft version of NEN 6253 using MWY medium, and the average of the results of the three Q-PCR techniques. The finely dotted line shows the relationship between the numbers detected with the two methods that have been compared. The other line is a linear trend line.

An initial glance at figure 3 shows that there is a wide spread in the analysis results. The spread is partially accounted for by disinfection in the systems examined (see explanation in section 3.1). In any case, it is clear from figure 3 that in 94% of the samples DNA copies were found in larger numbers when using Q-PCR than the numbers of colony-forming units that were found when using the cultivation method. This is possibly explained by the fact that, unlike with the cultivation method, corrections are made for the detection yield when Q-PCR is used. Poor correlation makes it impossible to establish a fixed relationship.

3.4 Comparison of cultivation methods

A total of five different cultivation techniques were used in this study (see table 3 and appendix VIII). The method according to NEN 6265:2007 using MWY medium was applied by two laboratories (lab IV and lab I).

The results of the 'normal' NEN 6265 using BCYE medium were disappointing because no result could be provided for 31 of the 52 samples (60%) owing to excessive additional growth on the culture medium. This is as expected, given the complex matrix of the cooling water and process water samples and is also the reason why the MWY medium was included in the NEN standard. Poor results were also obtained with the cultivation method using BCYE medium with a higher pH culture medium (pH 7.3) and samples at a higher incubation temperature (40 °C), which was specifically for *Legionella pneumophila* analysis. In that case, no result could be stated for 34 of 52 samples (65%) owing to excessive additional growth on the culture medium.

Using MWY medium in accordance with NEN 6265:2007 considerably reduced the amount of disruptive additional growth. This analytical method was used by lab IV as well as lab I, whereby it should be noted that lab I also made counts on day 3 and 5. The summary in appendix VIII shows that there was too much additional growth on the culture medium for respectively 15 (29%) and 16 (31%) of the 52 samples. That is half of the figure vis-à-vis the results when BCYE medium was used. Figure 4 shows the results of the two assessment methods using MWY medium alongside each other. It should be noted here that in the results of lab IV a figure of "< 100 cfu/l" is shown as 50 cfu/l (detection limit with a log value of 1.7) and in the results of lab I a figure of "< 500 cfu/l" is shown as 250 (log value 2.7).

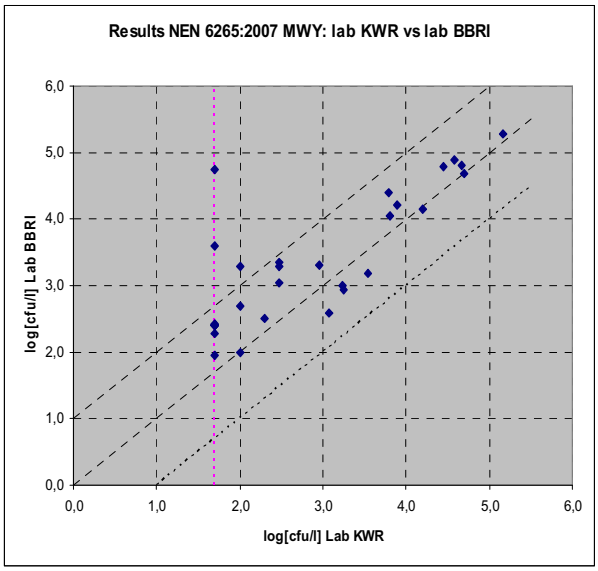


Figure 4. Comparison of the results of lab IV and lab I obtained using NEN 6265:2007 (MWY). The dotted lines show the relationship between the numbers detected using the two methods that have been compared (respectively a factor of 10 higher and a factor of 10 lower).

Two of the results shown in the graph differ markedly because they were below the detection limit in the analysis of lab IV whereas lab I stated figures of 4,000 and 65,000 cfu/l. These higher figures for these two specific samples appear to be more in line with the corresponding results obtained using the Q-PCR and ISO 11731 methods. Moreover, there is exceptionally good accordance between the results, whereby the yields were generally slightly higher for the detection method used by lab I. This may be a consequence of the extra treatment with acid applied during sample pretreatment.

Table 8 provides details of a qualitative assessment carried out in accordance with the ISO 16140 method (see section 2.4) of the results of the two NEN 6265:2007 methods using MWY medium vis-à-vis ISO 11731 as the reference method. This shows a high level of accordance although in the case of lab I the specificity of the detection method using MWY medium was relatively low. Low specificity may point to

a benefit of using MWY medium, as in cases in which the reference method is unable to detect anything, NEN6265:2007 using MWY medium is able to do so.

Table 8 Results of the qualitative assessment of the NEN 6265:2007 (MWY) vis-à-vis ISO 11731 as the reference method.

	NEN 6265:2007 (MWY) Lab IV	NEN 6265:2007 (MWY) Lab I
relative accuracy (AC)	81%	83%
relative specificity (SP)	89%	50%
relative sensitivity (SE)	79%	90%

Appendix VIII's summary of the cultivation methods shows that the cultivation method according to ISO 11731 performed best. In that case only 3 samples (5.7%) displayed excessive additional growth on the culture medium, so that no value could be stated. In the ISO method, lab I applied pretreatment with acid (in accordance with the standard) and also made counts more frequently after 3, 5, 7 and 10 days. This makes the method more laborious but produces a better result. In 37 of the 52 samples (71%) *Legionella* was detected and in 18 of those cases it was in concentrations of 10,000 cfu/l or higher. The comparison in figure 5 of the results from lab I obtained with ISO 11731 and those obtained with NEN 6265:2007 (MWY) indicates a turning point at 1,000 cfu/l. The ISO method's yield is higher when the sample contains higher numbers of legionella bacteria; the yield is lower when the numbers are lower. The significance of the measurement is lower for a figure below 1,000 cfu/litre.

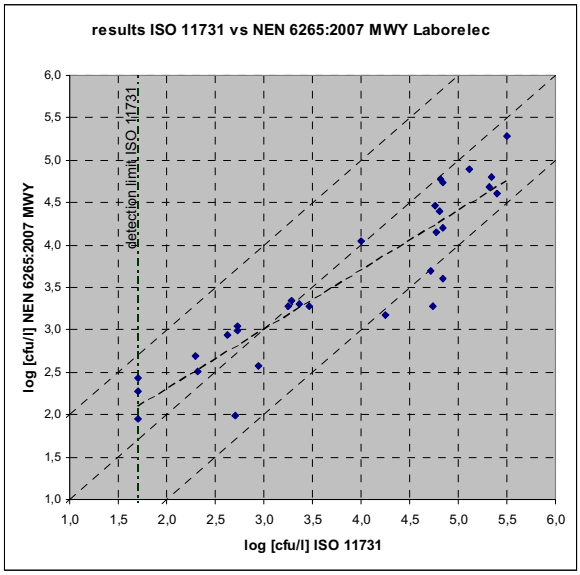


Figure 5. Comparison of the results from lab I obtained using ISO 11731 and NEN 6265:2007 (MWY). The dotted lines show the relationship between the numbers detected using the two methods that have been compared (respectively a factor of 10 higher and a factor of 10 lower).

The relationship between the two methods was also examined using variance analysis (see appendix VII). The zero hypothesis was tested in this analysis that the mean of the analysis results is the same for both methods. It is 95% certain that this hypothesis cannot be rejected. Statistically, this means that there were no significant differences between the results of the two methods (noting that the plates were also assessed for both methods after 3 and 5 days).

Finally, this study also applied a cultivation method specifically for *Legionella pneumophila* analysis in accordance with figure 1 (see also section 3.1). The method is based on the draft version of NEN6253 using MWY medium, with a different culture medium pH and incubation temperature to inhibit growth

of non-pneumophila varieties. The statements below are based on a comparison of results of this cultivation method and those lab I obtained with NEN 6265:2007 using MWY medium and the associated serotyping:

- The results of both analyses were positive for 13 samples and in accordance with serotyping in every case (i.e. the presence of *Legionella pneumophila* was confirmed by serotyping).
- In 6 samples *Legionella* was detected and the concentration of *Legionella pneumophila* was below the detection limit. However, only *Legionella pneumophila* sero-group 1 or sero-groups 2 – 14 were confirmed by serotyping, so this might point to a discrepancy.
- In 5 samples *Legionella* was detected and the concentration of *Legionella pneumophila* was below the detection limit, and serotyping did indeed point to the presence of non-pneumophila varieties.
- The results of both methods of analysis were below the detection limit for 6 of the samples.
- No result was stated for the remaining 22 samples, as there was too much additional growth on the culture medium in 13 cases for one of the methods and in 9 cases for both of the methods.

In any case, it follows from this that there was accordance between the analytical techniques for 24 of the 30 samples. Serotyping indicated possible poor accordance between the two methods for the remaining 6 samples but it should be noted here that serotyping always involves making a selection from the colonies on the culture medium.

3.5 Effect of cooling water disinfection on the analysis results

Cooling water disinfection is a normal part of conditioning cooling water systems. The main aim of disinfection is to prevent biofilm formation in cooling water systems. Biofilm formation in heat-exchangers reduces thermal transfer and can reduce the cooling capacity of cooling water sections, as it restricts air flow. Disinfection is also intended to inhibit *Legionella sp*. growth in order to reduce exposure risks in the surrounding area.
A distinction should be made in cooling water disinfection between continuous application of a disinfectant and discontinuous application by means of shock dosing. Extensive use is still made of sodium hypochlorite (NaOCl) for disinfection but a move is underway towards more sustainable disinfection, using hydrogen peroxide, ozone or physical techniques such as ultrasound or cavitation, for example. It is also relevant to distinguish between systems with and without filtration (full stream filtration or side stream filtration). Filtration is usually used to remove undissolved substances in the circulating cooling water. This can have a positive as well as a negative effect. On the one hand, removing undissolved substances may reduce the likelihood of the cooling system becoming microbiologically contaminated. On the other hand, microbiological accumulation in the actual filter leads to increased use of sodium hypochlorite and can reduce the effectiveness of disinfection.

In this study, samples were taken from 15 different cooling water systems in which the following disinfection strategies are used:

- No disinfection (1 system).
- Use of Sonoxide (1 system).
- Continuous addition of sodium hypochlorite (NaOCl) (3 systems).
- Discontinuous addition of sodium hypochlorite (NaOCl) / shock dosing (7 systems).
- Continuous addition of hydrogen peroxide (2 systems).
- Continuous addition of ozone (1 system).

Three systems also used filtration in a side stream (1 system) or in the entire circulating water flow (2 systems).

The limited scope of this study cannot justify any statistically sound statements on the effect of a particular disinfection strategy on the presence of legionella bacteria in the cooling water. Moreover, the participating companies selected cooling tower systems with a known legionella history, so the sample of industrial cooling water systems was emphatically not non-select.

If the results of using the ISO 11731 method are taken as the starting point, it is nevertheless clear that disinfection was practically never sufficiently effective in any cooling water system. Two systems of company C and one of company E formed an exception to this but it should be pointed out that extremely high doses of chlorine were used in the systems of company C during the sampling period, owing to a defective chlorine measurement system.

The following statements on the cooling water systems covered by this study apply, if the analysis results obtained using the ISO 11731 method are used as a basis for making a rough qualitative assessment of the performance of the aforementioned disinfection strategies:

- Practically all disinfection strategies that make use of a shock dose without sand filtration perform reasonably.
- All disinfection strategies involving the continuous addition of disinfectant in combination with a sand filter (full stream and/or side stream) perform poorly (ozone with side stream filtration and sodium hypochlorite with full stream filtration).
- However, disinfection strategies involving continuous addition without sand filtration perform reasonably (Sonoxide, sodium hypochlorite, and hydrogen peroxide).

This may point to an unwanted effect of using side stream or full stream filtration in the cooling water systems covered by this study. In practice, it may be assumed that considerable biofilm formation occurs on the large internal area of a sand filter. From the operational point of view that appears to be an advantage because in that case no biofilm forms on the heat-exchanger's pipes or on the cooling section. However, in the case of continuous disinfection or if filtration is not stopped during discontinuous disinfection, the biofilm in the sand bed may consume so much disinfectant that the amount of disinfectant received by the rest of the system after the sand bed is insufficient. Depending on the position where filtration takes place with respect to the point where disinfectant is added, this may lead to inadequate disinfection in parts of the system.

4 Evaluation and conclusions

4.1 General

Over a 6-week period this study used 10 different analytical techniques to examine a total of 52 samples from 15 cooling water systems and 4 process water systems for the presence of *Legionella* or *Legionella pneumophila*.

A standard monitoring protocol was drawn up prior to sampling. The aim of the protocol was to enable sampling to provide the most representative picture possible of legionella concentration in the cooling water or process water system at the point in the system where spraying is most likely to occur and aerosols are spread into the surrounding area. For example, it is therefore clear from this that discontinuously disinfected systems should be sampled at the end of the disinfection cycle. In cooling water systems, samples should preferably be taken of the falling water in the free space above the cooling tower basin. Following this, sampling from the cooling tower basin is preferable to sampling from a sampling tap on the outlet side of the circulation pipe, as close as possible to the basin.

As far as possible, participants in this study took samples in accordance with the monitoring protocol. However, it emerged that most systems were sampled by means of a sampling tap in the circulation pipe. This is understandable, as these sampling points have been fitted especially for that purpose and because this is the least complicated way of taking samples in practice. However, if the flow through the sampling tap is insufficient before starting to take samples, there is a risk of contaminating the sample with water and biofilm from the tap-out pipe that goes to the sampling point.

The results of the individual analytical methods and the comparison of the analytical methods in chapter 3 lead to the conclusion that the screening method for *Legionella pneumophila* shown in figure 1 is a useful method that could be employed in practice. The three Q-PCR techniques are subject to relatively little inhibition from the sample matrix. No statistically significant differences were found between the three different Q-PCR techniques (appendix VII). The methods also displayed a higher degree of accordance with the cultivation method according to ISO 11731, which performed well in this study (table 7). The high performance was partly attributable to the use of sample pretreatment with acid and frequent reading of the culture media during the incubation period. This makes the method considerably more laborious and expensive.

Cooling water systems with a known 'legionella history' were selected for this study. *Legionella* was therefore expected to be detected in a relatively large number of samples. This applies to both the non-specific cultivation methods (for example ISO 11731; 81% of the samples were positive) and the Q-PCR technique for *Legionella pneumophila* (an average of 75% of the cooling water samples were positive). Primarily this means that *Legionella pneumophila* occurs in relatively large amounts in the cooling water systems studied, unlike in the case of drinking water systems, in which non-pneumophila varieties are found more often[3]. It also means that using Q-PCR to measure *Legionella pneumophila* in cooling water samples does not directly result in fewer positive samples. Moreover, figure 3 shows that the number of copies of DNA per litre is generally higher than the number of colony-forming units per litre. These two aspects make routine use of Q-PCR in cooling water samples less attractive in practical situations. The method is still attractive in situations in which speed of analysis plays a role, such as when determining the effectiveness of measures in emergencies.

In the traditional method of NEN 6265 using BCYE medium, it was established that the culture medium is very susceptible to additional growth. This study has confirmed with regard to this aspect that using an MWY medium leads to a considerable improvement. The good accordance of the results of detection methods applied by lab IV and lab I according to NEN 6265:2007 using MWY medium also indicates that the method is readily reproducible (figure 4). These detection results also showed a high level of accordance with the results using the ISO 11731 method (table 8). The statistical study showed no

significant differences in the results (appendix VII). However, the comparison shows that for higher numbers of legionella bacteria the yield using the ISO method is slightly higher (figure 5) but is slightly lower when the numbers are lower. Finally, the results show that pretreating samples with acid in accordance with ISO 11731 leads to a considerably lower percentage of samples with too much disruptive additional growth on the culture medium (6% for ISO 11731 against 29% for NEN 6265:2007 using MWY medium). This undoubtedly demonstrates the importance of pretreatment with acid.

The Q-PCR method for specifically detecting *Legionella pneumophila* is also in line with a cultivation method for specifically detecting the same variety of legionella. The draft version of the NEN 6253 method with the BCYE medium used for it performed poorly in this study. As with the traditional NEN 6265 method, this method suffers from excessive problems from unwanted flora. However, using an MWY medium leads to a substantial improvement. The specific cultivation method for *Legionella pneumophila* then displays good accordance with the NEN 6265:2007 method using MWY medium and the associated serotyping. It was demonstrated in section 3.1 that the method to confirm a Q-PCR result can provide useful additional information which can be used as a basis for assessing the current situation in a cooling water or process water system (table 5). Pretreating a sample with acid and reading the culture media more often would probably further improve the method's yield – as was the case with the method of working in lab I using the ISO 11731 method.

In view of its high level of accordance with the results of Q-PCR and the ISO 11731 method and given the speed and simplicity of the test, the Fastpath™ method would appear to be a useful addition to the set of instruments available to the process operator responsible for the daily management of cooling water systems. The method's specificity makes it a useful addition to ATP measurements, colony number measurements or dip slides that are already used for monitoring water systems. The method's speed and simplicity make it exceptionally interesting for monitoring in emergencies. However, it is important to realise that specificity for *Legionella pneumophila* sero-group 1 means that the other sero-groups are not taken into account. Moreover, the information is of such a qualitative nature that it cannot replace the measurements that are normally made. However, as it accounts for 90% of registered cases worldwide, *Legionella pneumophila* sero-group 1 is the main cause of legionella pneumonia.

4.2 Process water in the paper and cardboard industry

As mentioned in section 1.3, Koninklijke VNP is especially interested in developing a detection method for *Legionella* or *Legionella pneumophila* in the process water used by the paper and cardboard industry. It is generally assumed that conditions in the process water are optimal for *Legionella* growth but detecting the organism in this difficult matrix continues to be a problem.

Detecting legionella bacteria in the paper industry's process water proved to be a problem in this study too. For example, when using the Fastpath™ method it was difficult to filter the required amount of water. When the Q-PCR technique (lab IV) was used, no results were stated on a number of occasions because the internal control was below the limit value for a reliable result. This indicates that PCR efficiency was too low, owing to an effect connected with the matrix (inhibition). Table 9 shows the results of the four most useful analytical methods for analysing the paper industry's process water. The values of the Pall Genesystems Q-PCR shown in the table have been recalculated in accordance with the draft version of NEN 6254. According to the original AFNOR standard, *Legionella pneumophila* may well have been detected (result above the limit of detection, LOD) for all the results (with the exception of those for OPIW 28 and 37) but the number of DNA copies could not be quantified (result below the limit of quantification, LOQ, as specified in the AFNOR standard). The other Q-PCR methods have higher detection limits, as a result of the small volume that was processed owing to problems with filtering the samples.

In the case of the paper industry's process water, the results obtained using the cultivation method according to ISO 11731 appear to be the most useful. However, the results indicate that the process water contains no *Legionella* or only relatively low concentrations of up to 1,000 cfu/l. Disruptive additional

growth at a level too high to permit a reliable result was only found in samples OPIW 30 and OPIW 38, which, like OPIW 46, came from the overflow of a dust chute. At the moment, although conditions in the process water appear to be optimal for *Legionella* growth, hardly any excessive *Legionella* growth was observed in the samples analysed for this study. Inhibition of *Legionella* growth and/or competition from other micro-organisms may play a role in this.

Table 9 Analysis results for samples from the paper and cardboard industry

sample no.	Q-PCR Lab IV	Q-PCR Lab II	Q-PCR Lab III	ISO 11731
	number of copies/l	number of copies/l	number of copies/l	cfu/l
OPIW 29	no result	3,430	no result	< 50
OPIW 39	406	537	< 1,200	510
OPIW 47	< 6,931	732	< 550	960
OPIW 30	no result	35,100	< 700	no result
OPIW 38	8,133	6,520	< 1,200	no result
OPIW 46	< 2,688	18,600	< 1,000	< 500
OPIW 28	< 2,430	< 680	< 1,400	< 50
OPIW 37	< 2,931	< 680	< 1,200	< 50
OPIW 48	no result	1,510	< 29,000	< 50

4.3 Pricing

Q-PCR technology is developing rapidly. More and more laboratories are capable of using the method and more and more samples are being presented for analysis. This leads to competition and a reduction in analysis costs. The price charged by a commercial laboratory for Q-PCR analysis of large numbers of samples (n > 20) is expected to work out in the range of € 40 to 60 per sample.
In comparison with analysing drinking water samples, the current practice of using the cultivation method on cooling water samples requires more time (+ 50%) for isolation and identification. When cultivating *Legionella pneumophila,* if treating the sample with acid increases the yield and more frequent counting leads to a better result, it will be necessary to take into account that the detection method will be even more labour-intensive and that the current cost of the cultivation method will increase. The price is expected to increase by 50 to 100% with respect to the current price for drinking water samples. However, the latter price can vary considerably in practice and is not always a true reflection of the quality required for a good analysis.

4.4 Conclusions and recommendations

Based on the results of the study, figure 6 is a more detailed version of the scheme shown in figure 1. The Fastpath™ method is a useful addition to the set of instruments available to the process operator for the daily management of cooling water systems. This method is faster in providing information on the possible presence of *Legionella pneumophila* sero-group 1 in cooling water and in that respect could initiate further analysis in accordance with the scheme in figure 6.
This study has shown that Q-PCR technology provides a fast, reliable and affordable technique for detecting *Legionella pneumophila* in cooling water and process water. (A restriction on using this technique is that the government has not made the step from detecting *Legionella* as a whole to detecting *Legionella pneumophila*.) The speed of the Q-PCR method is a major advantage for the industry. However, if it emerges that *Legionella pneumophila* is fairly common in cooling water systems, it will also be necessary to perform analyses regularly in accordance with the scheme in figure 6, using the cultivation method to confirm the viability of the legionella bacteria. This means that the time gain would be lost for some samples and, moreover, that performing screening in accordance with the scheme in figure 6

would be relatively expensive in comparison with only conducting an analysis using the cultivation method.
However, this study has shown that successfully using a cultivation method on cooling and process water samples will also lead to extra costs. Pretreating a sample with acid and counting more frequently for the specific detection of *Legionella pneumophila* make detection much more labour-intensive and therefore expensive. Of all the cultivation methods used, the cultivation method according to ISO 11731 performed well. However, counting was also more frequent in this case, to prevent additional growth on the culture medium from making it difficult to count colonies.

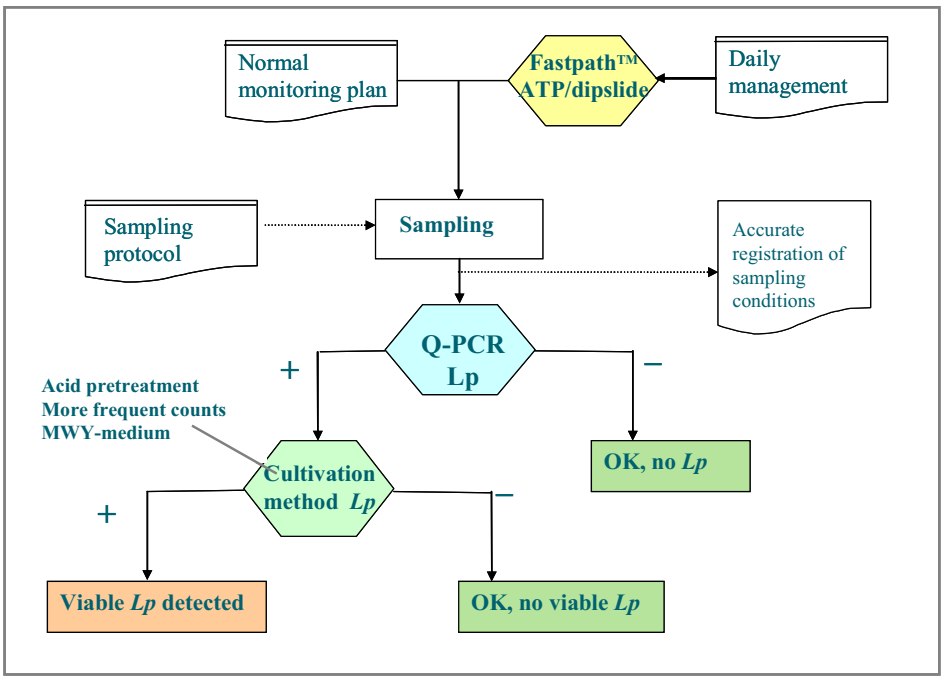


Figure 6. Modified scheme for screening for Legionella pneumophila in cooling water and process water on the basis of the results of this study.

The advice for selecting a cultivation method from the scheme shown in figure 6 is to

- examine whether the ISO 11731 method could be made suitable for detecting *Legionella pneumophila* and
- to further optimise the specific cultivation method for *Legionella pneumophila* using an MWY culture medium (based on NEN 6265:2007 and the draft version of NEN 6253) by applying pretreatment with acid and more frequent counting.

Furthermore, it would be advisable to check the performance of the two selective cultivation methods separately and in combination with Q-PCR (in terms of their microbiological yield and cost) by using them on a sufficiently large non-select sample of industrial cooling water systems. The results of the study will enable a final decision to be taken on the usefulness of advance screening using Q-PCR in accordance with the scheme in figure 6.

Conclusions:

- This study has demonstrated the usefulness and added value for analysing cooling water and process water samples in accordance with the scheme in figure 6. Besides providing managers of cooling water and process water systems with information faster, this new screening methodology also provides them with more specific information on the presence of *Legionella pneumophila* in the system. The choice of the specific Q-PCR for *Legionella pneumophila* in the scheme does not affect the result. The choice of the specific cultivation method should be based on the recommendations of this study and possibly the results of a further study.
- Variance analysis has shown for the cooling water and process water used in this study that there are no significant statistical differences between the results of three Q-PCR techniques performed by

three different laboratories in accordance with three different standards for detecting *Legionella pneumophila*.

- In most cases, the results of the Q-PCR techniques for the cooling water samples studied meet the requirement for a detection limit of at least 1,000 units/litre, based on table 12 of Health & Safety Information Sheet AI-32. If using Q-PCR technology systematically leads to more units per litre being detected than the number detected using the cultivation method, table 12 of the Health & Safety Information Sheet will have to be expanded for the interpretation of Q-PCR results. With regard to the specific situation at a company, when switching to Q-PCR, the method should preferably be used in parallel for a while with the cultivation method normally used up to that point, so that the results of the old and new method can be correlated. This can then be used as a basis for adjusting the action levels in the legionella control plan.
- The practical usefulness of the scheme in figure 6 may be limited by the observation that using Q-PCR for the specific detection of *Legionella pneumophila* in the cooling water samples analysed, does not lead to less positive samples than detection based on a cultivation method for *Legionella* in total. In comparison with drinking water systems, *Legionella pneumophila* is apparently fairly generally present in cooling water systems.
- This study has shown that applying NEN 6265 with BCYE medium for cooling water and process water samples leads to unsatisfactory results, owing to disruptive additional growth on the culture medium. NEN 6265 was revised for this in 2007 by also describing the MWY medium for samples with excessive additional growth.
- This study has shown that pretreating cooling water and process water samples with acid in accordance with ISO 11731:1998(E) and counting more frequently on day 3 and day 5 after incubation, leads to fewer outcomes for which no result can be stated on account of additional growth on the culture medium.
- Variance analysis has shown for the cooling water and process water used in this study that there are no significant statistical differences between the results of the cultivation method according to ISO 11731 and the cultivation method according to NEN 6265:2007 using an MWY culture medium, provided that the agar plates are also assessed on day 3 and day 5.
- Taking into account the method's limitations, the Fastpath™ method is a useful addition to the set of instruments available to the process operator responsible for the daily management of cooling water systems. The method's main benefits are speed, simplicity and the possibility of on-site implementation. However, it is important to realise that specificity in respect of *Legionella pneumophila* sero-group 1 means that the other sero-groups are not taken into account. (Nevertheless *Legionella pneumophila* sero-group 1 is the main cause of legionella pneumonia; up to 90% of registered cases worldwide). Moreover, the information is of such a qualitative nature that it cannot replace the measurements that are normally made.
- This study has not provided any indications of the presence of high concentrations of legionella bacteria in process water samples from the paper and cardboard industry. Nevertheless, the results indicate that *Legionella* or *Legionella pneumophila* can be detected, at least in a number of samples.
- It was notable for practically every cooling water system examined in this study that disinfection hardly ever consistently resulted in a legionella bacteria concentration of less than 1,000 cfu/l.
- The laboratories participating in this study have demonstrated that it is possible to achieve reliable results using a cultivation method for *Legionella* and/or *Legionella pneumophila*. A contributory factor to this was that these laboratories were experienced and did everything possible to achieve good analytical results, without attempting to cut costs or avoid difficulties. The interlaboratory comparisons organised by KWR also confirmed that a certain degree of reliability can be achieved using a cultivation method.

Recommendations:

- It is recommended on the basis of the experiences from this study that the specific cultivation method for *Legionella pneumophila,* with an MWY cultivation medium based on NEN 6265:2007 and the draft version of NEN 6253, should be further optimised by means of pretreatment with acid and more frequent counting.

- As good results in this study were obtained using the cultivation method according to ISO 11731, it would be advisable to examine the extent to which the method could also be made suitable for the specific cultivation of *Legionella pneumophila.*
- A further recommendation – assuming a sufficiently large non-select sample of industrial cooling water systems – is that the two specific cultivation methods for detecting *Legionella pneumophila* should be checked both separately and in combination with Q-PCR assessment to determine their performance in terms of yield and cost.
- It would be advisable to set up interlaboratory comparisons for the Q-PCR techniques, as is done for the cultivation method.
- The cultivation method in accordance with ISO 11731 is recommended for detecting *Legionella* in process water from the paper and cardboard industry, subject to pretreating the sample with acid and reading the culture medium counts more frequently after incubation.
- A further examination of the role that 'full stream' or 'side stream' filtration plays in the effectiveness of disinfection in cooling water systems is recommended. Relevant variables in this are the type of disinfectant and the dose and method of dosing (continuous/discontinuous).

5 References

1. Bart Wullings, Gerhard Wubbels, Harm Veenendaal and Dick van der Kooij. Snelle, kwantitatieve detectie van *Legionella pneumophila* met Q-PCR. H2O, nr5. 2007. pp 39 – 41

2. Harm Veenendaal and Dick van der Kooij. Een specifieke kweekmethode voor *Legionella pneumophila*. H2O, nr5. 2007. pp 36 – 38

3. Dick van der Kooij, Gerhard Wubbels and Bart Wullings. Legionellabacteriën in leidingwaterinstallaties behoren meestal tot de ongevaarlijke soort *Legionella anisa*. H2O, nr5. 2007. pp 33 – 35

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7. NEN 6265:2007. Water – detectie en telling van *Legionella*.

8. ISO 11731:1998(E). Water quality – detection and enumeration of *Legionella*.

9. draft NEN 6253:2008 nl. Water - detectie en kwantificering van *Legionella pneumophila* – Methode met selectieve kweekmedia.

10. draft NEN 6254:2008 nl. Water – detectie en kwantificering van *Legionella pneumophila* – Methode met kwantitatieve polymerase chain reaction (QPCR).

11. AFNOR. XPT90-471 Water quality- Detection and quantification of *Legionella* and/or *Legionella pneumophila* _by concentration and genic amplification _by polymerase chain reaction (PCR). April 2006

12. Applied Biosystems. Protocol v2.0. Detection and Quantification of *Legionella spp.* and *Legionella pneumophila*. 2007

I Appendix: Sampling protocol (partly in English)

Inleiding

Aanleiding

Dit protocol is opgesteld als onderdeel van het project OPIW 15 "Evaluatie van een snelle, betrouwbare en reproduceerbare screeningsmethodiek voor *Legionella*'s in koelwater en proceswater".
Op grond van de ervaringen met de toepassing van het protocol in dit onderzoek kan worden overwogen de methodiek verder te standaardiseren.

De input voor dit protocol is geleverd door een team bestaande uit de volgende personen: Antoine van Hoorn (Corus), Jack Smeets (KEMA Zuid), Jo Savelkoul, Ralph Lindeboom en Frank Oesterholt (allen Kiwa Industrie & Water).

Doelstelling

Dit protocol is bedoeld voor de deelnemers aan OPIW 15 en heeft als doel een monster/staal te verkrijgen dat een zo representatief mogelijk beeld geeft van de concentratie legionellabacteriën in het (circulerende) water van te onderzoeken object. Daarnaast moet het protocol leiden tot een gestandaardiseerde monstername/staalname binnen het project.

Tijdens het startoverleg is afgesproken dat elke projectdeelnemer 3 locaties selecteert (bijvoorbeeld 3 koelwatersystemen respectievelijk 3 proceswatersystemen, zoals in gebruik bij de productie van papier dan wel de productie van ijzer en staal) die vervolgens in de loop van het project 3 keer worden bemonsterd. Totaal 9 monsters per deelnemer.

Protocol

Conditions

Conditions for sampling of cooling water systems

For this research only samples should be taken from cooling water systems that have been continuously working during a period of at least 2 weeks.

On purpose of the research the water distribution in the cooling water tower has to be controlled once before starting the first sampling. A bad water distribution reveals positions with splash streams (e.g. caused by a broken distribution scale) and/or positions with just very small water contents (obstructed nozzles/sprinklers). (This inspection should be part of regular controls as these problems (i) could have negative influences on the capacity of the cooling water systems, (ii) could cause a high chance of pollution of the package and (iii) also the chance of growth of legionella bacteria.
Inspection preliminary to this research is of great importance because you can hardly execute/carry out good and representative samples in a cooling water tower with a bad water distribution. In case of bad water distribution the sampler and operator have to decide together to sample the cooling water tower by other methods (see preferences listed below).

Voorwaarden voor bemonstering van proceswatersystemen

Voor dit onderzoek dienen alleen proceswatersystemen te worden bemonsterd die gedurende een periode van ten minste 2 weken continu in bedrijf zijn geweest.
Bij voorkeur wordt een proceswatersysteem of een deel van een proceswatersysteem geselecteerd waaraan geen biocide wordt gedoseerd.

Preparation

The sampling has to be executed by an instructed sampler. Sampler is perfectly aware of the contents of this protocol.

The sampling has to be executed with sterilised bottles provided by KWR (content 1 litre). In a standard way these bottles contain a solution of sodiumthiosulphate for neutralisation of eventually present chlorine (or another oxidation biocide) and a NTA-solution for **complex formation** of eventual presence of heavy metals.

Execution

For this research sampling volumes are required bigger than 1 litre, so more bottles have to be filled. As expected three bottles have to be filled (total 3 litres) directly after each other.

As the bottles contain a small content of sodiumthiosulphate and NTA solution, these bottles preferably should not overflow during the sampling. We advise to fill these bottles for only 90%.

After the last sampling the water temperature has to be measured and registered.

KWR provides each run of sampling bottles with an information sheet for the registration of temperature and other relevant conditions under which circumstances the sampling has been done. The sampler should fill these sheets in (eventually in a mutual agreement with the operator).

Execution sampling cooling water systems

Cooling water systems discontinuously chorinated (disinfected) have to be sampled at the end of the disinfection cycle (i.e. just for the new shock dose).

For cooling water systems continuously disinfected the moment of sampling does not matter.

Sampling a cooling water system next sequence of preferences has to be used to determine the position of sampling.

1. Sampling of the falling water in the free space above the cooling water basin. *The position of sampling is minimal 1 metre from the edge of the cooling tower. A special tool adjusted at the circumstances with the help of which a sampling bottle can be positioned at the desired distance in the water curtain, has to be used.*
   If this way of sampling is not executable for practical reasons (e.g. too small louvres for the sampling bottle) and/or bad water distribution in the cooling tower (see paragraph 1.3) this way of sampling has to be cancelled. Than the next preference will be:
2. Sampling of water in the cooling water basin: *The position of sampling has to be 1 metre from the edge, maximum 10 till 20 centimetres under the water surface (avoid contact with the bottom) and on a position in the cooling water basin as close as possible to the suction pipe of the recirculation pump (water has to be in motion).*
   As for practical reasons this way of sampling is also not possible to execute due to too far distance between the sampling position and the cooling water basin or due to position points having a bad through-flow, this way of sampling has to be cancelled. In this case the next preference will be:

3 Taking samples from a sampling tap in a main water pipe after the recirculation pump (press side of the pump).*This way of sampling has to be carried out as close as possible to the cooling water tower. For this method a supplementary requirement is that the pipe to the sampling point (measured from the main water tap) has to be shorter than 3 metres. Before starting sampling the pipe has to be continuously streamed with full jets during at least 3 minutes in order to avoid wall effects*. In fact such a sampling pipe should be regularly flushed, e.g. as part of the procedure of flushing 'emergency showers'.

Uitvoering bemonstering proceswatersystemen

Proceswatersystemen die discontinue worden gedesinfecteerd dienen te worden bemonsterd aan het einde van de desinfectiecyclus (dat wil zeggen vlak voor de nieuwe dosering van desinfectiemiddel).

Voor proceswatersystemen die continue worden gedesinfecteerd, maakt het moment van monsterneming niet uit.

Papier- en kartonindustrie

In de Handleiding *Legionella* voor de papier- en kartonindustrie die door Kiwa Industrie & Water in opdracht van de Koninklijke VNP is opgesteld [1], is een prioriteitstelling opgenomen voor proceswaterlocaties in de papierfabriek. Deze prioriteitstelling is uitgevoerd op basis van het potentiële risico op blootstelling aan *Legionella* via aërosolen, op basis van de mate van aërosolvorming, de frequentie en duur van aanwezigheid van personeel en de kans op vermeerdering van *Legionella*.

Op grond van deze referentie dient voor de bemonstering van proceswater uit een papier- of kartonproductiebedrijf de volgende **voorkeursvolgorde** te worden gehanteerd voor de keuze van de positie van monsterneming (locaties die los staan van de papierproductieprocessen zoals hogedrukreinigers en schoonmaakhaspels zijn buiten beschouwing gelaten):

1. de ontwatering van de zeefpartij;
2. open sproeisysteem voorraadkuipen/indikkers proceswater gevoed;
   open pulper (stofvoorbereiding);
   gapvormer (fijnpapier/tissue stofomloop)
   sorteertrommel rejectreiniging (stofvoorbereiding);
   sproeisysteem viltreiniging proceswater gevoed (perspartij)
   sproeisysteem vilt- en zeefreiniging proceswater gevoed (zeef/nat-partij)

Bij de selectie van het monsterpunt dienen de volgende overwegingen te worden meegenomen:

- de prioriteit zoals hierboven aangegeven (1,2)
- een vrij vallende waterstroom heeft de voorkeur boven bemonstering via een tappunt (vergelijkbare situatie als bij de koeltoren);
- bij bemonstering van een tappunt dient dat tappunt niet door middel van een (rubber of kunststof) slang te zijn gekoppeld aan de installatie;

- bij bemonstering van een tappunt dient dat tappunt ten minste 5 liter water per minuut te leveren (uitgangspunt inwendige leidingdiameter max. 20 mm);
- bij bemonstering van een tappunt dient de uittapleiding naar het tappunt bij voorkeur zo kort mogelijk te zijn (< 1 meter). Om wandeffecten van de leiding en het tappunt te voorkomen dient de leiding minimaal 1 minuut bij volle straal te worden doorstroomd alvorens met de monsterneming wordt gestart.

IJzer- en staalindustrie

De ervaring heeft geleerd dat binnen de ijzer- en staalindustrie regelmatig relevante concentraties legionellabacteriën in proceswatersystemen aanwezig zijn. Ook hier wordt aan de hand van factoren als de mate van aërosolvorming, de frequentie en duur van aanwezigheid van personeel en de kans op vermeerdering van *Legionella* preventief een standaard monsternameprogramma uitgevoerd op een aantal verschillende locaties.

Enkele overwegingen voor de selectie van de bemonsteringslocatie die hierboven zijn opgenomen voor de papierindustrie gelden ook hier:

- een vrij vallende waterstroom heeft de voorkeur boven bemonstering via een tappunt (vergelijkbare situatie als bij de koeltoren);
- bij bemonstering van een tappunt dient dat tappunt niet door middel van een (rubber of kunststof) slang te zijn gekoppeld aan de installatie;
- bij bemonstering van een tappunt dient dat tappunt ten minste 5 liter water per minuut te leveren (uitgangspunt inwendige leidingdiameter max. 20 mm);
- bij bemonstering van een tappunt dient de uittapleiding naar het tappunt bij voorkeur zo kort mogelijk te zijn (< 1 meter). Om wandeffecten van de leiding en het tappunt te voorkomen dient de leiding minimaal 1 minuut bij volle straal te worden doorstroomd alvorens met de monsterneming wordt gestart.

Persoonlijke bescherming

Personal protection during sampling of cooling tower water.

During the sampling of cooling water systems following preferences 1 and 2 at least the regular, required personal protection outfits have to be used in combination with gloves an a mask of type FFP3. These masks have to meet the requirements following EN 149:2001.

> Until recently filtration masks where available only protecting against solids or liquids. At this moment all filtration masks are suitable for the protection against solid particles as well as liquid particles. The out of date qualifications written at the type of masks, were:
> S means Solids
> L means Liquids

Persoonlijke bescherming bij bemonstering proceswatersystemen

Bij de bemonstering van proceswatersystemen dienen de reguliere, voorgeschreven persoonlijke beschermingsmiddelen te worden gedragen, maar in ieder geval handschoenen.
Bij bemonstering van een vrij vallende waterstroom waarvoor een afgeschermde positie in het proces moet worden ingenomen, moet – in verband met de kans op blootstelling aan hoge concentraties aërosolen - worden overwogen om een gelaatsmasker van klasse FFP3 te dragen (zie opmerking paragraaf 2.4.1.)

Transport of the samples

Immediately after sampling each sample has to be put on ice or icepacks and placed in a cooling box. On the same day of sampling, the samples have to be delivered (if necessary by courier) before 12.00 AM at Kiwa Water Research to the attention of Harm Veenendaal (chief of the microbiology laboratory).

Referenties

[1] Handleiding *Legionella* voor de papier- en kartonindustrie in Nederland. KWR 07.020. Kiwa Industrie & Water, 2007.

II Appendix: Information sheet

Information sheet monstername OPIW 15

This sheet should be filled in after sampling of a cooling water or process water system. It provides information about the sampling itself and the relevant system conditions at the time of sampling. (See other side for footnotes).

General Information	
Date/time	…/ …./2008 …: ….hrs
Name sampler/telephone nr	
Company name	
Description cooling water or process water system	
Description used sampling method[1] (see protocol sampling)	
Description used codes on sampling bottle for this sample	
Number of filled bottles	
Water temperature after sampling	……˚C
Visual description samples[2]	
Information in detail sampling system	
Description disinfection procedure cooling- or process water system[3]	
Expired time after last chlorination	
Actual chloride concentration or last measured chloride concentration + date and time	
Position blow down (open/closed) during sampling	
Last measured ATP value for the system + date/time[4]	
Used ATP action level[4]	
Last measured colony count + date/time[4]	
Used action level for colony count[4]	
Last measured legionella concentration + date/time[4]	
Hydraulic residence time in the system = volume system/blow down	
Type of make up water	
Are sand filters being used in the cooling water systems? Side stream of full stream?	

Notes:

1) Describe the way the sampling was executed. Therefore we refer to the protocol's sequence of preferences for sampling.
2) Describe remarkable things of the taken water samples, e.g. troubled or clear water, many suspended particles, sediment in the bottle, odour and colour, etc. etc.
3) What type(s) of disinfection method(s) has (have) been used? Do they disinfect continuously or discontinuously. What doses have been used and how frequently?
4) Gather these data only when these are applicable, e.g. when ATP measurements or dip slides have been used as protection of the system. Describe in this case when the last measurement took place (date). In both cases please mention also which action levels have been used.

III Appendix: Overview research results

			KWR							lab II				lab I/lab III			
date 2008	company	sample code	BCYE + AB NEN 6265	MWY NEN 6265:2007	BCYE pnue pH 7,3 & temp 40C	MWY pneu pH 7,3 & temp 40C	PCR (Lp) concept o-NEN 6254	Fastpath (Lp1) Nalco		PCR (Lp) AFNOR	recalculated according to NEN	ISO 11731		MWY NEN 6265:2007		PCR (Lp)	
			cfu/l	cfu/l	cfu/l	cfu/l	kopieën/l	result	volume analysed (ml)	GU/l	GU/l	cfu/l	Serotype	cfu/l	Serotype	kopieën/l	
26-aug	A	OPIW 7	1.800	7.800	5.400	1.600	223.798	positive (6)	250	<3.330	1.020	70.000	a+b+c	16.000	a+b+c	3.100	
16-sep	A	OPIW 25	10.200	38.500	(3)	(1)	196.796	positive (6)	250	18.200	18.200	130.000	a+b+c	79.000	a+b+c	17.000	
29-sep	A	OPIW 44	(1)	15.600	3.400	6.500	87.914	negative	250	27.500	27.500	60.000	a+b+c	14.000	a+b+c	-	(5)
26-aug	A	OPIW 8	300	300	500	<100	79.863	positive (6)	250	<3.330	259	1.800	b	1.900	a+b	9.000	
16-sep	A	OPIW 26	(1)	146.200	(3)	27.920	133.443	positive (6)	250	25.300	25.300	320.000	b	190.000	b	163.000	
29-sep	A	OPIW 43	37.900	46.600	27.000	50.300	85.136	negative	250	69.900	69.900	220.000	b	64.000	b	79.200	
26-aug	A	OPIW 9	(1)	(1)	(1)	<100	5.626	positive (6)	250	<3.330	631	2.900	b+c	1.900	a+b	<480	
16-sep	A	OPIW 27	(1)	300	(3)	1.040	1.164	negative	250	<4.380	668	1.900	b+c	2.200	b+c	6.700	
29-sep	A	OPIW 45	(1)	900	(1)	<100	1.469	negative	250	< 83.300	23.200	2.300	b+c	2.000	b+c	<720	
26-aug	B	OPIW 4	720	3.500	500	600	822.344	negative	250	32.000	32.000	18.000	b	1.500	b	400.000	
16-sep	B	OPIW 22	600	1.200	(3)	600	335.753	negative	250	57.000	57.000	870	b	380	b	268.600	
23-sep	B	OPIW 31	100	300	<100	500	127.073	negative	250	24.100	24.100	530	a+b	1.100	b	13.200	
26-aug	B	OPIW 6	(1)	1.900	(1)	1.040	8.800	positive	250	<83.300	30.400	4.100	a+b	(1)		10.000	(4)
16-sep	B	OPIW 23	(1)	480	(3)	960	1.691	positive	250	<2.130	<2.130	< 50		(1)		<1.700	
23-sep	B	OPIW 33	(1)	<100	(1)	<100	2.063	positive	250	<13.900	889	3.600	a+b	(1)		-	(5)
26-aug	B	OPIW 5	<100	(1)	(1)	(1)	-	positive (6)	250	816.000	816.000	11.000	a+b	(1)		88.000	
16-sep	B	OPIW 24	4.080	100	(3)	<100	1.633.306	negative	250	60.000	60.000	55.000	a+b	1.900	a	752.500	
23-sep	B	OPIW 32	(1)	(1)	(1)	(1)	637.144	positive (6)	250	123.000	123.000	65.000	a+b	(1)		352.000	
2-sep	C	OPIW 15	<100	<100	<100	<100	<591	negative	250	<3.330	3.160	98	c	<500		<800	
29-sep	C	OPIW 40	<100	<100	<100	<100	<551	negative	250	<3.330	695	< 50		< 500		<720	
6-okt	C	OPIW 51	<100	<100	<100	<100	<655	negative	250	<83.300	2.320	< 50		< 500		950	
2-sep	C	OPIW 13	(1)	28.100	(1)	16.800	22.717	negative	250	42.100	42.100	65.000	a+b	60.000	a+b	5.700	
29-sep	C	OPIW 41	500	1.700	200	<100	664	positive (6)	250	< 4.200	691	530	b	980	a+b	<1.200	
6-okt	C	OPIW 50	1.375	1.800	300	700	2.736	positive (6)	250	<83.300	911	420	a+b	860	a+b	1.500	
2-sep	C	OPIW 14	100	100	(1)	(1)	<549	negative	250	<3.330	1.960	500	b	98	c	<1.000	
29-sep	C	OPIW 42	633	200	<100	<100	<612	negative	250	6.990	6.990	210	c	320	b+c	<1.200	
6-okt	C	OPIW 49	400	100	<100	<100	711	negative	250	<3.330	1.340	200	b+c	490	a+b+c	1.575	
2-sep	D	OPIW 10	(1)	(1)	(1)	120.000	32.696	positive	250	13.900	13.900	250.000	a+b	40.000	b	13.300	
16-sep	D	OPIW 19	(1)	50.000	(3)	8.900	75.855	positive	250	45.100	45.100	210.000	a+b+c	49.000	a+b	850	
26-aug	D	OPIW 1	(1)	(1)	(1)	(1)	< 699	positive	250	<680	< 680	540	b	(1)		<480	
2-sep	D	OPIW 11	(1)	(1)	(1)	(1)	5.060	positive	250	6.860	6.860	52.000	a+b	4.900	b	21.400	
16-sep	D	OPIW 20	(1)	(1)	(3)	<100	19.763	positive	250	<4.200	181	57.000	a+b	29.000	a+b	27.400	
2-sep	D	OPIW 12	(1)	(1)	(1)	2.233	1.786	positive	250	<5.550	1.080	1.500	a+b	(1)		<800	
16-sep	D	OPIW 21	(1)	<100	(3)	<100	<508	positive	250	<46.000	5.510	< 50		88	a	2.000	
2-sep	E	OPIW 18	(1)	6.400	(1)	25.333	29.762	positive (6)	250	< 11.100	5.890	10.000	b	11.000	b+c	-	(5)
23-sep	E	OPIW 35	(1)	<100	(1)	<100	-	positive	150	<13.900	1.560	< 50		< 500		<1.500	
6-okt	E	OPIW 53	(1)	6.100	(1)	(1)	161.853	positive	150	125.000	125.000	64.000	a+b+c	25.000	a+b	<900	
2-sep	E	OPIW 16	<100	<100	(1)	<100	<848	negative	250	<680	<680	< 50		190	b+c	<800	
23-sep	E	OPIW 36	<100	<100	<100	<100	<844	negative	250	<3.330	623	96	c	< 500		2.900	
6-okt	E	OPIW 54	<100	<100	(1)	<100	<905	negative	250	<8.330	1.070	< 50		270	b	<1.600	
2-sep	E	OPIW 17	(1)	(1)	10.000	(1)	12.857	positive	250	<5.550	701	(1)		(1)		3.100	(4)
23-sep	E	OPIW 34	(1)	<100	(1)	(1)	174.733	positive	250	32.000	32.000	70.000	a+b	4.000	b	48.000	(4)
6-okt	E	OPIW 52	(1)	<100	(1)	(1)	117.841	positive	250	<110.000	7.540	69.000 (2)	a+b	55.000 (2)	a+b	128.000	
23-sep	F	OPIW 29	(1)	(1)	(1)	(1)	-	positive	75	<13.900	3.430	< 50		(1)		-	(5)
29-sep	F	OPIW 39	(1)	(1)	(1)	(1)	406	negative	250	< 4.630	537	510	c	(1)		<1.200	
6-okt	F	OPIW 47	(1)	<100	(1)	<100	<6931	positive	250	< 5.550	732	960	b+c	< 500		<550	
23-sep	F	OPIW 30	(1)	(1)	(1)	(1)	-	positive	40	< 83.300	35.100	(1)		(1)		<700	
29-sep	F	OPIW 38	(1)	(1)	(1)	(1)	8.133	positive	40	< 33.300	6.520	(1)		(1)		<1.200	
6-okt	F	OPIW 46	(1)	(1)	(1)	(1)	<2688	positive	25	< 83.300	18.600	< 500		(1)		<1.000	
23-sep	F	OPIW 28	<100	<100	<100	<100	<2430	negative	50	<680	< 680	< 50		(1)		<1.400	
29-sep	F	OPIW 37	(1)	(1)	<100	<100	<2931	negative	40	<680	< 680	< 50		(1)		<1.200	
6-okt	F	OPIW 48	(1)	<100	<100	<100	-	negative	10	< 6.660	1.510	< 50		(1)		<29.000	

(1) No Legionella found, result not reliable due to too much side growth on the agar medium
(2) Estimated value, real number possibly higher due to much side growth on the agar medium
(3) By mistake inoculated on BCYE-AB, pH 7.3. No Legionella found, result not reliable due to too much side growth on the agar medium
(4) Analysis inhibited by the samples matrix. Real number of *Legionella pneumophila* is probably higher
(5) No *Legionella pneumophila* found. Analysis inhibited by the samples matrix; not reliable result.
(6) Difficult to visibly detect line; line is very thin
(7) *Legionella pneumophila* found, but result can not be quantified.
(8) Colonies (partly) confirmed with PCR
(9) Though negativ at confirmation on plate, presence of *Legionella* is suspected
(10) The output of the internal control sample is too low to give a reliable quantitative result
(11) *Legionella pneumophila* found, but the output of the internal control sample is too low to give a reliable quantitative result
(12) *Legionella pneumophila* concentration is indicative; below the lower limit for a reliable quantitative result

Serotypes: a = *Legionella pneumophila* serogroep 1.
b = *Legionella pneumophila* serogroep 2-14.
c = *Legionella* bacteria non *pneumophila.*

IV Appendix: Q-PCR *Lp* followed by a specific cultivation method for *Lp*

			lab IV					desinfectie	
date 2008	company	sample code	PCR (Lp) concept o-NEN 6254			MWY pneu pH 7,3 & temp 40C			
			kopieën/l		sd	cfu/l	sd		
26-aug	A	OPIW 7	2,24E+05		20410,91	1600 (2)	1746	0,1 - 0,2 Cl cont + 1 ppm ClO2 1 u/day	full stream sand filtration
16-sep	A	OPIW 25	2,0E+05		21749,99	(1)		0,1 - 0,2 Cl cont + 1 ppm ClO2 1 u/day	full stream sand filtration
29-sep	A	OPIW 44	8,79E+04		2321,93	6500	1458	0,1 - 0,2 Cl cont + 1 ppm ClO2 1 u/day	full stream sand filtration
26-aug	A	OPIW 8	7,99E+04		4183,07	<100		0,4 - 0,5 ppm sodiumhypochlorite cont	full stream sand filtration
16-sep	A	OPIW 26	1,3E+05		16380,87	27920 (8)	9008	0,4 - 0,5 ppm sodiumhypochlorite cont	full stream sand filtration
29-sep	A	OPIW 43	8,51E+04		2834,72	50300	4192	0,4 - 0,5 ppm sodiumhypochlorite cont	full stream sand filtration
26-aug	A	OPIW 9	5,63E+03		1693,57	<100		Sonoxide B35	
16-sep	A	OPIW 27	1,2E+03		256,61	1040 (8)	876	Sonoxide B35	
29-sep	A	OPIW 45	1,47E+03		1333,99	<100		Sonoxide B35	
26-aug	B	OPIW 4	8,22E+05		75572,04	600 (8)	652	no disinfection allowed	
16-sep	B	OPIW 22	3,4E+05		38319,38	600	652	no disinfection allowed	
23-sep	B	OPIW 31	1,27E+05		16420,04	500	354	no disinfection allowed	
26-aug	B	OPIW 6	8,80E+03		478,06	1040	727	0,10 ppm actual Cl2 conc.	autom. chloringssysteem 2 * 2 hour/day 2 ppm
16-sep	B	OPIW 23	1,7E+03		195,14	960 (8)	669	0,10 ppm actual Cl2 conc.	autom. chloringssysteem 2 * 2 hour/day 2 ppm
23-sep	B	OPIW 33	2,06E+03		1497,68	<100		0,15 ppm actual Cl2 conc.	autom. chloringssysteem 2 * 2 hour/day 2 ppm
26-aug	B	OPIW 5	-	(11)		(1)		last shock dose 4 mnd ago; 2 ppm	shock dose op basis van hoge TBC/ATP
16-sep	B	OPIW 24	1,6E+06		8173,55	<100		last shock dose 5 mnd ago	shock dose op basis van hoge TBC/ATP
23-sep	B	OPIW 32	6,37E+05		10501,85	(1)		last shock dose 5 mnd ago	shock dose op basis van hoge TBC/ATP
2-sep	C	OPIW 15	<591			<100		0,5 ppm Cl 15 min/24 hour	real concentration: 0 ppm
29-sep	C	OPIW 40	<551			<100		0,5 ppm Cl 15 min/24 hour	real concentration: 32 ppm
6-okt	C	OPIW 51	<655			<100		0,5 ppm Cl 15 min/24 hour	real concentration: 13 ppm
2-sep	C	OPIW 13	2,27E+04		2215,60	16800	8362	0,5 ppm Cl2 15 min per 8 hour	real concentration: 0,13 ppm
29-sep	C	OPIW 41	6,64E+02		379,95	<100		0,5 ppm Cl2 15 min per 8 hour	real concentration: 4,5 ppm
6-okt	C	OPIW 50	2,74E+03		627,12	700	1095	0,5 ppm Cl2 15 min per 8 hour	real concentration: 4,2 ppm
2-sep	C	OPIW 14	<549			(1)		0,5 ppm Cl 15 min/24 hour	real concentration: 0,2 ppm
29-sep	C	OPIW 42	<612			<100		0,5 ppm Cl 15 min/24 hour	real concentration: 15 ppm
6-okt	C	OPIW 49	7,11E+02	(12)	696,19	<100		0,5 ppm Cl 15 min/24 hour	real concentration: 1,5 ppm
2-sep	D	OPIW 10	3,27E+04		770,64	120000 (2)		disinfection with NaOCl	continuous chloring 1,25 ppm free chlorine
16-sep	D	OPIW 19	7,6E+04		4193,87	8900 (8)	10071	disinfection with NaOCl	continuous chloring 1,25 ppm free chlorine
26-aug	D	OPIW 1	< 699			(1)		disinfection with NaOCl	shock dose once every 2 weeks
2-sep	D	OPIW 11	5,06E+03		448,97	(1)		disinfection with NaOCl	continuous chloring 1,25 ppm free chlorine
16-sep	D	OPIW 20	2,0E+04		4326,95	<100		disinfection with NaOCl	continuous chloring 1,25 ppm free chlorine
2-sep	D	OPIW 12	1,79E+03		8,28	2233 (2)	1365	disinfection with NaOCl	shock dose/ last 1 week ago (25/8)
16-sep	D	OPIW 21	<508			<100		disinfection with NaOCl	shock dose
2-sep	E	OPIW 18	2,98E+04		9661,27	25333 (2)	4726	continu H2O2	
23-sep	E	OPIW 35	-	(11)		<100		continu H2O2	
6-okt	E	OPIW 53	1,6E+05		3392,34	(1)		continu H2O2	
2-sep	E	OPIW 16	<848			<100		continu H2O2	
23-sep	E	OPIW 36	<844			<100		continu H2O2	
6-okt	E	OPIW 54	<905			<100		continu H2O2	
2-sep	E	OPIW 17	1,29E+04		3147,68	(1)		continu ozone dose	side stream filtration
23-sep	E	OPIW 34	1,75E+05		20725,33	(1)		continu ozone dose	side stream filtration
6-okt	E	OPIW 52	1,2E+05		11670,29	(1)		continu ozone dose	side stream filtration
23-sep	F	OPIW 29	-	(11)		(1)		none	
29-sep	F	OPIW 39	4,06E+02	(12)	182,27	(1)		none	
6-okt	F	OPIW 47	<6931			<100		none	
23-sep	F	OPIW 30	-	(11)		(1)		none	
29-sep	F	OPIW 38	8,13E+03	(12)	479,00	(1)		none	
6-okt	F	OPIW 46	<2688			(1)		none	
23-sep	F	OPIW 28	<2430			<100		hypochlorite during cleaning; 5 days ago	
29-sep	F	OPIW 37	<2931			<100		hypochlorite during cleaning; 8 days ago	
6-okt	F	OPIW 48	-	(10)		<100		hypochlorite during cleaning; 6 days ago	

(1) No Legionella found, result not reliable due to too much side growth on the agar medium
(2) Estmated value, real number possibly higher due to much side growth on the agar medium
(4) Analysis inhibited by the samples matrix. Real number of *Legionella pneumophila* is probably higher
(5) No *Legionella pneumophila* found. Analysis inhibited by the samples matrix; not reliable result.
(8) Colonies (partly) confirmed with PCR
(10) The output of the internal control sample is too low to give a reliable quantitative result
(11) *Legionella pneumophila* found, but the output of the internal control sample is too low to give a reliable quantitative result
(12) *Legionella pneumophila* concentration is indicative; below the lower limit for a reliable quantitative result

V Appendix: Comparison Fastpath and Q-PCR/ISO 11731

			lab IV					lab I	
date 2008	company	sample code	PCR (Lp) concept o-NEN 6254			Fastpath (Lp1) Nalco		ISO	NEN 6265
			kopieën/l		sd	result	volume analysed (ml)	Serotype	Serotype
26-aug	A	OPIW 7	2,24E+05		20410,91	positive (6)	250	a+b+c	a+b+c
16-sep	A	OPIW 25	2,0E+05		21749,99	positive (6)	250	a+b+c	a+b+c
29-sep	A	OPIW 44	8,79E+04		2321,93	negative	250	a+b+c	a+b+c
26-aug	A	OPIW 8	7,99E+04		4183,07	positive (6)	250	b	a+b
16-sep	A	OPIW 26	1,3E+05		16380,87	positive (6)	250	b	b
29-sep	A	OPIW 43	8,51E+04		2834,72	negative	250	b	b
26-aug	A	OPIW 9	5,63E+03		1693,57	positive (6)	250	b+c	a+b
16-sep	A	OPIW 27	1,2E+03		256,61	negative	250	b+c	b+c
29-sep	A	OPIW 45	1,47E+03		1333,99	negative	250	b+c	b+c
26-aug	B	OPIW 4	8,22E+05		75572,04	negative	250	b	b
16-sep	B	OPIW 22	3,4E+05		38319,38	negative	250	b	b
23-sep	B	OPIW 31	1,27E+05		16420,04	negative	250	a+b	b
26-aug	B	OPIW 6	8,80E+03		478,06	positive	250	a+b	
16-sep	B	OPIW 23	1,7E+03		195,14	positive	250		
23-sep	B	OPIW 33	2,06E+03		1497,68	positive	250	a+b	
26-aug	B	OPIW 5	-	(11)		positive (6)	250	a+b	
16-sep	B	OPIW 24	1,6E+06		8173,55	negative	250	a+b	a
23-sep	B	OPIW 32	6,37E+05		10501,85	positive (6)	250	a+b	
2-sep	C	OPIW 15	<591			negative	250	c	
29-sep	C	OPIW 40	<551			negative	250		
6-okt	C	OPIW 51	<655			negative	250		
2-sep	C	OPIW 13	2,27E+04		2215,60	negative	250	a+b	a+b
29-sep	C	OPIW 41	6,64E+02		379,95	positive (6)	250	b	a+b
6-okt	C	OPIW 50	2,74E+03		627,12	positive (6)	250	a+b	a+b
2-sep	C	OPIW 14	<549			negative	250	b	c
29-sep	C	OPIW 42	<612			negative	250	c	b+c
6-okt	C	OPIW 49	7,11E+02	(12)	696,19	negative	250	b+c	a+b+c
2-sep	D	OPIW 10	3,27E+04		770,64	positive	250	a+b	b
16-sep	D	OPIW 19	7,6E+04		4193,87	positive	250	a+b+c	a+b
26-aug	D	OPIW 1	< 699			positive	250	b	
2-sep	D	OPIW 11	5,06E+03		448,97	positive	250	a+b	b
16-sep	D	OPIW 20	2,0E+04		4326,95	positive	250	a+b	a+b
2-sep	D	OPIW 12	1,79E+03		8,28	positive	250	a+b	
16-sep	D	OPIW 21	<508			positive	250		a
2-sep	E	OPIW 18	2,98E+04		9661,27	positive (6)	250	b	b+c
23-sep	E	OPIW 35	-	(11)		positive	150		
6-okt	E	OPIW 53	1,6E+05		3392,34	positive	150	a+b+c	a+b
2-sep	E	OPIW 16	<848			negative	250		b+c
23-sep	E	OPIW 36	<844			negative	250	c	
6-okt	E	OPIW 54	<905			negative	250		b
2-sep	E	OPIW 17	1,29E+04		3147,68	positive	250		
23-sep	E	OPIW 34	1,75E+05		20725,33	positive	250	a+b	b
6-okt	E	OPIW 52	1,2E+05		11670,29	positive	250	a+b	a+b
23-sep	F	OPIW 29	-	(11)		positive	75		
29-sep	F	OPIW 39	4,06E+02	(12)	182,27	negative	250	c	
6-okt	F	OPIW 47	<6931			positive	250	b+c	
23-sep	F	OPIW 30	-	(11)		positive	40		
29-sep	F	OPIW 38	8,13E+03	(12)	479,00	positive	40		
6-okt	F	OPIW 46	<2688			positive	25		
23-sep	F	OPIW 28	<2430			negative	50		
29-sep	F	OPIW 37	<2931			negative	40		
6-okt	F	OPIW 48	-	(10)		negative	10		

Fathpath
52 samples
5 times false negative
4 times false positive

47 samples
PCR = reference method
Fastpath = alternatieve method

	Fastpath +	Fastpath -	
PCR +	28	5	
PCR -	4	10	
no result PCR		5	
relative accuracy	AC		81%
relative specificy	SP		71%
relative sensitivity	SE		85%

Attention! Truly negative results with Fastpath are judged as Fastpath+
This means that during serotyping no Lp SG1 was found.

			lab IV		lab I		
date 2008	company	sample code	Fastpath (Lp1) Nalco		ISO 11731		NEN 6265
			result	volume analysed (ml)	cfu/l	Serotype	Serotype
26-aug	A	OPIW 7	positive (6)	250	70.000	a+b+c	a+b+c
16-sep	A	OPIW 25	positive (6)	250	130.000	a+b+c	a+b+c
29-sep	A	OPIW 44	negative	250	60.000	a+b+c	a+b+c
26-aug	A	OPIW 8	positive (6)	250	1.800	b	a+b
16-sep	A	OPIW 26	positive (6)	250	320.000	b	b
29-sep	A	OPIW 43	negative	250	220.000	b	b
26-aug	A	OPIW 9	positive (6)	250	2.900	b+c	a+b
16-sep	A	OPIW 27	negative	250	1.900	b+c	b+c
29-sep	A	OPIW 45	negative	250	2.300	b+c	b+c
26-aug	B	OPIW 4	negative	250	18.000	b	b
16-sep	B	OPIW 22	negative	250	870	b	b
23-sep	B	OPIW 31	negative	250	530	a+b	b
26-aug	B	OPIW 6	positive	250	4.100	a+b	
16-sep	B	OPIW 23	positive	250	< 50		
23-sep	B	OPIW 33	positive	250	3.600	a+b	
26-aug	B	OPIW 5	positive (6)	250	11.000	a+b	
16-sep	B	OPIW 24	negative	250	55.000	a+b	a
23-sep	B	OPIW 32	positive (6)	250	65.000	a+b	
2-sep	C	OPIW 15	negative	250	98	c	
29-sep	C	OPIW 40	negative	250	< 50		
6-okt	C	OPIW 51	negative	250	< 50		
2-sep	C	OPIW 13	negative	250	65.000	a+b	a+b
29-sep	C	OPIW 41	positive (6)	250	530	b	a+b
6-okt	C	OPIW 50	positive (6)	250	420	a+b	a+b
2-sep	C	OPIW 14	negative	250	500	b	c
29-sep	C	OPIW 42	negative	250	210	c	b+c
6-okt	C	OPIW 49	negative	250	200	b+c	a+b+c
2-sep	D	OPIW 10	positive	250	250.000	a+b	b
16-sep	D	OPIW 19	positive	250	210.000	a+b+c	a+b
26-aug	D	OPIW 1	positive	250	540	b	
2-sep	D	OPIW 11	positive	250	52.000	a+b	b
16-sep	D	OPIW 20	positive	250	57.000	a+b	a+b
2-sep	D	OPIW 12	positive	250	1.500	a+b	
16-sep	D	OPIW 21	positive	250	< 50		a
2-sep	E	OPIW 18	positive (6)	250	10.000	b	b+c
23-sep	E	OPIW 35	positive	150	< 50		
6-okt	E	OPIW 53	positive	150	64.000	a+b+c	a+b
2-sep	E	OPIW 16	negative	250	< 50		b+c
23-sep	E	OPIW 36	negative	250	96	c	
6-okt	E	OPIW 54	negative	250	< 50		b
2-sep	E	OPIW 17	positive	250	(1)		
23-sep	E	OPIW 34	positive	250	70.000	a+b	b
6-okt	E	OPIW 52	positive	250	69.000 (2)	a+b	a+b
23-sep	F	OPIW 29	positive	75	< 50		
29-sep	F	OPIW 39	negative	250	510	c	
6-okt	F	OPIW 47	positive	250	960	b+c	
23-sep	F	OPIW 30	positive	40	(1)		
29-sep	F	OPIW 38	positive	40	(1)		
6-okt	F	OPIW 46	positive	25	< 500		
23-sep	F	OPIW 28	negative	50	< 50		
29-sep	F	OPIW 37	negative	40	< 50		
6-okt	F	OPIW 48	negative	10	< 50		

Fathpath
52 samples
5 times false negative
5 times false positive

49 samples

ISO = reference method
Fastpath = alternatieve method

	Fastpath +	Fastpath -
ISO +	32	5
ISO -	5	7
no result ISO		3

relative accuracy	AC	80%
relative specificy	SP	58%
relative sensitivity	SE	86%

Attention! Truly negative results with Fastpath are judged as Fastpath+
This means that during serotyping no Lp SG1 was found.

VI Appendix: overview Q-PCR results

			lab IV					lab II					lab III		
date 2008	company	sample code	PCR (Lp) concept o-NEN 6254					PCR recalculated according to NEN					PCR (Lp)		
			kopieën/l	log		sd	log sd	kopieën/l	log	sd	log sd	log	kopieën/l		
26-aug	A	OPIW 7	2,24E+05	5,3		20410,91	4,3	1,02E+03	3,0	273	2,4	3,5	3,10E+03		
16-sep	A	OPIW 25	2,0E+05	5,3		21749,99	4,3	1,82E+04	4,3	799	2,9	4,2	1,70E+04		
29-sep	A	OPIW 44	8,79E+04	4,9		2321,93	3,4	2,75E+04	4,4	6214	3,8		-		(5)
26-aug	A	OPIW 8	7,99E+04	4,9		4183,07	3,6	2,59E+02	2,4	15	1,2	4,0	9,00E+03		
16-sep	A	OPIW 26	1,3E+05	5,1		16380,87	4,2	2,53E+04	4,4	1791	3,3	5,2	1,63E+05		
29-sep	A	OPIW 43	8,51E+04	4,9		2834,72	3,5	6,99E+04	4,8	12423	4,1	4,9	7,92E+04		
26-aug	A	OPIW 9	5,63E+03	3,8		1693,57	3,2	6,31E+02	2,8	369	2,6	2,4	<480		
16-sep	A	OPIW 27	1,2E+03	3,1		256,61	2,4	6,68E+02	2,8	71	1,9	3,8	6,70E+03		
29-sep	A	OPIW 45	1,47E+03	3,2		1333,99	3,1	2,32E+04	4,4	3616	3,6	2,6	<720		
26-aug	B	OPIW 4	8,22E+05	5,9		75572,04	4,9	3,20E+04	4,5	965	3,0	5,6	4,00E+05		
16-sep	B	OPIW 22	3,4E+05	5,5		38319,38	4,6	5,70E+04	4,8	2927	3,5	5,4	2,69E+05		
23-sep	B	OPIW 31	1,27E+05	5,1		16420,04	4,2	2,41E+04	4,4	7891	3,9	4,1	1,32E+04		
26-aug	B	OPIW 6	8,80E+03	3,9		478,06	2,7	3,04E+04	4,5	2266	3,4	4,0	1,00E+04		(4)
16-sep	B	OPIW 23	1,7E+03	3,2		195,14	2,3	< 2.13E+03	3,0			2,9	<1700		
23-sep	B	OPIW 33	2,06E+03	3,3		1497,68	3,2	8,89E+02	2,9	694	2,8		-		(5)
26-aug	B	OPIW 5	-		(11)			8,16E+05	5,9	54747	4,7	4,9	8,80E+04		
16-sep	B	OPIW 24	1,6E+06	6,2		8173,55	3,9	6,00E+04	4,8	13193	4,1	5,9	7,53E+05		
23-sep	B	OPIW 32	6,37E+05	5,8		10501,85	4,0	1,23E+05	5,1	38684	4,6	5,5	3,52E+05		
2-sep	C	OPIW 15	<591	2,5				3,16E+03	3,5	1373	3,1	2,6	<800		
29-sep	C	OPIW 40	<551	2,4				6,95E+02	2,8	194	2,3	2,6	<720		
6-okt	C	OPIW 51	<655	2,5				2,32E+03	3,4			3,0	9,50E+02		
2-sep	C	OPIW 13	2,27E+04	4,4		2215,60	3,3	4,21E+04	4,6	11393	4,1	3,8	5,70E+03		
29-sep	C	OPIW 41	6,64E+02	2,8		379,95	2,6	6,91E+02	2,8	148	2,2	2,8	<1200		
6-okt	C	OPIW 50	2,74E+03	3,4		627,12	2,8	9,11E+02	3,0	406	2,6	3,2	1,50E+03		
2-sep	C	OPIW 14	<549	2,4				1,96E+03	3,3	947	3,0	2,7	<1000		
29-sep	C	OPIW 42	<612	2,5				6,99E+03	3,8	2776	3,4	2,8	<1200		
6-okt	C	OPIW 49	7,11E+02	2,9	(12)	696,19	2,8	1,34E+03	3,1	552	2,7	3,2	1,58E+03		
2-sep	D	OPIW 10	3,27E+04	4,5		770,64	2,9	1,39E+04	4,1	3282	3,5	4,1	1,33E+04		
16-sep	D	OPIW 19	7,6E+04	4,9		4193,87	3,6	4,51E+04	4,7	20165	4,3	2,9	8,50E+02		
26-aug	D	OPIW 1	< 699	2,5				< 680	2,5			2,4	<480		
2-sep	D	OPIW 11	5,06E+03	3,7		448,97	2,7	6,86E+03	3,8	2829	3,5	4,3	2,14E+04		
16-sep	D	OPIW 20	2,0E+04	4,3		4326,95	3,6	1,81E+02	2,3	102	2,0	4,4	2,74E+04		
2-sep	D	OPIW 12	1,79E+03	3,3		8,28	0,9	1,08E+03	3,0	354	2,5	2,6	<800		
16-sep	D	OPIW 21	<508	2,4				5,51E+03	3,7	1568	3,2	3,3	2,00E+03		
2-sep	E	OPIW 18	2,98E+04	4,5		9661,27	4,0	5,89E+03	3,8	3270	3,5		-		(5)
23-sep	E	OPIW 35	-		(11)			1,56E+03	3,2	703	2,8	2,9	<1500		
6-okt	E	OPIW 53	1,6E+05	5,2		3392,34	3,5	1,25E+05	5,1	15729	4,2	2,6	<900		
2-sep	E	OPIW 16	<848	2,6				<680	2,5			2,6	<800		
23-sep	E	OPIW 36	<844	2,6				6,23E+02	2,8	269	2,4	3,5	2,90E+03		
6-okt	E	OPIW 54	<905	2,6				1,07E+03	3,0	303	2,5	2,9	<1600		
2-sep	E	OPIW 17	1,29E+04	4,1		3147,68	3,5	7,01E+02	2,8	308	2,5	3,5	3,10E+03		(4)
23-sep	E	OPIW 34	1,75E+05	5,2		20725,33	4,3	3,20E+04	4,5	6625	3,8	4,7	4,80E+04		(4)
6-okt	E	OPIW 52	1,2E+05	5,1		11670,29	4,1	7,54E+03	3,9	3078	3,5	5,1	1,28E+05		
23-sep	F	OPIW 29	-		(11)			3,43E+03	3,5	1767	3,2		-		(5)
29-sep	F	OPIW 39	4,06E+02	2,6	(12)	182,27	2,3	5,37E+02	2,7	174	2,2	2,8	<1200		
6-okt	F	OPIW 47	<6931	3,5				7,32E+02	2,9	155	2,2	2,4	<550		
23-sep	F	OPIW 30	-		(11)			3,51E+04	4,5	6603	3,8	2,5	<700		
29-sep	F	OPIW 38	8,13E+03	3,9	(12)	479,00	2,7	6,52E+03	3,8	1457	3,2	2,8	<1200		
6-okt	F	OPIW 46	<2688	3,1				1,86E+04	4,3	10284	4,0	2,7	<1000		
23-sep	F	OPIW 28	<2430	3,1				< 6.80E+02	2,5			2,9	<1400		
29-sep	F	OPIW 37	<2931	3,2				< 6.80E+02	2,5			2,8	<1200		
6-okt	F	OPIW 48	-		(10)			1,51E+03	3,2	416	2,6	4,2	<29000		

Attention: truly negativ results of Q-PCR are judged as +
This means that no Lp was found during serotyping

45 samples		
ISO = reference method		
QPCR KWR = alternatieve method		
	ISO +	ISO -
QPCR KWR +	33	1
QPCR KWR -	3	8
no result		7
relative accuracy	AC	91%
relative specificy	SP	89%
relative sensitivity	SE	92%

ISO vs D

45 samples		
ISO = reference method		
QPCR D = alternatieve method		
	ISO +	ISO -
QPCR Labore+	26	2
QPCR Labore-	8	9
no result		7
relative accuracy	AC	78%
relative specificy	SP	82%
relative sensitivity	SE	76%

ISO vs GS

49 samples		
GS = reference method		
QPCR GS = alternatieve method		
	ISO +	ISO -
QPCR GS +	36	8
QPCR GS -	1	4
no result		3
relative accuracy	AC	82%
relative specificy	SP	33%
relative sensitivity	SE	97%

(4) Analysis inhibited by the samples matrix. Real number of *Legionella pneumophila* is probably higher
(5) No *Legionella pneumophila* found. Analysis inhibited by the samples matrix; not reliable result.
(7) *Legionella pneumophila* found, but result can not be quantified.
(10) The output of the internal control sample is too low to give a reliable quantitative result
(11) *Legionella pneumophila* found, but the output of the internal control sample is too low to give a reliable quantitative result
(12) *Legionella pneumophila* concentration is indicative; below the lower limit for a reliable quantitative result

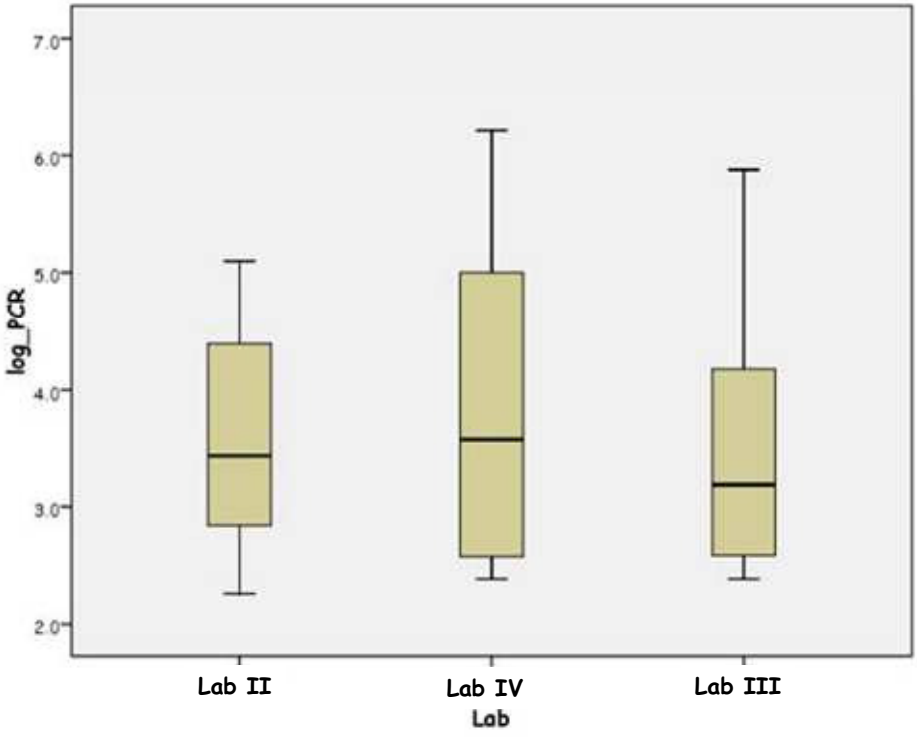
VII Appendix: Details statistical analysis

Test on differences between methods of Q-PCR analysis
Three laboratories – laboratory II, III and IV - each use a different method to analyse on Q-PCR. To examine the differences between the results of the three methods, we took 52 samples of cooling water and process water, coming from 20 locations at 6 sites (plants). These were all locations with a history of Legionella pneumophila occurrences. Most locations were sampled at three different periods, some at two different periods and some at only one period. Each sample was analysed by all three labs.

We tested statistically on differences between the results of the three labs, using analysis of variance (anova). If a sample result of one or more of the labs was missing, the results of that sample were not used. This was the case for 8 samples, so 44 samples remained for the analysis. Censored data were set at half the reporting limit. If the sample results of two or more labs were censored, they were set at half the lowest reporting limit for that sample, to avoid artificial differences between the labs. After that, each result was transformed by taking its logarithm. This was done to better meet the underlying assumption of analysis of variance that the residuals of the anova-model come from a normal distribution.

Figure 1 shows the boxplots of the logarithms of the results of the 44 samples for each lab.

Figure 1: Boxplots of the logarithms of the results of the 44 samples for each lab.



As can be seen from figure 1 the centres of the three boxplots do not differ much. However, the upper tails of the boxplots of lab IV and lab III are longer than that of lab II. This means that the high results of lab II tend to be lower than the high results of lab III and IV.

Using analysis of variance, we tested the null hypothesis that the mean of the logarithm of the results is the same for the three labs. The alternative hypothesis is that these means are not the same. We declared site, location and sample to be random factors and lab to be a fixed factor. Location was declared nested within site and sample nested within location. The F-test on the significance of the factor lab resulted in a p-value of 0.083, which means that the null hypothesis is not rejected (with 95% confidence). **Therefore,**

we did not find statistical significant differences between the means of the logarithms of the results of the three labs.
The residuals of the anova model conform to normality (with 95% confidence), as the Shapiro-Wilk test on normality of these residuals has a p-value of 0.321. So this condition for the use of anova was met.

Table 1 shows for each lab the estimated mean of the logarithms of the results and the lower and upper bound of the 95% confidence interval of the estimated mean.

Table 1: For each lab the estimated mean of the logarithms of the results and the lower and upper bound of the 95% confidence interval of the estimated mean.

Dependent Variable:log_PCR

Lab	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
II	3.584[a]	.092	3.402	3.766
IV	3.789[a]	.092	3.607	3.971
III	3.505[a]	.092	3.323	3.687

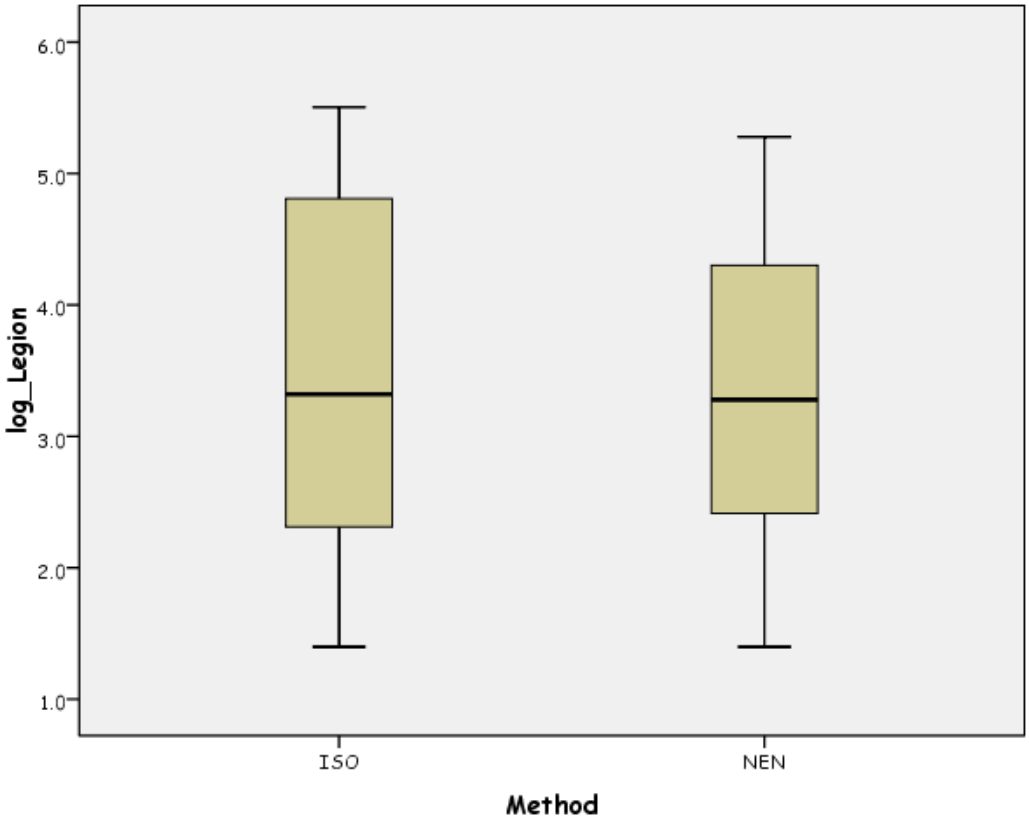
a. Based on modified population marginal mean.

As can be seen from table 1 the confidence intervals of the three means show overlap.

Test on differences between methods of total Legionella analysis
To examine the differences between two methods to determine the concentration of total Legionella – ISO 11731 and NEN 6265:2007 (MWY) - we used the same 52 samples of cooling water and process water as described before. Each sample was analysed by both methods.
We tested statistically on differences between the results of the two methods, using analysis of variance (anova). If a sample result of one method was missing, the results of that sample were not used. This was the case for 16 samples, so 36 samples remained for the analysis. Censored data were set at half the reporting limit. If the sample results of both methods were censored, they were set at half the lowest reporting limit for that sample, to avoid artificial differences between the methods. After that, each result was transformed by taking its logarithm. This was done to better meet the underlying assumption of analysis of variance, that the residuals of the anova-model come from a normal distribution.

Figure 2 shows the boxplots of the logarithms of the results of the 36 samples for each method.

Figure 2: Boxplots of the logarithms of the results of the 36 samples for each method.



As can be seen from figure 2 the centres of the two boxplots do not differ much.

Using analysis of variance, we tested the null hypothesis that the mean of the logarithm of the results is the same for the two methods. The alternative hypothesis is that these means are not the same. We declared site, location and sample to be random factors and method to be a fixed factor. Location was declared nested within site and sample nested within location. The F-test on the significance of the factor method resulted in a p-value of 0.070, which means that the null hypothesis is not rejected (with 95% confidence). **Therefore, we did not find a statistical significant difference between the means of the logarithms of the results of the two methods.**
The residuals of the anova model conform to normality (with 95% confidence), as the Shapiro-Wilk test on normality of these residuals has a p-value of 0.909. So this condition for the use of anova was met.

Table 2 shows for each method the estimated mean of the logarithms of the results and the lower and upper bound of the 95% confidence interval of the estimated mean.

Table 2: For each method the estimated mean of the logarithms of the results and the lower and upper bound of the 95% confidence interval of the estimated mean.

Dependent Variable:log_Legion

Method	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ISO	3.475[a]	.067	3.340	3.611
NEN	3.299[a]	.067	3.164	3.435

a. Based on modified population marginal mean.

As can be seen from table 2 the confidence intervals of the two means show overlap.

VIII Appendix: Comparison cultivation methods

			lab IV								lab I				
date 2008	company	sample code	BCYE + AB NEN 6265		MWY NEN 6265:2007		BCYE pnue pH 7,3 & temp 40C		MWY pneu pH 7,3 & temp 40C		ISO 11731		MWY NEN 6265:2007		
			cfu/l	sd	cfu/l	sd	cfu/l	sd	cfu/l	sd	cfu/l	Serotype	cfu/l	Serotype	
26-aug	A	OPIW 7	1800 (2)	1151	7800	2439	5400 (8)	2966	1600 (2)	1746	70.000	a+b+c	16.000	a+b+c	
16-sep	A	OPIW 25	10200	5461	38500	6124	(3)		(1)		130.000	a+b+c	79.000	a+b+c	
29-sep	A	OPIW 44	(1)		15600	2104	3400	3029	6500	1458	60.000	a+b+c	14.000	a+b+c	(5)
26-aug	A	OPIW 8	300	274	300	447	500	612	<100		1.800	b	1.900	a+b	
16-sep	A	OPIW 26	(1)		146200	32823	(3)		27920 (8)	9008	320.000	b	190.000	b	
29-sep	A	OPIW 43	37900 (2)	15441	46600	9562	27000 (9)	20905	50300	4192	220.000	b	64.000	b	
26-aug	A	OPIW 9	(1)		(1)		(1)		<100		2.900	b+c	1.900	a+b	
16-sep	A	OPIW 27	(1)		300 (8)	489	(3)		1040 (8)	876	1.900	b+c	2.200	b+c	
29-sep	A	OPIW 45	(1)		900	548	(1)		<100		2.300	b+c	2.000	b+c	
26-aug	B	OPIW 4	720 (8)	179	3500	2598	500 (8)	612	600 (8)	652	18.000	b	1.500	b	
16-sep	B	OPIW 22	600 (2)	1342	1200	1304	(3)		600	652	870	b	380	b	
23-sep	B	OPIW 31	100 (2)	137	300	447	<100		500	354	530	a+b	1.100	b	
26-aug	B	OPIW 6	(1)		1900	1245	(1)		1040	727	4.100	a+b	(1)		(4)
16-sep	B	OPIW 23	(1)		480 (8)	716	(3)		960 (8)	669	< 50		(1)		
23-sep	B	OPIW 33	(1)		<100		(1)		<100		3.600	a+b	(1)		(5)
26-aug	B	OPIW 5	<100		(1)		(1)		(1)		11.000	a+b	(1)		
16-sep	B	OPIW 24	4080	2349	100 (8)	137	(3)		<100		55.000	a+b	1.900	a	
23-sep	B	OPIW 32	(1)		(1)		(1)		(1)		65.000	a+b	(1)		
2-sep	C	OPIW 15	<100		<100		<100		<100		98	c	<500		
29-sep	C	OPIW 40	<100		<100		<100		<100		< 50		< 500		
6-okt	C	OPIW 51	<100		<100		<100		<100		< 50		< 500		
2-sep	C	OPIW 13	(1)		28100	8073	(1)		16800	8362	65.000	a+b	60.000	a+b	
29-sep	C	OPIW 41	500 (2)	600	1700	1823	200	447	<100		530	b	980	a+b	
6-okt	C	OPIW 50	1375	1031	1800	1304	300	314	700	1095	420	a+b	860	a+b	
2-sep	C	OPIW 14	100 (2)	224	100	224	(1)		(1)		500	b	98	c	
29-sep	C	OPIW 42	633 (2)	351	200	274	<100		<100		210	c	320	b+c	
6-okt	C	OPIW 49	400	418	100	224	<100		<100		200	b+c	490	a+b+c	
2-sep	D	OPIW 10	(1)		(1)		(1)		120000 (2)		250.000	a+b	40.000	b	
16-sep	D	OPIW 19	(1)		50000 (2)		(3)		8900 (8)	10071	210.000	a+b+c	49.000	a+b	
26-aug	D	OPIW 1	(1)		(1)		(1)		(1)		540	b	(1)		
2-sep	D	OPIW 11	(1)		(1)		(1)		(1)		52.000	a+b	4.900	b	
16-sep	D	OPIW 20	(1)		(1)		(3)		<100		57.000	a+b	29.000	a+b	
2-sep	D	OPIW 12	(1)		(1)		(1)		2233 (2)	1365	1.500	a+b	(1)		
16-sep	D	OPIW 21	(1)		<100		(3)		<100		< 50		88	a	
2-sep	E	OPIW 18	(1)		6400 (2)	3525	(1)		25333 (2)	4726	10.000	b	11.000	b+c	(5)
23-sep	E	OPIW 35	(1)		<100		(1)		<100		< 50		< 500		
6-okt	E	OPIW 53	(1)		6100 (8)	3380	(1)		(1)		64.000	a+b+c	25.000	a+b	
2-sep	E	OPIW 16	<100		<100		(1)		<100		< 50		190	b+c	
23-sep	E	OPIW 36	<100		<100		<100		<100		96	c	< 500		
6-okt	E	OPIW 54	<100		<100		(1)		<100		< 50		270	b	
2-sep	E	OPIW 17	(1)		(1)		10000 (2)		(1)		(1)		(1)		(4)
23-sep	E	OPIW 34	(1)		<100		(1)		(1)		70.000	a+b	4.000	b	(4)
6-okt	E	OPIW 52	(1)		<100		(1)		(1)		69.000 (2)	a+b	55.000 (2)	a+b	
23-sep	F	OPIW 29	(1)		(1)		(1)		(1)		< 50		(1)		(5)
29-sep	F	OPIW 39	(1)		(1)		(1)		(1)		510	c	(1)		
6-okt	F	OPIW 47	(1)		<100		(1)		<100		960	b+c	< 500		
23-sep	F	OPIW 30	(1)		(1)		(1)		(1)		(1)		(1)		
29-sep	F	OPIW 38	(1)		(1)		(1)		(1)		(1)		(1)		
6-okt	F	OPIW 46	(1)		(1)		(1)		(1)		< 500		(1)		
23-sep	F	OPIW 28	<100		<100		<100		<100		< 50		(1)		
29-sep	F	OPIW 37	(1)		(1)		<100		<100		< 50		(1)		
6-okt	F	OPIW 48	(1)		<100		<100		<100		< 50		(1)		

(1) No Legionella found, result not reliable due to too much side growth on the agar medium
(2) Estmated value, real number possibly higher due to much side growth on the agar medium
(3) By mistake inoculated on BCYE-AB, pH 7.3. No Legionella found, result not reliable due to too much side growth on the agar medium
(4) Analysis inhibited by the samples matrix. Real number of *Legionella pneumophila* is probably higher
(5) No *Legionella pneumophila* found. Analysis inhibited by the samples matrix; not reliable result.
(6) Difficult to visibly detect line; line is very thin
(7) *Legionella pneumophila* found, but result can not be quantified.
(8) Colonies (partly) confirmed with PCR
(9) Though negativ at confirmation on plate, presence of *Legionella* is suspected

Serotypes: a = *Legionella pneumophila* serogroep 1.
b = *Legionella pneumophila* serogroep 2-14.
c = *Legionella* bacteria non *pneumophila.*

			lab IV			lab I/labIII			
date 2008	company	sample code	MWY NEN 6265:2007			MWY NEN 6265:2007			
			cfu/l		sd	cfu/l		Serotype	
26-aug	A	OPIW 7	7800	3,9	2439	16.000	4,2	a+b+c	
16-sep	A	OPIW 25	38500	4,6	6124	79.000	4,9	a+b+c	
29-sep	A	OPIW 44	15600	4,2	2104	14.000	4,1	a+b+c	(5)
26-aug	A	OPIW 8	300	2,5	447	1.900	3,3	a+b	
16-sep	A	OPIW 26	146200	5,2	32823	190.000	5,3	b	
29-sep	A	OPIW 43	46600	4,7	9562	64.000	4,8	b	
26-aug	A	OPIW 9	(1)			1.900	3,3	a+b	
16-sep	A	OPIW 27	300	2,5	489	2.200	3,3	b+c	
29-sep	A	OPIW 45	900	3,0	548	2.000	3,3	b+c	
26-aug	B	OPIW 4	3500	3,5	2598	1.500	3,2	b	
16-sep	B	OPIW 22	1200	3,1	1304	380	2,6	b	
23-sep	B	OPIW 31	300	2,5	447	1.100	3,0	b	
26-aug	B	OPIW 6	1900	3,3	1245	(1)			(4)
16-sep	B	OPIW 23	480	2,7	716	(1)			
23-sep	B	OPIW 33	50	1,7		(1)			(5)
26-aug	B	OPIW 5	(1)			(1)			
16-sep	B	OPIW 24	100	2,0	137	1.900	3,3	a	
23-sep	B	OPIW 32	(1)			(1)			
2-sep	C	OPIW 15	50	1,7		250	2,4		
29-sep	C	OPIW 40	50	1,7		250	2,4		
6-okt	C	OPIW 51	50	1,7		250	2,4		
2-sep	C	OPIW 13	28100	4,4	8073	60.000	4,8	a+b	
29-sep	C	OPIW 41	1700	3,2	1823	980	3,0	a+b	
6-okt	C	OPIW 50	1800	3,3	1304	860	2,9	a+b	
2-sep	C	OPIW 14	100	2,0	224	98	2,0	c	
29-sep	C	OPIW 42	200	2,3	274	320	2,5	b+c	
6-okt	C	OPIW 49	100	2,0	224	490	2,7	a+b+c	
2-sep	D	OPIW 10	(1)			40.000	4,6	b	
16-sep	D	OPIW 19	50000	4,7		49.000	4,7	a+b	
26-aug	D	OPIW 1	(1)			(1)			
2-sep	D	OPIW 11	(1)			4.900	3,7	b	
16-sep	D	OPIW 20	(1)			29.000	4,5	a+b	
2-sep	D	OPIW 12	(1)			(1)			
16-sep	D	OPIW 21	50	1,7		88	1,9	a	
2-sep	E	OPIW 18	6400	3,8	3525	11.000	4,0	b+c	(5)
23-sep	E	OPIW 35	50	1,7		250	2,4		
6-okt	E	OPIW 53	6100	3,8	3380	25.000	4,4	a+b	
2-sep	E	OPIW 16	50	1,7		190	2,3	b+c	
23-sep	E	OPIW 36	50	1,7		250	2,4		
6-okt	E	OPIW 54	50	1,7		270	2,4	b	
2-sep	E	OPIW 17	(1)			(1)			(4)
23-sep	E	OPIW 34	50	1,7		4.000	3,6	b	(4)
6-okt	E	OPIW 52	50	1,7		55.000	4,7	a+b	
23-sep	F	OPIW 29	(1)			(1)			(5)
29-sep	F	OPIW 39	(1)			(1)			
6-okt	F	OPIW 47	50	1,7		250	2,4		
23-sep	F	OPIW 30	(1)			(1)			
29-sep	F	OPIW 38	(1)			(1)			
6-okt	F	OPIW 46	(1)			(1)			
23-sep	F	OPIW 28	50	1,7		(1)			
29-sep	F	OPIW 37	(1)			(1)			
6-okt	F	OPIW 48	50	1,7		(1)			

conclusions

52 samples

MWY KWR

15 times (1) too much side growth 29%

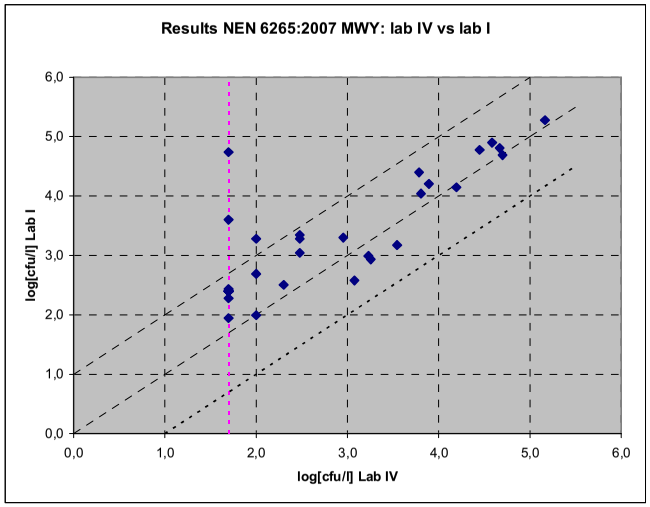
MWY D

16 times (1) too much side growth 31%

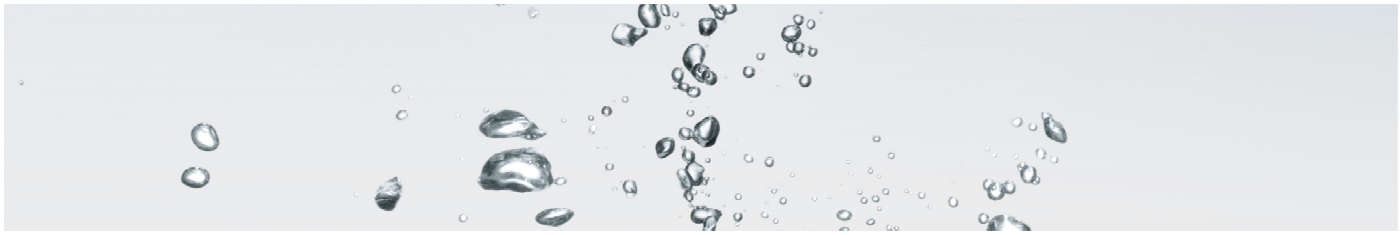
comparance

11 times results differ 21%
9 times too much side growth for at least 1 of 2 methods 17%
2 times < 100 versus > 100 4%

In graphic all WTCB results < 500 as 250 ; KWR results < 100 as 50



1,7	0	0	0	1	
1,7	1	1	1	2	0
1,7	2	5	5	6	4
1,7	3	5,5	5,5		4,5
1,7	6				



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