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Analysis of ISO 11731:2017 method to assess Legionella pneumophila in water with high background

And how it differentiates from its earlier variant ISO 11731:1998

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Abstract

Legionella pneumophila is a human pathogen commonly found in natural and artificial aquatic environments and can cause a condition called legionellosis. Monitoring for legionellae is therefore important for protecting public health and identifying its environmental sources is a way to prevent illness. This has resulted in development of several control strategies to identify these sources. One of these strategies is to construct a valid method to detect *Legionella pneumophila* and monitoring these methods is a way to ensure the method remain effective at tracing infection.

The current version of standardized method is called ISO 11731:2017 and supersedes its former version called ISO 11731:1998. The former version uses a combination of heat and acid solution treatment to reduce interfering microorganisms in water with high background, whereas the current version separates the treatment by subdividing the sample in three parts. One part is subjected to heat treatment, one with acid solution treatment and one remains untreated. Therefore, the aim of this study is to analyse how this difference in method strategy will affect detection of *Legionella pneumophila* between the current and its former version of ISO 11731. To do this, this study divided the experiment into two parts: experiment A was aimed at evaluating the validity of the method and experiment B was designed to study repeatability in terms of dispersion and performance data range. For experiment A: 14 samples were tested using both ISO 11731:2017 and 11731:1998 to see how the results differentiated. Six are natural samples and was appointed based on their previous results that showed positive for Legionella. Four samples were spiked with different serotypes of Legionella and the remaining four were spiked with both Legionella and Legionella-inhibited bacteria. For experiment B, three certified reference material with different concentration of *Legionella pneumophila* serotype 1 was tested in repeatability conditions with each sample producing ten replicates.

In conclusion, based on results assessed in this study ISO 11731:1998 was more suitable to analyse water with higher concentration of interfering microorganisms. By a combination of heat and acid solution treatment: it maximizes the reduction of interfering microorganisms which facilitates Legionella to cultivate on agar. ISO 11731:2017 was more efficient in recovering different serotypes of Legionella. Although, there were a significant increase in dispersion and performance data range results in ISO 11731:2017. This indicates that since there is an additional dilution step added in acid solution treatment: it increases the risk of human error and therefore a greater vulnerability to the method.

List of abbreviations

L. *pneumophila* : Legionella pneumophila

cfu : Colony forming units

Table of Contents

1. Introduction

Legionella pneumophila from the family of *Legionellaceae* is a human pathogen found in freshwater environments [7]. The bacteria were first recognized in 1976 after a large outbreak of fatal pneumonia during a convention of legionnaires in US, Philadelphia and has since been known as Legionnaire's Disease [3]. For infection tracking a Swedish resident has obligation to report to the Public Health Agency of Sweden called Folkhälsomyndigheten if the person has been contaminated with *L. pneumophila*. Some of the symptoms to the disease are fever, headache, cough, diarrhoea, and muscle ache [1]. *L. pneumophila* has its growth temperature between 25 and 42 degrees and thrives in warm and humid environments [7]. Approximately 90% of the diseases caused by *Legionella pneumophila* can be prevented by having a better water control and is considered as a preventable illness [7]. This resulted to several guidelines and control strategies has been developed aimed at reducing the risk of legionellosis. One of such strategies is to develop an effective and valid method to detect *L. pneumophila* and identify environmental sources that poses a risk of legionellosis. Monitoring such methods is thus important so that these control measures remain effective and tracing infection sources more accurate.

Standardized method for quantification of *L. pneumophila* was established by European Committee for Standardization, CEN, [10] for environmental detection. Agar plate culture is the gold standard method for detection of *L. pneumophila* and is used for specific diagnostic operation [7], it does however have its limitations. One of the major limitations to this method is *L. pneumophila* is inhibited to cultivate on agar if there are other interfering microorganisms present in sample risking creating false negative results [8]. To avoid this, CEN included heat and acid solution treatment to eliminate as much of these interfering microorganisms as possible [10].

One of the laboratories that practices the standardized method for analysing *L. pneumophila* is SGS Analytics Sweden AB whose method is accredited by Swedac, the national accreditation body in Sweden [2]. The current version of standardized method called SS-EN ISO 11731:2017 supersedes the former version called ISO 11731:1998 [10]. The former version used a combination of heat and acid solution treatment for water samples with high concentration of interfering microorganisms. Whereas the present version involves

subdividing the sample into three portions: one portion untreated, one portion for treatment with heat and one portion for treatment with acid solution. It has not yet been explored how this will affect in identifying Legionella in sample. Therefore, the aim of this study is to analyse how this difference in method strategy will affect detection of *Legionella pneumophila* between the current and its former version of ISO 11731.

2. Background

2.1 Brief overview of Legionella and L. *pneumophila*

Summer in 1976 a celebration of the Declaration of Independence took place in Philadelphia with 182 members of Pennsylvania America Legion. The convention that should have been the focus developed instead to an epidemic of fatal pneumonia, where 29 of 182 members died after they returned [3]. The magnitude of the Philadelphia epidemic contributed to the recognition of Legionella species and its human pathogenesis, and the one to discover the bacterium causing Legionnaire's Disease was Joseph McDade who subsequently named it *Legionella pneumophila* [3].

Family *Legionellaceae* consist of one single genus *Legionella*, based on studies of 16S rRNA analysis [7]. It confirms the family *Legionellaceae* to be a single monophyletic subgroup among the subdivision of ɣ2-Proteobacteria since the 16S rRNA sequence of family *Legionellaceae* is <95% identical [7]. Today Legionella comprises more than 58 species with 70 different serogroups [8]. All species are susceptible to legionellosis, however *Legionella pneumophila* is alone responsible to 95% of cases of Legionnaire's Disease diagnosed worldwide [7]. The specie can be subdivided into 16 serogroups and most confirmed cases of Legionnaire's Disease is caused by *L. pneumophila* serogroup one [9].

As mentioned earlier, legionellosis is a preventable illness and controlling as well as eliminating the bacterium in its natural habitat will prevent cases of the disease. In 2021, Public Health Agency of Sweden reported 168 cases with legionella infection and the majority fell ill from L. *pneumophila* serogroup 1[1]. Most cases are traced back to human made aquatic environments [7] such as evaporative cooling system and hot and cold-water distributions systems [10]. Or, in general associated equipment such as spa pools, dental units,

air conditioning units etc. [10]. For the sake of public health, it is important monitoring these environmental sources that poses a risk of legionellosis.

2.2 Molecular mechanism of legionella toxicity

As previously mentioned, L. *pneumophila* is the main specie to cause Legionnaire´s Disease worldwide, thus the bacterium represents best the molecular pathogenesis of Legionella. The central feature of L. *pneumophila* pathogenesis is its ability to multiply within macrophages without causing cytolysis when entering the host cell [6]. The bacteria initiate its toxicity by entering the cell through phagocytosis [6], an immune process where a phagocyte ingests harmful particles and eliminates them. L. *pneumophila* enters the cell by binding to inducing extracellular protein called opsonin and a complement component C3 protein, who binds to CR1 and CR3 receptors of the phagocyte and initiates phagocytosis [6]. The macrophage engulfs the bacterium and enters as intracellular vesicle called phagosome where it normally fuses with a lysosome degrading the phagosome. However, L. *pneumophila* phagosome does not fuse with lysosome, a process promoted by lysosomal enzymes and vacuolar acidification and hence avoids degradation [6]. Instead, the bacterium replicates through binary fission within the macrophage creating an enlarged phagosome [6]. Finally, L. *pneumophila* infection results in necrosis to the host cell releasing the newly formed L. *pneumophila* bacteria cells for further cell invasion.

It has been known that there are bacterial surface structures which promote intracellular infection and virulence caused by L. *pneumophila* and this study will mention several of them. One is a temperature-regulated, bundle-forming IV pilus of legionella that promotes the invasion of host cell by bacterial attachment [6]. Another surface structure is peptidoglycan-linked porin, a protein binding site for the C3 protein that mediates phagocytosis process. And lastly a 24 kDa macrophages infectivity potentiator, Mip, protein that has been recognized to be necessary for the initial stages of L. *pneumophila* virulence infection to macrophages, protozoa and mammalian cells [11]. Because of the importance in *Mip* protein and its role in L. *pneumophila* infection, the gene targeting the *Mip* protein plays a leading role in identification of L. *pneumophila* using the PCR method.

2.3 Quantitative method for detection of legionella: real-time PCR and *mip* gene

The *mip* protein has proven to be essential virulence infection factor of L. *pneumophila*. Thus, a PCR method for amplification and identification of *mip* gene was developed to identify L. *pneumophila* in water samples and clinical specimen. *Mip* gene of L. *pneumophila* is searched using Uniprot and is represented as followed:

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   atgaagatga aattggtgac tgcagctgtt atggggcttg caatgtcaac agcaatggct
 61 gcaaccgatg ccacatcatt agctacagac aaggataagt tgtcttatag cattggtgcc
121 gatttgggga agaattttaa aaatcaaggc atagatgtta atccggaagc aatggctaaa
181 ggcatgcaag acgctatgag tggcgctcaa ttggctttaa ccgaacagca aatgaaagac
241 gttcttaaca agtttcagaa agatttgatg gcaaagcgta ctgctgaatt caataagaaa
301 gcggatgaaa ataaagtaaa aggggaagcc tttttaactg aaaacaaaaa caagccaggc
361 gttgttgtat tgccaagtgg tttgcaatac aaagtaatca atgctggaaa tggtgttaaa
421 cccggtaaat cggatacagt cactgtcgaa tacactggtc gtctgattga tggtaccgtt
481 tttgacagta ccgaaaaaac tggtaagcca gcaacttttc aggtttcaca agttatccca
541 ggatggacag aagctttgca attgatgcca gctggatcaa cttgggaaat ttatgttcca
601 tcaggtcttg catatggccc acgtagcgtt ggcggaccta ttggcccaaa tgaaacttta
661 atatttaaaa ttcacttaat ttcagtgaaa aaatcatctt aa
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DNA material from L. *pneumophila* in water sample is prepared before initiating PCR. Firstly, the bacteria are concentrated from water sample, normally by membrane filtration, and then placed in a tube with DNA free water [16]. The tube is homogenized and then incubated in 37ºC for 30 min [16]. Next step is to prepare cell extract from bacteria which is normally through chemical methods. One way is to add EDTA, ethylenediamine tetraacetate, and detergent SDS, sodium dodecyl sulphate, where EDTA removes magnesium ions disrupting cell wall whereas SDS removes lipid molecules weakening the cell wall [12]. With these chemicals added: it will cause the cell to burst. Next step is removal of cell debris which is done by removal of pellet after centrifugation. Then, purification of DNA material from cell extract is prepared. One method for this is using silica gel membrane that binds DNA in presence of guanidinium thiocyanate retaining DNA in a column, resulting proteins and other biochemicals are immediately eluted. DNA can then be recovered by adding water which destabilizes the interaction between DNA and silica [12].

PCR, polymerase chain reaction, is a technique that copies billions of a specific DNA sequence [12]. It is carried out in a single test tube mixing DNA with a set of reagents and then placed in a thermal cycler enabling the mixture to be incubated in series of temperature. The target of which DNA template to amplify is determined by the design of primers used. The target genome for this PCR amplification is the 650-bp sequence gene coding for the

macrophage infectivity protein, *mip*. The primers specific for all serogroups of L. *pneumophila* was designed by Mahubuni et al [13] and consist of the following sequence:

5´-GCT ACA GAC AAG GAT AAG TTG -3´
5´-GTT TTG TAT GAC TTT AAT TCA- 3´

Detection and quantification of the sample can then be performed in accordance with 2012, ISO/TS 12869 [1] with PCR result in Genomic Unit per m^3 or GU/m^3.

This study will however not use this method for analysing *Legionella pneumophila* in water samples. The aim of this study is to compare the difference in gold-standard method cultivating Legionella bacteria between ISO 11731:1998 and 11731:2017 since it is the standard practice in quantifying living bacteria. Therefore, using the PCR method for this study will not be applicable.

2.4 ISO standards and guidelines to analyse microorganisms using culture techniques

ISO is derived from the Greek word "isos" which means equal [14]. The acronym representing the organization for international standardization would be different depending on different language it is translated to. For example, IOS in English or OIN in French, thus the founders decided the short form to be ISO [14].

ISO organization was founded in 1947 by total of 67 technical committees with different expertise of subjects. The first ISO standard was published in 1951 named ISO/R 1:1951 *Standard reference temperature for industrial length measurements* and by 1955, 68 other ISO standards had been created [14]. In 1971 ISO creates its two first committees in environmental field called Air quality and Water Quality where they focus on standardizing measurement methods, sampling and reporting the air and water quality characteristics [14]. In 1996 ISO published the environmental management system standard called ISO 14001 which is a tool for companies and organizations to help them identify and control their environmental impact.

Analysing microorganisms using microbiological methods are one of the aspects that addresses the quality of water. One of such methods is using specified culture media for

detection and enumeration of specific microorganisms to cultivate on. ISO has presented general requirements and guidelines for microbiological examination by culture, particularly in preparation of sample, culture media, apparatus, and glassware. The general principle of culture method technique consists of assessing a certain known volume of water sample by membrane filtration or centrifugation, on or directly into culture medium. It is assumed after incubation the target microorganisms will be present either as a colony visible on the culture medium or by changing observable properties of liquid medium [16]. One of the requirements that ISO standard states is the general measurement specific for target microorganism must be followed such as its ideal storage temperature, incubation and culture media temperature and its incubation times. Sterilization and decontamination protocol must also be fulfilled in accordance with ISO 8199 standards, as well as sampling and sample handling is executed under ISO 19458 [10].

The general principle for enumeration on culture technique is that each colony is considered to have originated from one single microorganism or a clump of microorganisms present in test sample. Taking the volume of test sample and number of colonies formed into account, the results is therefore expressed as colony-forming units, cfu or colony-forming particles, cfp at a given volume such as 1 ml or 100 ml. In this study, the results are presented as cfu/100 ml as this study uses SGS Analytics Sweden AB protocol to assess results.

2.5 SS-EN ISO 11731 Water quality – Enumeration of Legionella

This ISO standard specifies culture method for isolation and enumeration of Legionella in water samples. It is applicable to different kinds of water including industrial waste, natural water and drinking water. The standard uses related matrices separating the kinds of water into; water with low background, water with high background and water with extremely high background [10]. This study will focus on assessing water with high background, as the new ISO standard has specifically altered the design of this method.

L. *pneumophila* was first isolated using Mueller-Hinton agar added with haemoglobin and a supplement containing L-cysteine [7]. It was found later that iron from haemoglobin and amino acid L-cysteine was the essential components for a more effective recovery of L. *pneumophila* [7]. Since then, the medium has been improved several times and eventually resulting to adding charcoal for detoxification of the medium as well as yeast extract as a

source of amino acid [7]. These two main components led the development of buffered charcoal-yeast extract, or BCYE agar enriched with α-ketoglutarate which is the medium currently used today for isolation of L. *pneumophila* [7].

ISO 11731 states the potential use of four different types of BCYE agar depending on the nature of water quality. Since this study analyses water with high concentration of interfering organisms, as recommended by ISO 11731, this study has determined to use GVPC agar [10]. GVPC, short for Glycine-vancomycin-polymyxin-cycloheximide, is the recommended agar due to its much-improved quality in recovering *Legionella* spp. and for its selectivity properties [4].

As mentioned earlier, culture method remains the gold standard for diagnostic procedure mainly due to its high specificity near 100% [7]. In most cases this requires a concentration technique by membrane filtration using a funnel through a vacuum with a membrane filter of 47-142mm in diameter and rated pore sizes of 0,2µm. Acid treatment may be included if needed which is done directly on membrane filter for 5±0,5min and a washing procedure is proceeded afterward using sterilized dilute solution. The membrane filter is then placed directly on agar plate and incubated [10]. However, this study will use the method assigned for water with high background of which involves no membrane filtration and demands the use of both heat and acid treatment to the sample. The difference between the new and previous ISO 11731 standard for water with high background will be described in more detail under chapter 2.5 *Verification SS-EN ISO11731:2017 Water Quality – Enumeration of Legionella*.

The agar plates are incubated in 36±2ºC for 10-14 days. They are examined two times during its incubation period where the first reading occurs on either day 2,3,4 or 5 followed by the second reading which occurs at the end of incubation period between 7-10 days. Legionella generally has white-grey colour with ground-glass appearance. There are also several species of Legionella that fluoresce under ultraviolet light as brilliant white. Colonies of L. *pneumophila* may also appear dull green and sometimes with a hint of yellowish colour [10]. However, new species of Legionella might possess different characteristics than to those described above. Hence identifying Legionella has proven to be difficult as it can appear with

many other characteristics, and it takes hard work and professional skills to examine BCYE agar for the bacteria [8]. This study will therefore use an experienced examiner that has more than 5 years of experience of identifying and numerating Legionella on culture medium.

2.6 Verification of SS-EN ISO11731:2017 Water quality – Enumeration of Legionella

Before proceeding with the experimental chapter of this study, a verification plan is drafted and approved by QA manager at SGS Analytics Sweden AB before the experiment is initiated. See appendix A for *Verifieringsplan för Legionella i vatten med hög bakgrundsflora (sötvatten/dricksvatten)*. This verification plan regards only the method that analyses Legionella in water samples with high concentration of interfering microorganisms, as it was only this method whose design was altered [10].

As mentioned earlier, method of analysing Legionella in water with high background does not use the concentration technique of membrane filtration, which are normally the case [10]. In ISO 11731:1998, the method instructs to place the sample in one sterile test tube of which undergoes heat treatment by placing the test tube in a water bath of 50ºC for 30 min eliminating microorganisms that are heat sensitive [10]. When the sample has reached room temperature after heat treatment, it is diluted with acid solution to a 1:2 relationship, lets it rest for 5±0,5min eliminating microorganisms that are low-pH sensitive before direct plating on agar. Whereas in ISO 11731:2017, the method instructs to subdivide the sample into three parts where two of them are placed in sterile test tubes. One test tube undergoes heat treatment as described above. Second test tube undergoes acid solution treatment as described above but dilutes the sample with 1:10 relationship instead. This change was made to ensure the pH remains low in the sample during acid solution treatment regardless of the initial pH of the sample [10], improving the validity of acid solution treatment. Lastly the third part is un-treated and is directly plated on agar. A flowchart is developed to show more clearly the difference in method between 11731:2017 and 11731:1998:

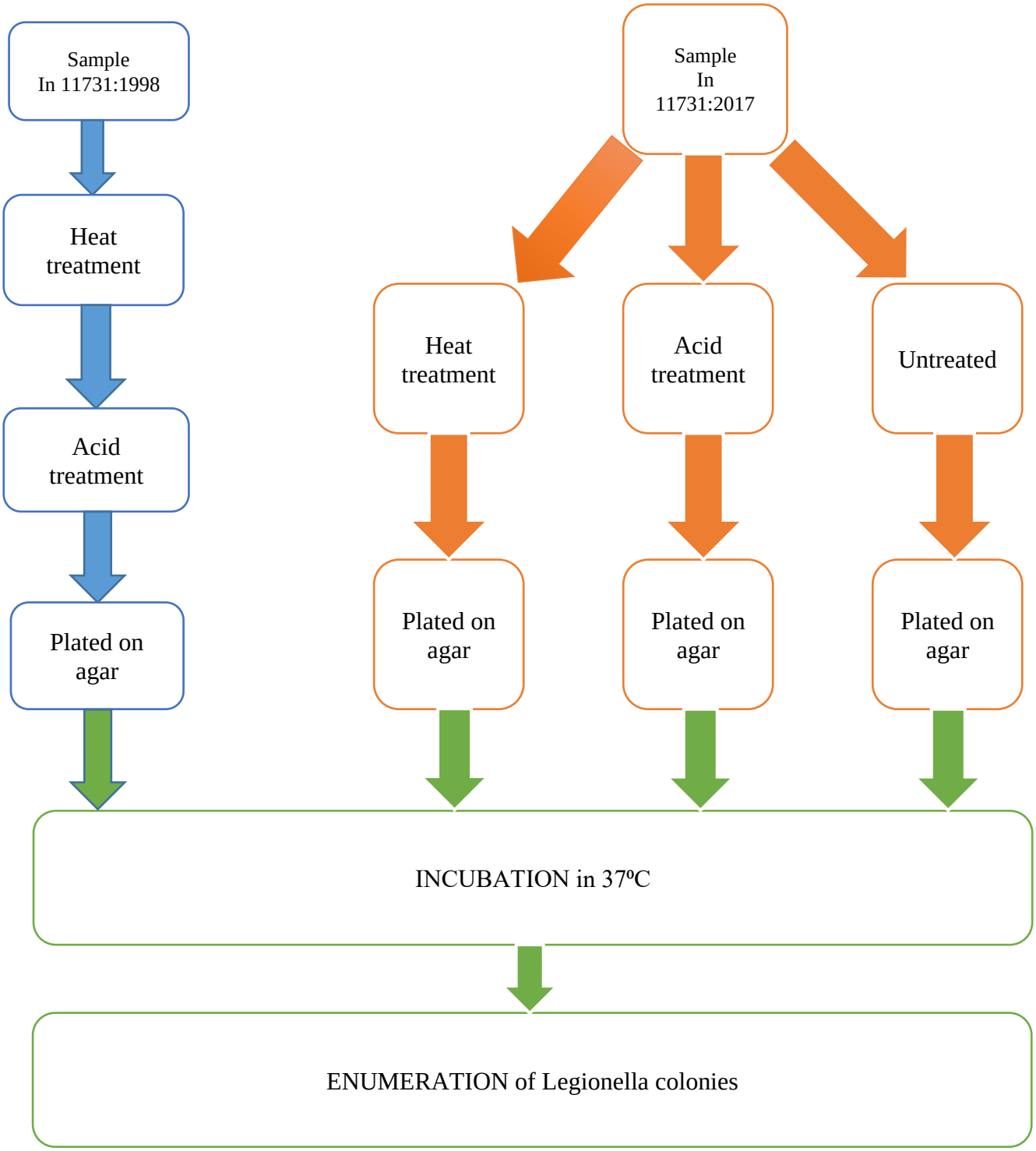

Figure 1. Flowchart presenting the difference in method between ISO 11731:2017 and ISO 11731:1998

Monitoring for legionellosis has become one of public health regulations worldwide [1] and it is important that these control methods of detecting L. *pneumophila* stay effective, valid and that they produce reliable results. ISO 11731 was developed for this purpose and changing the design of the method may affect monitoring legionellosis infection for public health

supervision. Thus, the aim of this study is analysing the difference between ISO 11731:2017 and ISO 11731:1998 in terms validity and repeatability performance of the method. Validity is measured by comparing the results between ISO 11731:2017 and ISO 11731:1998 with known amount of Legionella. Repeatability performance will be measured by analysing replicates from the same sample in repeatability conditions i.e., by the same technician on the same day in approximate the same time. The results from replicates will then be analysed in terms of their mean, variance, performance data range, and over-dispersion by applying Poisson index of dispersion. These results will then be compared with results assessed by SGS Analytics Sweden AB using the same reference material but tested using 11731:1998.

3. Experimental

The experiment of this study is conducted at SGS in Karlstad at their microbiology laboratory facility for one day. It is divided into two parts: experiment A and B, where experiment A is aimed to evaluate the validity of the method whereas experiment B is designed to evaluate repeatability of the method. For experiment A, six natural water samples and four spiked samples are analysed where each sample has known amount cfu of Legionella, results that has been assessed by the staff at SGS Analytics Sweden in Karlstad. The natural samples are selected based on their previous results that has tested positive for Legionella with results that spans from 1000-200 000 cfu/100 ml. Though, test results are not complete until five days after analysis at minimum after first reading and so the storage time will exceed of what is recommended by ISO 19458. Supervisor at SGS Analytics Sweden AB in Karlstad did consider this and determined that since Legionella are tenacious bacteria, they will have no issue surviving the extended storage time before this study starts to assess the sample which was arranged to be within two weeks. However, one sample was stored for three weeks before analysed. Furthermore, three samples were selected before the initial test results was complete and one sample was selected regardless of having too low concentration of Legionella and interfering microorganisms. This will be more explained in section 5 *Discussion*.

The nature of the spiked samples contains different types of L. *pneumophila* spiked with water with high background. One sample is spiked with serotype 1, one sample with serotype 2-14, one serotype that is known to be sensitive and more challenging to isolate: with L. *pneumophila* species and lastly one sample with a mixture of serotype 2-14 and L. *pneumophila* species. The four samples that are spiked with Legionella as previous mentioned

were further spiked with Legionella-inhibited bacteria with a significant higher concentration than Legionella and are then proceeded to be tested: this is to test how well ISO 11731:2017 method can recover Legionella if there are much more Legionella-inhibited bacteria present in the sample in comparison to ISO 11731:1998. For experiment B, only ISO 11731:2017 is tested and this study uses three samples with ten replicates for each sample. The nature of the samples is certified reference material called *Legionella pneumophila* CRM12821M with different concentration of legionella, see appendix E for more information of reference material used in this study.

As mentioned earlier, this study uses GVPC agar due to its improved properties of detection and selectivity for isolating of L. *pneumophila*. See appendix B for batch number of purchased GVPC agar plates used in this study. Sterile test tubes, heated water bath of 50ºC, dilute and acid solution is prepared in accordance with ISO 11731. See appendix C and D on how dilute and acid solution was prepared respectively. Apparatus as well as glassware are handled and sterilized in accordance with ISO 8199. Identification and numeration of Legionella bacteria colonies on GVPC plates is performed manually through a microscope. This is handled by an examinator from the microbiology department at SGS Analytics Sweden AB in Karlstad that has more than five years of experience of identifying and numerating Legionella colonies on GVPC plates.

3.1 Experiment A

For ISO 11731:1998, 5 ml of test sample is put in one test tube, placed in the water bath of 50ºC for 30 minutes, lets it rest until it reaches room temperature before adding 5 ml of acid solution and lets it be for 5±0,5 min. After homogenizing, 0,2 ml of the solution is direct plated to a GVPC agar plate. This batch corresponds to a dilution -1 (as in 10 times less in reference to 1 ml). Then 2 ml is taken from the test tube and is diluted with 8 ml of dilute solution in a separated test tube, mixed, and then 0,1 ml is direct plated on a GVPC agar plate. This batch corresponds to a dilution of -2 (as in 100 times less in reference to 1 ml). This procedure is repeated in all test samples. To prepare for ISO 11731:2017 for experiment A, 2 ml of test sample was added in one test tube. The test tube was placed in water bath of 50ºC for 30 min, lets it rest until the sample reaches room temperature before direct plating of 0,1 ml on a GVPC agar plate with only heat treatment resulting a dilution of -1. Second test tube is added with 1 ml of sample and 9 ml of acid solution, lets it be for 5±0,5min, homogenizing,

then 0,1 ml is direct plated to a GVPC agar plate with only acid solution treatment resulting to a dilution of -2. Lastly, 0,1 ml of untreated material is taken from test sample and is direct plated on a GVPC agar plate which gives a result of -1 dilution. This procedure is repeated for all test samples. Again, all agar plates from ISO 11731:2017 corresponds to a dilution of -1 except for the plates with acid solution treatment which corresponds to a dilution of -2.

3.2 Experiment B

In experiment B three certified reference material with different concentration of L. *pneumophila* called sample A, B and C are used. For ISO 11731:2017, the procedure is the same from experiment A, however ten replicates are produced in each sample of A, B and C. The GVPC plates will be proceeded with two readings, first reading on day five and second reading on day eight. Every colony that represents L. *pneumophila* will be counted and then presented in a table of results. For colonies that appear dubious to L. *pneumophila* during the first reading will also be presented in the table and a "(B)" comment is included. These colonies are tested for confirmation using blood agar plate. If these bacteria colonies that are tested for Legionella have bacteria growth on blood agar plates, it is assessed as negative confirmation since Legionella are one of the very few bacteria that do not cultivate on blood [10]. These blood agar plates are read at the same time during the second reading of the GVPC plates. Agar plates with more than 200 colonies of Legionella will not be counted and is presented as >200 colonies. Agar plates with overgrowth of bacteria that have different characteristics than what is typical for Legionella and/or have negative confirmation on blood agar plate are presented as "degrowth" and do not represent Legionella bacteria. If more than one type of Legionella is found on agar plate, the colonies will be counted individually for each type. If Legionella colonies have fluorescence, they are most likely made from L. *pneumophila* species and "fluoresce" comment will be included.

4. Results

4.1 Results from experiment A

Table 1. First reading from experiment A using ISO 11731:2017

Amount of cfu expected to be found (cfu/0,1 ml)	Type **of sample**	**Heat treatment (cfu/0,1 ml)**	**Acid Treatment (cfu/0,1 ml)**	**Untreated (cfu/0,1 ml)**
20-40	***Jacuzzi (natural)***	0	0	1
~50	***Doggy (natural)***	3+33 (two types)	10+10+degrowth	1+1+degrowth (two types)
~1-10	***Kitchen (natural)***	0	0	0
0-10	***Natural 1 (natural)***	0	0	2
0-10	***Natural 2 (natural)***	0	0	2 (B)
0-10	***Natural 3 (natural)***	degrowth	degrowth	6(B)
19-49	***L. pneum 1 (spiked)***	7	50	24
~55	***L. pneum species (spiked)***	50	10(fluoresce)	8(fluoresce)
~63	***L. pneum 2-14 (spiked)***	31	50	46
~53-63	***L. pneum mix (spiked)***	30(fluoresce)+18 (two types)	10(fluoresce)+50 (two types)	9(fluoresce)+34 (two types)
19-49	***L. pneum 1 (spiked with inhibited bacteria)***	>200 (B)	50	Degrowth
~55	***L. pneum species (spiked with inhibited bacteria)***	4(B)+68(B) (Both types are dubious)	0	Degrowth
~63	***L. pneum 2-14 (spiked with inhibited bacteria)***	145 (B)	0	Degrowth
~53-63	***L. pneum mix (spiked with inhibited bacteria)***	143 (B)	30	Degrowth

Table 2. Second reading from experiment A using ISO 11731:2017

Amount of cfu expected to be found	Type **of sample**	**Heat treatment (cfu/0,1 ml)**	**Acid Treatment (cfu/0,1 ml)**	**Untreated (cfu/0,1 ml)**
20-40	***Jacuzzi (natural)***	0	0	1
~30	***Doggy (natural)***	3+45 (two types)	10+10	18+3+degrowth (two types)
~1-10	***Kitchen (natural)***	0	0	0
0-10	***Epoc Drilling 1 (natural)***	0	0	0 (Blood agar confirmed no legionella)
0-10	***Natural 2 (natural)***	0	0	0 (degrowth)
0-10	***Natural 3 (natural)***	degrowth	degrowth	0 (degrowth)
19-49	***L. pneum 1 (spiked)***	8	60	24
~55	***L. pneum species (spiked)***	51	10(fluoresce)+50	10(fluoresce)
~63	***L. pneum 2-14 (spiked)***	31	70	49
~53-63	***L. pneum mix (spiked)***	31(fluoresce)+23 (two types)	10(fluoresce)+50 (two types)	10(fluoresce)+34 (two types)
19-49	***L. pneum 1 (spiked with inhibited bacteria)***	Degrowth (Blood agar confirmed no legionella)	Degrowth (Blood agar confirmed no legionella)	Degrowth
~55	***L. pneum species (spiked with inhibited bacteria)***	Degrowth (Blood agar confirmed no legionella)	Degrowth	Degrowth
~63	***L. pneum 2-14 (spiked with inhibited bacteria)***	Degrowth (Blood agar confirmed no legionella)	Degrowth	Degrowth
~53-63	***L. pneum mix (spiked with inhibited bacteria)***	Degrowth (Blood agar confirmed no legionella)	30	Degrowth

Table 3. First reading from experiment A using ISO 11731:1998

Amount of cfu expected to be found	Type **of sample**	**Heat and acid treatment (cfu/0,1 ml)**	**Heat and acid treatment (cfu/0,01 ml)**
20-40	***Jacuzzi (natural)***	3	0
~30	***Doggy (natural)***	3+52 (Two types)	5
~1-10	***Kitchen (natural)***	2	0
0-10	***Natural 1 (natural)***	0	0
0-10	***Natural 2 (natural)***	0	0
0-10	***Natural 3 (natural)***	Degrowth	0
19-49	***L. pneum 1 (spiked)***	3	1
~55	***L. pneum species (spiked)***	3	0
~63	***L. pneum 2-14 (spiked)***	29	1
~53-63	***L. pneum mix (spiked)***	6(fluoresce)+6 (Two types)	0
19-49	***L. pneum 1 (spiked with inhibited bacteria)***	3 (B)	0
~55	***L. pneum species (spiked with inhibited bacteria)***	0	0
~63	***L. pneum 2-14 (spiked with inhibited bacteria)***	2(B)+25(B)	0
~53-63	***L. pneum mix (spiked with inhibited bacteria)***	1(Fluoresce)+1	4 (B)

Table 4. Second reading from experiment A using ISO 11731:1998

Amount of cfu expected to be found	**Type of sample**	**Heat and acid treatment (cfu/0,1 ml)**	**Heat and acid treatment (cfu/0,01 ml)**
20-40	***Jacuzzi (natural)***	3	0
~30	***Doggy (natural)***	3+54 (Two types)	5
~1-10	***Kitchen (natural)***	2	0
0-10	***Natural 1 (natural)***	0	0
0-10	***Natural 2 (natural)***	0	0
0-10	***Natural 3 (natural)***	Degrowth	Degrowth
19-49	***L. pneum 1 (spiked)***	4	1
~55	***L. pneum species (spiked)***	3	0
~63	***L. pneum 2-14 (spiked)***	34	2
~53-63	***L. pneum mix (spiked)***	6(fluoresce)+8 (Two types)	0
19-49	***L. pneum 1 (spiked with inhibited bacteria)***	3 Degrowth (Blood agar confirmed no legionella)	0
~55	***L. pneum species (spiked with inhibited bacteria)***	Degrowth	0
~63	***L. pneum 2-14 (spiked with inhibited bacteria)***	Degrowth (Blood agar confirmed no legionella)	0
~53-63	***L. pneum mix (spiked with inhibited bacteria)***	1(Fluoresce) +1+degrowth	Degrowth (Blood agar confirmed no legionella)

Table 5. Final results from recovering Legionella using both ISO 11731:2017 and 11731:1998 presented in cfu/100 ml as standardized. If no Legionella colonies was found, results are presented as <1000 cfu/100 ml since detection level starts at 1000 cfu/100 ml if one colony is found on agar plate with 0,1 ml material used. If there were too much degrowth on agar plate so that Legionella was inhibited to cultivate due to too high concentration of interfering microorganisms, the result is presented as IC, inconclusive.

Circa Amount of cfu expected to be found (cfu/100 ml)	**Type of sample**	**Colonies recovered using 11731:2017 (cfu/100 ml)**	**Colonies recovered using 11731:1998 (cfu/100 ml)**
~2000-4000	***Jacuzzi***	1000	3000
~50 000	***Doggy***	48000	57000
~1-10 000	***Kitchen***	<1000	2000
0	***Natural 1***	<1000	<1000
0	***Natural 2***	<1000	<1000
0	***Natural 3***	IC	IC
~19000-49 000	***L. pneum 1***	60000	10000
~55 000	***L. pneum spp***	60000	3000
~63 000	***L. pneum 2-14***	40000	34000
~53-63 000	***L. pneum mix***	56000	14000
IC	***L. pneum 1 Inhibited***	IC	<1000
IC	***L. pneum spp Inhibited***	IC	IC
IC	***L. pneum 2-14 Inhibited***	IC	IC
IC	***L. pneum mix Inhibited***	30000	2000

Natural samples “Jacuzzi”, “Doggy” and “Kitchen” shows that 11731:1998 method recovers more Legionella colonies compared to using 11731:2017 method. Unfortunately, natural sample “Natural” 1-3 did not contain any Legionella, despite how historical results showed otherwise. However, every spiked sample “L. *pneumophila* serotype 1”, “L. *pneumophila* serotype 2-14”, “L. *pneumophila* species” and “L. *pneumophila* mix” presents results where colonies recovered using 11731:2017 shows to be more efficient compared to using 11731:1998 method. Furthermore, their results of colonies found is significantly closer to the actual colonies that can be found than the method using 11731:1998.

Results from samples that were spiked with Legionella-inhibited bacteria displays the difficulty to recover Legionella using both the current design of method and its precedent. Results from using 11731:2017 contained too much of non-target bacteria on agar plates

preventing Legionella to cultivate, which is especially displayed in sample "L. *pneumophila* 1 Inhibited", "L. *pneumophila* 2-14 Inhibited" and "L. *pneumophila* species Inhibited".

4.2 Results from experiment B

Table 6. Final reading from experiment B in sample A tested using ISO 11731:2017

	Acid Treatment (cfu/0,1 ml)	**Heat treatment (cfu/0,1 ml)**	**Untreated (cfu/0,1 ml)**
	130	36	103
	70	36	125
	90	39	88
	140	54	102
	50	34	119
	120	33	98
	110	23	106
	50	49	88
	140	48	80
	100	56	107
Mean	**100**	**40,8**	**101,6**
Standard Deviation	**34,32**	**10,53**	**13,95**
Variance	**1177,78**	**110,84**	**194,49**

Table 7. Final reading from experiment B in sample B tested using ISO 11731:2017

	Acid Treatment (cfu/0,1 ml)	**Heat treatment (cfu/0,1 ml)**	**Untreated (cfu/0,1 ml)**
	40	19	50
	50	14	70
	90	18	58
	30	25	59
	20	20	63
	40	24	57
	10	24	66
	90	18	55
	80	22	56
	10	24	30
Mean	**46**	**20,8**	**56,4**
Standard Deviation	**30,98**	**3,58**	**10,35**
Variance	**960**	**12,84**	**118,93**

Table 8. Final reading from experiment B in sample C tested using ISO 11731:2017

	Acid Treatment (cfu/0,1 ml)	**Heat treatment (cfu/0,1 ml)**	**Untreated (cfu/0,1 ml)**
	60	24	61
	40	27	66
	30	30	67
	60	28	63
	40	29	54
	40	30	73
	20	29	63
	70	32	66
	30	35	60
	30	21	59
Mean	**42**	**28,5**	**63,2**
Standard Deviation	**16,19**	**3,92**	**5,2**
Variance	**262,22**	**15,39**	**27,07**

Table 9. Results from reference material tested using ISO 11731:1998

Sample reference	Colonies recovered (cfu/0,1 ml)
	137
	97
	97
	85
	86
	167
	125
	72
	143
	86
Mean	**109,5**
Standard Deviation	**31,36**
Variance	**983,17**

According to ISO 13843:2017 Requirements of establishing performance characteristics of quantitative microbiological methods, over-dispersion is determined first by calculating the observed value and then compare it to the critical 0,05 probability distribution. According to Chi square distribution, the critical 0,05 probability value for (10-1) degrees of freedom is 16,919. If the observed value is greater than the critical 0,05 probability value, there is a significant over-dispersion detected [17]. Performance data range is calculated by dividing the

difference of highest and lowest value by the average and then multiply it by 100. These data will then be compared to the accepted performance data stated by ISO 11731.

Table 10. Calculated Observed value and in sample A

Sample A	Arithmetic mean	Variance	Observed value	Performance data range (%)
Acid treatment	100	1177,78	106	80,0
Heat treatment	40,8	110,84	24,45	80,1
Untreated	101,6	194,49	17,23	44,3

Table 11. Calculated Observed value and relative operational variance in sample B

Sample B	Arithmetic mean	Variance	Observed value	Performance data range (%)
Acid treatment	46	960	187,83	173,9
Heat treatment	20,8	12,84	5,56	52,9
Untreated	56,4	118,93	18,98	70,9

Table 12. Calculated Observed value and relative operational variance in sample C

Sample C	Arithmetic mean	Variance	Observed value	Performance data range (%)
Acid treatment	42	262,22	56,19	95,3
Heat treatment	3,92	15,39	4,86	49,1
Untreated	5,2	27,06	3,85	30,0

Table 13. Calculated Observed value and relative operational variance in reference sample using 11731:1998

Sample reference	Arithmetic mean	Variance	Observed value	Performance data range (%)
	109,5	983,17	80,81	74,9

Results from experiment B shows that the observed value from every sample that has been treated with acid solution is much greater than critical 0,05 probability value of 16,919 and consequently has significant over-dispersion. However, samples that has been treated with heat or untreated samples show results that are either close or lower than the critical 0,05 probability value, therefore only a slight or no significant over-dispersion is detected in these types of series of repeated measurement. Results from reference sample shows, as every sample with acid solution treatment, have an observed value that greatly exceeds the critical 0,05 probability value. This will be further discussed in the next chapter of *Discussion.*

5. Discussion

5.1 Discussion concerning results from experiment A

As stated earlier, natural samples "Jacuzzi," "Doggy" and "Kitchen" shows that 11731:1998 method recovers more Legionella colonies compared to using 11731:2017 method. Thus, it is conceivable to argue that the former design of method may be more efficient, however, it is essential to indicate Legionella colonies recovered was much lower to the actual amount of Legionella in the sample. According to table 5, the expected cfu/100 ml to be found was 2-40000, 50000 and 1-10000 in "Jacuzzi", "Doggy" and "Kitchen" samples respectively. Using 11731:2017 method only 1000, 48 000 and <1000 respectively cfu/100 ml was recovered. Whereas in using 11731:1998 method 2000, 57 000 and 2000 respectively cfu/100 ml was recovered One explanation to this is that natural samples tested in this study may not represent standardized natural samples. According to the guidelines stated by ISO 19458: samples can be stored at maximum 72 hours before testing. Moreover, supervisor at SGS analytics Sweden AB assessed that two weeks of storage time should not significantly affect the concentration of Legionella in sample since the bacteria is very tenacious. However, "Jacuzzi" sample was stored for three weeks before tested for this study which gravely crosses the guidelines on storage of sampling. Unfortunately, the verification plan was delayed while reviewing and must be approved before any experimental testing can be initiated, that ended with further extended storage time to this sample. When the initial analysis was made at SGS, more than 200 colonies was recovered from testing 0,1 ml of sample using the 11731:1998 method and the final readings of the results that follows was >200000cfu/100 ml. But since the storage time exceeds of what is accepted by the time this study assessed the sample: it is possible Legionella bacteria in sample has deceased during this period. This makes it more challenging to determine the actual amount of Legionella in "Jacuzzi" sample and drawing any conclusions based on these results is therefore discarded.

Another sample that is arguably unreliable is "Kitchen": when the initial analysis was made at SGS, only 46 colonies was recovered when 100 ml of sample was tested for water with low background. This implicates that the content of Legionella and interfering microorganisms was too low when it was tested for water with high background and thus the validity of the results from this sample is questionable. Unfortunately, there was a lack of samples with high background containing Legionella to be found at SGS during this period and this sample, as

well as “Jacuzzi” sample, was included in the experiment for this study to tackle this issue. It is however commendable that the former design of method 11731:1998 was able to isolate Legionella, with 2 cfu/0,1 ml, despite the risk that the concentration of Legionella in sample was too low. This indicates 11731:1998 may be more effective than 11731:2017 for samples with low concentration of Legionella and interfering microorganisms. Though, it is advisable that further studies should be conducted before drawing any conclusions.

“Doggy” sample did however have appropriate amount of Legionella to be tested, and as the results shows, 11731:1998 method with 57 000 cfu/100 ml recovered more Legionella colonies than 11731:2017 method with 48 000 cfu/100 ml as well as the expected amount cfu to be found which was circa 50 000 cfu/100 ml. This indicates that the former design of method is more effective at isolating Legionella since it is desirable to recover fundamentally all Legionella bacteria that can be found in sample. One explanation to this is that initially, there may have been a high concentration of degrowth in “Doggy” sample and subjecting to only one treatment in 11731:2017 to reduce degrowth could be insufficient, preventing Legionella to cultivate on agar more effectively. In 11731:1998 where the sample is treated with both heat and acid solution, reduces degrowth sufficiently enough for Legionella bacteria to thrive on agar more efficiently. However, results from every subsample of 11731:2017 shows there are precisely two serotypes of Legionella in sample. This indicates that 11731:2017 can give a stronger statement of possible different serotypes that can be found when every subsample presents result that is the same. 11731:1998 could only present in one subsample that there were two serotypes of Legionella, conveying a lesser reliable result of possible different serotypes.

Results from both methods shows that Natural samples did not contain any Legionella bacteria. Despite their previous samples taken from the same sampling point was confirmed positive for Legionella, these set of samples tested for this study did not. Unfortunately, time became a limiting factor when these samples were appointed for this study and could have been avoided if the initial results from SGS test analysis were received ahead. However, results show that Natural 3 sample did contain high concentration of degrowth bacteria. So, despite experiencing both heat and acid solution treatment in 11731:1998, maximizing the reduction of degrowth, degrowth bacteria still flourished in first and second reading. Thus, the

old design presents more reliable results that the samples may not contain Legionella compared to results by 11731:2017 method. Since samples were only subjected to one treatment: degrowth bacteria were not maximal reduced. This makes it is more difficult to evaluate the results if there are no Legionella colonies recovered. Can it be as a cause of being inhibited by abundance of degrowth still in sample, or because sample truly did not contain any Legionella? Therefore, results from Natural 3 indicates that in cases where samples have high concentration of degrowth bacteria and no Legionella, 11731:1998 can produce more dependable results that are easier to evaluate compared to 11731:2017 method.

It is clear in results that spiked samples, 11731:2017 method was more effective in recovering Legionella compared to 11731:1998. In samples Legionella type 1, type spp, type 2-14 and type mix had 60000, 60000, 40000 and 56000 cfu/100 ml respectively colonies recovered using 11731:2017 method. In 11731:1998 method only 10000, 3000, 34000 and 14000 cfu/100 ml respectively was recovered, when the expected amount was 19-49000, 55000, 63000 and 53-63000 cfu/100 ml respectively. One theory that explains this phenomenon is that there were not enough of interfering microorganisms in these spiked sample since not a single degrowth colony was detected on the agar plates whose subsample was untreated, despite that the water sample was categorized as water with high background at SGS Analytics Sweden in Karlstad. If the concentration of interfering microorganisms were low, not much of microorganisms needed to be reduced under heat or acid solution treatment and Legionella bacteria could cultivate on agar more successfully. This is displayed especially in results from Legionella species who are known to be a type that are more sensitive, as the results shows, cultivated more successfully when the sample underwent only one or no treatment. However, it is important to discuss the probability of natural samples with similar character as these spiked samples. It is often water who are classified as water with high background normally have a high concentration of microorganisms and particles e.g., industrial water. It is highly debatable if method 11731:2017 will display equivalent results as this study presents if natural samples in fact have high concentration of interfering microorganisms. Therefore, drawing any conclusions based on these results is greatly discouraged.

As mentioned earlier, results from samples that were spiked with Legionella-inhibited bacteria was difficult to analyse since there were overgrowth of degrowth bacteria cultivated on agar plate already during the first reading assessed by 11731:2017 method. Therefore, it is important to address this difficulty to assess samples with similar components, especially when this method is designed to analyse water with high background. Subjecting the samples with only one type of treatment (heat or acid) may not be sufficient to reduce the concentration of non-target bacteria. This subsequently makes it more challenging to recover Legionella from the sample and therefore more difficult to evaluate (much like Natural 3). Results from 11731:1998 did not display such nature but only several degrowth colonies was detected indicating reduction of non-target bacteria occurred at maximum. Thus, the likelihood of Legionella being inhibited by non-target bacteria to cultivate on agar is less when no Legionella colony was formed.

Surprisingly, in sample "L. *pneumophila* mix Inhibited" did manage to recover three Legionella colonies of one type on agar plate with acid treatment during the first reading. However, notify that the same sample that was subjected to both heat and acid treatment managed to recover both two types also during the first reading, where one of them fluoresce. Legionella types that fluoresce who are known to be more sensitive and consequently more challenging to recover from sample [10], was still recovered using 11731:1998. This suggests these types of L. *pneumophila* have higher likelihood of recovering when sufficient removal of concentration of non-target bacteria occurs and therefore more appropriate to use 11731:1998 method.

5.2 Discussion concerning results from experiment B

As mentioned earlier, the observed values from results that has undergone acid solution treatment with 106, 187,8 and 56,2 from sample A, B and C respectively all exceed greatly the critical 0,05 probability value of 16,9 and consequently has significant over-dispersion. One explanation to this occurrence could be that since such small amount is analysed from sample using acid solution treatment (0,01 ml), a small error in volume follows a great inaccuracy in results. For instance, if dilution step was not properly homogenized and 0,011 ml of sample is analysed instead of 0,01 ml, there is already a 10% error in volume which gives significant error in results. Hence, acid solution treatment is very sensitive to amount of

volume analysed which increases the risk of over-dispersion as displayed in the results from sample A, B and C. However, results from heat treatment and untreated have observed values that are slightly above, slightly under or significantly under the critical 0,05 probability value. Therefore, a slight over-dispersion or insignificant over-dispersion respectively was detected in these types of series of repeated measurement.

In comparison to the observed value by reference sample assessed using ISO 11731:1998 with 80,8 where it also significantly exceeds the critical value 0,05 probability value of 16,9 suggests repeatability of performance data agrees more strongly with 11731:2017 method. However, it is important to point out the data from reference sample assessed by SGS Analytics Sweden in Karlstad do not meet the requirements to acknowledge that the data is derived from repeatable conditions stated by ISO 13843 but are derived from reproducible conditions. According to ISO 13841, repeatable conditions are attained when the data that consist of ten replicates are from the same sample, analysed by the same technician on the same day at approximate the same time. Data that are assessed by SGS were collected in reproducibility conditions where the set of replicates are not from the same reference sample, were not assessed by the same technician and were not analysed at the same day but all data were tested on different days. Thus, calculated observed value from reference sample using ISO 11731:1998 is disputable and comparing dispersion with 11731:2017 that meets the requirements of repeatable conditions will be inaccurate. Unfortunately, verification plan set by SGS did not include to assess repeatability of 11731:1998 since the method is already accredited by Swedac. And so, no set of data to test repeatability of 11731:1998 was formed in this study. One suggestion for future studies is to conduct an interlaboratory study to examine the reproducibility of ISO 11731:2017 at the same laboratory at SGS. The dispersion can then be evaluated in reproducibility conditions to see if it is improved or declined in 11731:2017 method compared to 11731:1998.

Due to change in acid treatment in the latest design of method, interlaboratory study was carried out by CEN to determine new parameters of performance characteristics for ISO 11731:2017, one of which includes performance data range. The interlaboratory study shows that precision data concerning sample with high concentration of interfering microorganisms who is direct plated after dilution, had a performance data range at 78,5% [10]. Performance

data range presented in this study from sample A and C whose range was at 80,0% and 95,3% respectively, closely meets performance data range of interlaboratory study. However, acid treatment data from sample B who have performance data range at 173,9% which significantly exceeds the performance data range presented by CEN. One explanation to this is, as mentioned earlier, the acid solution treatment is an overly sensitive pre-treatment step where small error in amount of volume analysed due to insufficient homogenized diluted sample, can produce great error in result. In sample B with performance data range that is considerably greater than what CEN presented, suggests that the sample was not properly homogenized during the dilution process. This will generate a more widespread data points and therefore a greater performance data range. However, such error of human factor is common, and the risk of such misstep increases as dilution series increases. Based on these grounds, it is therefore important to acknowledge the increased vulnerability in 11731:2017 acid solution treatment to human error when an additional dilution step is included.

6. Conclusion

Results from natural samples shows that method 11731:1998 recovers Legionella more efficiently compared to 11731:2017. However, the natural samples in this study may not represent standardized natural samples when they were tested in this study. One sample was stored for three weeks before tested which greatly exceeds the accepted storage time stated by ISO 19458. Another natural sample was unsuitable for this study due to its low concentration of Legionella and interfering microorganisms. Additional three natural samples did not even contain any Legionella. Hence, these flaws in test samples consequently makes it challenging to draw any conclusions based on their results.

Results from natural sample “Doggy” shows that 11731:1998 method recovers more Legionella colonies than 11731:2017 as well as the feasible amount cfu that could be found. Although, every subsample by 11731:2017 method was able to recover two serotypes of Legionella, indicating strongly that the sample contained precisely two serotypes. 11731:1998 could only isolate two serotypes in one subsample which presents a weaker statement of possible number of serotypes to be found in sample. Hence, both methods show advantages and disadvantages where 11731:1998 is potentially more efficient at isolating amount of Legionella, whereas 11731:2017 is more appropriate to adapt when there are potential

different serotypes of Legionella in sample. It is however important to point out that such samples are impossible to predict and therefore much more challenging to determine when 11731:2017 method is more suitable.

Results from natural samples “Natural” 1-3 shows that they did not contain any Legionella, but only degrowth bacteria was detected from sample Natural 3. Since 11731:2017 subjects the sample with only one treatment, reduction of degrowth is poor and the remaining interfering microorganisms will inhibit Legionella to grow on agar if there is in fact Legionella in sample. Hence, it is more difficult to assess results from 11731:2017 when reduction of degrowth is insufficient. Whereas 11731:1998 who subjects the sample with both heat and acid solution treatment, maximizes the reduction of interfering microorganisms. If no Legionella is recovered: possibility of Legionella being inhibited to cultivate on agar is less and therefore easer to evaluate. Much like results from samples containing Legionella and Legionella-inhibited bacteria. It was difficult to analyse since too much degrowth was cultivated on these agar plates, so no Legionella colony was isolated. This is especially directed at results assessed by 11731:2017 when non-target bacteria were not sufficiently reduced. This consequently makes it more challenging for Legionella to cultivate on agar and therefore more difficult to evaluate, like results from Natural 3.

Results from every spiked sample present result that shows 11731:2017 to be more efficient in recovering Legionella compared to 11731:1998. However, no degrowth bacteria was detected on these agar plates. This indicates that the concentration of interfering microorganisms in these spiked samples may be too low since it is expected that degrowth bacteria should be detected on untreated subsamples if water with high background is tested. Thus, these samples do not accurately represent water samples with high background if no degrowth bacteria is detected in untreated subsamples. Since no degrowth bacteria was detected, it is questionable if 11731:2017 will display equivalent results in samples that in fact do have high background and drawing any conclusions based on these results should be discarded.

In ISO 11731-2017 with subsample that has undergone acid solution treatment produced results that has significant over-dispersion during assessment in repeatability conditions.

Performance data range was also evaluated in experiment B where sample B acid solution treatment significantly exceeds the accepted parameters of performance data range. In acid solution treatment where such small volume is analysed, it will be more sensitive if there is a small error in volume analysed which follows a great inaccuracy in results, risking of producing results with significant over-dispersion. Moreover, when another dilution step is added in 11731-2017 method during acid solution treatment, it increases the risk of human error of insufficient homogenized sample and will therefore generate a greater performance data range, as displayed in results. It is thus important to acknowledge the increased vulnerability in 11731:2017 acid solution treatment to human error.

In conclusion, based on results assessed in this study: the most efficient method to use is heavily based on the nature of the sample. If water with high concentration of interfering microorganisms is assessed, it will be more suitable to apply ISO 11731:1998. Since this method can maximize the reduction of interfering microorganisms: it will facilitate Legionella to cultivate on agar. In ISO 11731:2017 where subsample is subjected to only one treatment, it imposes a risk of insufficient reduction of interfering microorganisms to occur and thus will be more challenging to evaluate the results. However, results assessed in this study indicates that 11731:2017 can give a stronger statement of possible different serotypes found when every subsample presents result that is the same. Although ISO 11731:2017 in acid solution treatment where an additional dilution step is added: it increases the risk of human error and therefore a greater vulnerability to the method.

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8. Appendix

Appendix A: Verifieringsplan för Legionella I vatten med hög bakrundsflora (sötvatten/dricksvatten)

Verifieringsplan för Legionella i vatten med hög bakgrundsflora (sötvatten/dricksvatten)

Normativa referenser

Verifieringsplanen och dess omfattning sker efter den interna rutinen *GMR 37 Verifiering av mikrobiologiska analysmetoder* och SS-EN ISO 13843:2017.

Ny version av standardmetod för analys av legionella i kyltornsvatten ska verifieras i referens till *Svensk Standard SS-EN ISO 11731:2017 – Vattenundersökningar - Kvantifiering av Legionella*.

Förändringens omfattning

Analysmetod för undersökning av legionella ska ändras enligt nya SS-EN ISO 11731:2017 Svensk Standard då den nuvarande analysmetod som används är refererad enligt en tidigare version; ISO 11731:1998 samt SS-EN ISO 11731-2 2008.

Under denna verifieringsprocess kommer direkt rackling på agar platta som idag syra och värmebehandlas vid analys av vatten med hög bakrundflora att ändras enligt Annex J Figur J.1 – Decision matrix (SS-EN ISO 11731:2017). Förändringen omfattar bland annat att ändra förhållandet vid syrebehandling av prov från 1:2 till 1:10 mellan prov och syrabuffert. Andra förändringar som kommer verifieras är;

- en GVPC platta kommer racklas med 0,1 ml värmebehandlat prov vilket resulterar -1 spädning,
- en GVPC platta kommer racklas med 0,1 ml syrabehandlat prov med 1:10 spädning vilket resulterar -2 spädning,
- och en GVPC platta kommer racklas med 0,1 ml obehandlat prov vilket resulterar -1 spädning.

I jämförelse med nuvarande analysmetoden då provet genomgår både med syra och värmebehandling innan rackling på GVPC med både -1 samt -2 spädning. Vi väljer att behålla membranfiltrering med syrabehandling som den är idag, vilket också är godkänt förfarande för vatten med hög bakrundsflora enligt SS-EN ISO 11731:2017. Resultatet kommer rapporteras i cfu/L i stället för nuvarande cfu/100 ml.

Planering

Verifieringen planeras att göras inom två veckor under september/oktober 2022 med en vecka för laborativt arbete och en vecka avsedd för avläsning, beräkning samt slutsats av resultat.
En jämförelse ska göras mellan den nuvarande metoden och den nya standarden. Minst fem olika provmaterial som är antingen naturligt kontaminerade (riktiga prover), dricksvatten som är spetsade med ytvatten eller slam eller certifierat referensmaterial ska analyseras med ett försök per prov (inga replikat). Halten ska ligga på 20–200 cfu per 90mm agar platta.
För repeterbarhet kommer försöket att utföras på tre olika provmaterial som är naturligt kontaminerade, med känd halt på 20–200 cfu, med 10 försök per prov. Detta görs under vecka ett och under vecka två sker avläsning av proven.
Mätosäkerheten kommer beräknas enligt SS-EN 13843:2017 och *GMR 37 Verifiering av mikrobiologiska analysmetoder* under vecka två på totalt 30 plattor som är rekommenderat enligt SS-EN 13843:2017. Endast ett laboratorium, SGS Analytics Sweden AB i Karlstad, kommer att beröras. SGS Analytics Sweden AB i Karlstad kommer även vara det enda laboratorium som ansöker ackreditering för den nya standardmetoden.

Risker och möjligheter

En risk som den nya standardmetoden kan komma att medföra är en ökning av mänskliga misstag genom förväxling. En odlingsplatta för varje syrabehandling, värmebehandling och icke-behandlat prov kommer att ansättas vilket resulterar att det finns risk för laboranten att förväxla odlingsplatta med fel behandling. Risken planeras att minimeras genom att plattor kommer vara uppdelade efter enskild behandling och märkta med etiketter från LIMS enligt sin respektive behandling (syra/värme/obehandlat). I jämförelse med den nuvarande standardmetoden där syra- och värmebehandlingarna kombineras så är denna risk mindre.
Möjligheten med den nya standardmetoden är genom den nya spädningen 1:10 kunna bibehålla låg pH nivå under syrabehandling av provet, oavsett provets initiala pH.
En risk med att använda en kombination av syra och värmebehandling på ett prov ger ett lägre resultat. Denna risk kan minskas genom en del av provet syrabehandlas, en del värmebehandlas och en del lämnas obehandlad för de legionella arter som är mer känsliga för syra och värme.

Referensstammar/referensvialer

Legionella pneumophila serogrupp 1 (CRM12821M) kommer att användas

Aktuella provtyper/ provarter

Denna förändring beror endast provtypen legionella med provart dricksvatten och sötvatten (vatten med hög bakrundsflora/kyltornsvatten).

Bestämning av riktighet (endast vid kvantitativ analys)

Test: En jämförelse ska göras mellan den nuvarande analysmetoden och den nya standarden. Detta görs med minst fem olika provmaterial som är antingen naturligt kontaminerade (riktiga prover), dricksvatten som är spetsade med ytvatten eller slam eller certifierat referensmaterial som ska analyseras med ett försök per prov. Halten ska ligga på 20–200 cfu per 90mm agar platta.
Krav: Provets art måste vara naturligt kontaminerat, dricksvatten som är spetsade med ytvatten eller slam eller certifierat referensmaterial med känd halt av 20–200 cfu per 90mm agar platta. Om spetsat prov används, bör det komma från minst två olika källor.

Bestämning av repeterbarhet och intern reproducerbarhet (endast vid kvantitativ analys)

Test: - För repeterbarhet kommer verifieringsförsöket att utföras på tre olika provmaterial som är naturligt kontaminerad (riktiga prover) med okänd mängd av aktuella bakterier. Provmaterialen kommer väljas ut med hänsyn till provers tidigare resultat. 10 försök kommer göras per prov.

För intern reproducerbarhet kommer minst två olika laboranter att analysera på minst 30 olika prover som är naturligt kontaminerad enligt SS-EN ISO 13843:2017 delkapitel 6.4.3 Intralaboratory reproducibility. Proven kommer ansättas under olika tillfällen och sedan jämföras enligt SS-EN ISO 13843:2017 Table 6.
Krav: - Provets art måste vara naturligt kontaminerat, känd halt av 20–200 cfu per 90mm agar platta.

Bestämning av mätosäkerhet

Mätosäkerheten ska kontrolleras en gång under fyraårsperiod enligt rutin. Data kommer beräknas enligt Appendix 10J Beräkning av mätosäkerhet inom mikrobiologi -Vatten och beräknas enligt SS-EN ISO 13843:2017
Test:
Under försökts gång kommer 30 plattor med legionellaväxt räknas av flera laboranter (en gång per laborant) enligt SS-EN ISO 13843:2017.
Krav: - Det är rekommenderat att 30 plattor ska räknas två gånger av en enskild laborant eller en gång av flera laboranter enligt SS-EN ISO 13843:2017.

Kontrolldiagram och -listor

Referensmaterial Legionella *pneumophila* CRM12821M kommer att användas till kontrolldiagrammet som kommer upprättas enligt laboratoriets rutiner. Referensmaterial ansätts minst en gång i månaden och laboratoriet deltar i kompetensprövningar från PHE två till tre gånger per år. Kontrolldiagrammets gränser kommer initialt vara anpassade till den mätosäkerhet som bestäms enligt *GMR 37 Verifiering av mikrobiologiska analysmetoder,* avsnitt 5.2.4.

Specificitet samt interferenser och störningar

Tillväxt av legionella kan hämmas av andra störande mikroorganismer och kan därför bli en interferens under avläsning och kvantifiering vid resultatberäkning.

Avfallshantering och kemikalier

Inga nya material eller kemikalier introduceras under förändringen av standardmetoden så ingen miljörutin behöver uppdateras eller riskbedömning som behöver genomföras.

Appendix B Batch number of GVPC agar plates

Appendix C Preparation of acid solution

Stock solutions

16,4 ml of concentrated HCl is mixed with 1000 ml of MilliQ-water give 0.2M HCl

14,9 g of KCl is dissolved in 1000 ml of MilliQ-water give 0.2M KCl

56 g KOH is dissolved in 1000 ml distilled water give 1M KOH

All stock solutions are autoclaved, 121±3 ºC in 15±1min

When all solutions are autoclaved, 39 ml of 0,2M HCl and 250 ml of 0,2M KCl are mixed in a beaker with magnet stirrer. Adjust the pH to 2,2±0,2 using 1M KOH solution. Store the acid solution in a dark glass bottle in room temperature.

Appendix D Preparation of diluent

8,5g of sodium chloride is dissolved in 1000 ml of distilled water. Adjust the pH to 7,0±0,2 using NaOH or HCl. Autoclave in 121±3 ºC for 15±1min and then store in glass bottle in room temperature.

Certificate Page 1 of 5 Certificate version 01

Certificate of Analysis – LENTICULE® discs (microbiological CRM)

Legionella pneumophila CRM12821M

Product no.:	**CRM12821M**
Lot no.:	**BCCF9874**
Description of CRM:	Bacteria in a pure culture preserved in a tablet format (LENTICULE® discs)
Expiry date:	**SEP 2022**
Storage:	**-20 ± 5 °C**; store the mylar bag containing the plastic vials with the LENTICULE® discs unopened
Starting material:	**NCTC 12821 batch 6** (freeze-dried microorganism in a glass ampoule)

9.3E+03 cfu per disc	0.042	**3.5E+03 - 2.5E+04 cfu per disc**
Conditions:	**Legionella GVPC / aerobic / 36 °C / 6 days**	
Date of testing:	13 JUL 2021	
cfu: colony forming units The measurement of uncertainty (MoU) originates from the standard deviation (SD) resulting from the geometric mean value obtained during homogeneity testing. The expected range takes into account media batch to batch variability, which is done by multiplying within batch standard deviation by 1.6.		

Metrological traceability:	Details see "Certification process details" on page 2.
Measurement method:	The certified value is established by plate counting.
Intended, correct use & handling instructions:	Please follow the instructions given in "General instructions for intended uses of this reference material" on page 3.
Health and safety information:	Please refer to the Safety Data Sheet (link on page 3) for detailed information about the nature of any hazard and appropriate precautions to be taken.
Accreditation:	Sigma-Aldrich Production GmbH is accredited by the Swiss accreditation authority SAS as registered reference material producer SRMS 0001 in accordance with ISO 17034 [2] and registered testing laboratory STS 0490 according to ISO/IEC 17025 [1].
Certificate issue date:	**28 SEP 2021**