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DI URBINO
CARLO BO

Department of Biomolecular Sciences

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Development of a real-time PCR method for the detection and/or quantification of viable *Legionella* spp. and *L. pneumophila* in sanitary and thermal water samples

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Supervisor:

Prof. Mauro Magnani

Ph.D. student:

Dr. Veronica Ceppetelli

Co-Advisor:

Prof. Giulia Amagliani

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1. Introduction

The first reported cases of infection by bacteria of *Legionella* genus occurred as a severe outbreak in July 1976 at a Bellevue-Stratford Hotel hosting the 58th annual convention of the American Legion in Philadelphia, Pennsylvania. Two hundred twenty-one convention attendees were infected, and 34 died. The disease was found to be caused by the bacterium *Legionella pneumophila* belonging to the family Legionellaceae, which was isolated in Hotel's air conditioning system [Torrisi *et al*., 2012]. Subsequently, the atypical pneumonia caused by *L. pneumophila* was designated as Legionnaires' disease.

Legionella spp. is also responsible for a less severe form of infection named Pontiac fever. Since then, sporadic and epidemic cases have dramatically increased in industrialized countries and this can be attributed both to the improvement of diagnostic tools and to the increase in opportunities for exposure to the causative agent [Guidelines, 2015].

In 1983, the World Health Organization (WHO) established a National Legionellosis Registry and in 1986 in Europe, the European Working Group for *Legionella* Infections (EWGLI) was formed with the aim to identify cases of infection in travelers, detecting epidemic outbreaks and notifying the competent authorities of the countries involved. In 1987, the Group established a surveillance scheme (EWGLINET) for the detection of the cases in people who travelled and stayed in hotels and resorts. Since 2010, the international surveillance program has been coordinated by the European Center for Disease Prevention and Control (ECDC) and called the European Legionnaires' Disease Surveillance Network (ELDSNet) [WHO, 2007].

In 2017 the ECDC reported 8 624 confirmed cases of Legionnaires' disease in Europe with a 30% increase in the number of reported cases compared with 2016. *L. pneumophila* serogroup 1 was the most commonly identified species, responsible for 79% of culture-confirmed cases. Legionnaires' disease remains an important cause of potentially preventable morbidity and mortality in Europe and there is no indication of decreasing burden [ECDC, 2019].

Some people are at higher risk including people 50 years or older, smokers and heavy drinkers, those suffering from chronic respiratory or kidney disease, and people whose

immune system is impaired [Farnham *et al*., 2014]. Nosocomial Legionnaires' disease is an important problem as it has been estimated that 20–30% of legionellosis are nosocomial infections and that they are associated with a contamination of the health-care water networks [Tai *et al*., 2012]. The complexity of hospital's water systems and the vulnerability of hospitalized patients increase the risk for *Legionella* spp. transmission and severe outcomes. In hospitals in addition to the water system, health practices concerning the airways (e.g. ventilation, aspiration, devices for artificial respiration and dental tools) can increase the risk of infection [Montagna *et al*., 2018].

Legionellae naturally occur in environmental water sources and are well adapted to man-made water systems, are often found in water system of buildings, cooling towers, evaporative condensers, and dental unit waterlines [Bonetta *et al*., 2017].

Legionellae are difficult to control in their natural sources due to their resistance to disinfectants, their ability to associate with biofilm and parasitism in protozoa. The risk assessment for *Legionella* is particularly important for public health officials and managers responsible for maintenance of water distribution systems of industrial or public buildings [Whiley *et al.*, 2014]. Current risk assessment model is established on culture-based enumeration on selective media, that represents the reference method for *Legionella* control. Nevertheless, aside from the fact that this method requires up to 14 days for analysis, detection of *Legionella* from water samples is further confounded as the presence of disinfectants and other water treatment chemicals may render *Legionella* viable but not culturable (VBNC), leading to an unrealistically low number of visible colonies or false negatives, particularly in systems that are treated with monochloramine [Kirschner, 2016; Taylor *et al.*, 2014]. This aspect has an important implication for Public Health especially in health care settings where high-risk patients may be susceptible to low concentrations of *L. pneumophila* in water systems: VBNC cells can be responsible for sporadic infection and outbreaks as they are able to resuscitate and preserve the virulence characteristics [Marinelli *et al.*, 2017].

The only culture independent method that has achieved the status of a standard is quantitative real-time PCR (qPCR). This standard NF T90-471:2010 was firstly developed in France and then published as ISO/TS 12869 in 2012 [Anonymous, 2010; Anonymous, 2012]. The procedure is based on water sample filtration, DNA extraction followed by real-time PCR detection and/or quantification. DNA-based detection methods can elucidate the presence of *Legionella* spp. within few hours, with high sensitivity and specificity.

However, DNA detection can overestimate the risk of infection owing to the amplification of DNA deriving from dead cells.

A promising approach for a rapid detection of viable *Legionella* cells is viability-PCR, based on sample pre-treatment with photoactivable nucleic acid intercalating dyes such as Ethidium Monoazide (EMA), Propidium Monoazide (PMA) and commercial derivatives, such as PEMAX (a mix of photo-reactive azide forms of phenanthridium) prior to DNA extraction and PCR amplification [Thanh *et al.*, 2017]. EMA and PMA are conventional dyes developed respectively in 2003 by Nogva and co-workers [Nogva *et al*., 2003] and in 2006 by Nocker [Nocker *et al*., 2016] that allow to differentiate between viable and dead cells on the basis of membrane integrity: the dyes penetrate only membrane compromised dead cells but not intact live cells. Once inside a cell, the dyes can covalently link to the nucleic acid through a photo-activation step, with the result that the amplification of the nucleic acid by PCR is inhibited. However, differentiation based on membrane integrity is not always sufficient: for example, some disinfection procedures applied to water such as UV treatment or solar disinfection do not affect primarily the cellular membranes [Cangelosi *et al*., 2014; Kirschner, 2016].

To overcome this limitation, it is necessary to extend the concept of viability so that cells must not only have intact membranes, but they must also be functional and active. In this case, "active" can be defined as capable of maintaining bacterial homeostasis using an active transport mechanism that requires ATP. The PEMAX dye (Geniul, Spain) is based on double-dye technology: a mixture of EMA and PMA dyes that allows to selectively amplify viable cells with both active metabolisms and intact cell membrane structure [Thanh *et al*., 2017; Codony *et al*., 2015; Augusti *et al.*, 2017]. Several authors proposed methods for live/dead *Legionella* spp. differentiation based on EMA and or PMA dyes in combination with qPCR [Scaturro *et al*., 2016; Ditommaso *et al.*, 2014; Mansi *et al*., 2014], but none was completely effective and considered validated. In 2016 a multicentre study was organized by Scaturro *et al*., with the aim to evaluate the efficacy of PMA-qPCR for the quantification of *Legionella* spp. cells. However they concluded that even if the method is easily applicable, there are some limitations linked to PMA molecule that affect the efficiency of the protocol, thus further efforts are necessary for the routine use.

On the base of our knowledge up to date, PEMAX dye has never been applied in combination with qPCR for the quantification of live *Legionella* spp. cells from sanitary and thermal water samples and for these reasons we focused our attention on PEMAX dye.

1.1. Taxonomy and general characteristics

The family Legionellaceae consists of the single genus *Legionella*. Some investigators (Garrity et al., 1980; Brown et al., 1981) have proposed placing the legionellae in three separate genera — *Legionella, Fluoribacter and Tatlockia* — on the basis of low DNA hybridization values between some *Legionella* species [Fox *et al.*, 1993]. However, studies based on the analysis of 16S rRNA have confirmed that the Legionellaceae family includes the single genus *Legionella*. To date about 61 species of *Legionella* are known, divided into over 70 serogroups (Table 1), [http://www.bacterio.net/legionella.htmL]. *Legionella pneumophila* is the species most frequently associated with human disease and includes 16 serogroups (sg) [Montagna *et al.*, 2018; Mekkour *et al*., 2013; WHO, 2007]. Legionellae are facultative intracellular Gram-negative bacteria, aerobic, asporigenous bacilli, acapsulated, generally mobile due to the presence of one or more flagella, with dimensions ranging from 0.3 to 0.9 μm in width and from 1.5 to 6 μm in length. In culture *Legionella* forms long filamentous structures up to 20 μm [WHO, 2007]. Unlike most Gram-negative bacteria, the cell wall contains high amounts of branched-chain fatty acids and ubiquinones which make cellular staining difficult [Mekkour *et al*., 2013]. Concerning the biochemical properties, legionellae are urease negative, catalase positive and utilize amino acids as a source of carbon and energy rather than carbohydrates including cysteine, arginine, isoleucine and methionine. Most species produce beta-lactamases and liquefy gelatine [Fields *et al*., 2002]. *Legionella* spp. growth is stimulated by iron compounds and its cultivation in artificial media requires the addition of specific compounds such as L-cysteine, arginine, leucine, isoleucine, threonine, valine, methionine, phenylalanine, tyrosine and serine, and the addition of trace elements such as iron, calcium, cobalt, copper, magnesium, manganese, molybdenum, nickel, vanadium and zinc [Whiley *et al.*, 2014]. Some legionellae cannot be grown on routine *Legionella* culture media and have been termed *Legionella*-like amoebal pathogens (LLAPs) [WHO, 2007]. Historically, the term Legionella-like amoebal pathogens was introduced to designate obligate intracellular parasites of free-living amoebae which were closely related to the legionellae. The term of LLAPs then has been retained for historical reasons, as most of these species have now been recognized to belong phylogenetically to the *Legionella* genus. Moreover, most of them are currently able to grow on BCYE agar because of the improvement in the quality

of media and possibly because of a progressive adaptation by successive subcultures on amoebae [Lamoth *et al*., 2010].

Table 1: List of *Legionella* species and serogroups.

Species name	n. of Serogroups	Species name	n. of Serogroups
L. adelaidensis		*L. maceachernii*	
L. anisa		*L. massiliensis*	
L. beliardensis		*L. micdadei*	
L. birminghamensis		*L. moravica*	
L. bozemanii	2	*L. nagasakiensis*	
L. brunenti		*L. nautarum*	
L. busanensis		*L. oakridgensis*	
L. cardiaca		*L. parisiensis*	
L. cherrii		*L. pittsburghensis*	
L. cincinnatiensis		*L. pneumophila*	16
L. drancourtii		*L. pneumophila subsp. fraseri*	
L. dresdenensis		*L. pneumophila subsp. pneumophila*	
L. drozanskii		*L. quateirensis*	
L. dumoffii		*L. quinlivanii*	2
L. erythra	2	*L. rowbothamii*	
L. fairfieldensis		*L. rubrilucens*	
L. fallonii		*L. sainthelensi*	2
L. feeleii		*L. santicrucis*	
L. geestiana		*L. shakespearei*	
L. gormanii		*L. spiritensis*	2
L. gratiana		*L. steelei*	
L. gresilensis		*L. steigerwaltii*	2
L. hackeliae	2	*L. taurinensis*	
L. impletisoli		*L. tucsonensis*	
L. israelensis		*L. tunisiensis*	
L. jamestowniensis		*L. wadsworthii*	
L. jordanis		*L. waltersii*	
L. lansingensis		*L. worsleiensis*	
L. londiniensis	2	*L. yabuuchiae*	
L. longbeachae	2	*L. lytica*	

1.2 Ecology and environmental sources of *Legionella* spp.

The pathogenesis and ecology of *Legionella* spp. are inherently related. Legionellae are widely distributed in natural aquatic environments such as lake and river surfaces, thermal springs in which it is generally found at low concentrations. Some species have also been found in sea water and *L. longbeacheae* has frequently been isolated from potting soil. This species is the leading cause of legionellosis in Australia and occurs in gardeners and those exposed to commercial potting soil [Mekkour *et al*., 2013].

From these environments, the bacterium reaches artificial sources such as water distribution system of large buildings, humidification systems, swimming pools, decorative fountains, responsible for transmission to humans. The presence of organic sediments, rust, deposits of materials facilitates their settlement [Fields *et al*., 2002].

Legionella spp. can survive in a wide range of temperatures, but it prefers aquatic environments with temperatures between 25 and 42°C with optimal values around 35°C. *Legionella* spp. is thermotolerant and able to withstand temperatures of 50°C for several hours, while around 70°C it is rapidly destroyed. At values below 20°C *Legionella* spp. survives but is not more able to replicate, however when temperatures return favourable, cells begin to proliferate [Mekkour *et al*., 2013; WHO, 2007]. Most cases of legionellosis can be traced to human-made environments where water temperature is higher than ambient one.

The effect of pH on *Legionella* survival was also investigated: the bacterium has been isolated in the environment at both acid and alkaline pH values. Katz *et al.* demonstrated a 2 logs reduction in the number of *L. pneumophila* cells in mineral water after being subjected to a pH change from 4 to 7 for a month, and a 6-log decline at pH 8. Furthermore, Sheehan *et al.* isolated four *Legionella* species protected inside protozoa in the geothermal sources of the Yellowstone National Park with a pH equal to 2.7 [Katz *et al*., 1987; Sheehan *et al.*, 2005].

Association with amoebae

The levels of nutrients that the legionellae require are rarely found in fresh water and may be supplied, directly or indirectly, by other species of bacteria or other associated

microorganisms in the form of dissolved organic constituents, through the excess of production of organic nutrients or through decay of the microorganisms. Legionellae survive in aquatic and moist soil environments as intracellular parasites of free-living protozoa: the aquatic microbial flora is generally made up of different species of bacteria, fungi and protozoa, and within these microbial communities *Legionella* finds an environment favourable to its development.

Legionellae have been shown to multiply in 14 species of amoebae, two species of ciliated protozoa, and one species of slime mould (Table 2) which serve as a reservoir for the multiplication and survival of legionellae and contributes to the maintenance of the pathogenetic potential and invasiveness (Figure 1) [Lau *et al*., 2008].

Table 2: Protozoan species found to harbour intracellular *Legionella* spp.

Category	***Species***	***References***
Amoeba	*Acanthamoeba castellani*	Rowbotham *et al*., 1980
	Acanthamoeba culbertsoni	Fields *et al*., 1989
	Acanthamoeba hatchetti	Breiman*et al.,* 1990b
	Acanthamoeba polyphaga	Rowbotham *et al.,*1980
	Acanthamoeba palestinensis	Rowbotham *et al*., 1986
	Acanthamoeba royreba	Tyndall *et al*., 1982
	Amoeba proteus strain x D	Park *et al*., 2004
	Comandonia operculata	Breiman *et al.,* 1990b
	Echinamoeba exudans	Fields *et al.,* 1989
	Filamoeba nolandi	Breiman *et al*., 1990b
	Hartmannella spp.	Fields *et al*., 1989
	Hartmannella cantabrigiensis	Rowbotham *et al*., 1986 Breiman *et al*., 1990b
	Hartmannella vermiformis	Rowbotham, *et al.*, 1986; Fields *et al.,* 1989; Breiman *et al.,* 1990b
	Naegleria fowleri	Newsome *et al.,*1985
	Naegleria gruberi	Rowbotham *et al*., 1980
	Naegleria jadini	Rowbotham *et al.,* 1980
	Naegleria lovaniensis	Tyndall *et al.,* 1982
	Paratetramitus jugosis	Breiman *et al.,*1990b
	Vahlkampfia spp.	Breiman *et al.,*1990b
	Vahlkampfia jugosa	Rowbotham *et al*., 1986
	Vahlkampfia ustiana	Breiman *et al*., 1990b Fields *et al.,* 1984
Ciliate	*Tetrahymena pyriformis*	
	Tetrahymena thermophila	Kikuhara *et al*., 1994
Slime Mould	*Dictyostelium discoideum*	Hagele *et al.,* 2000

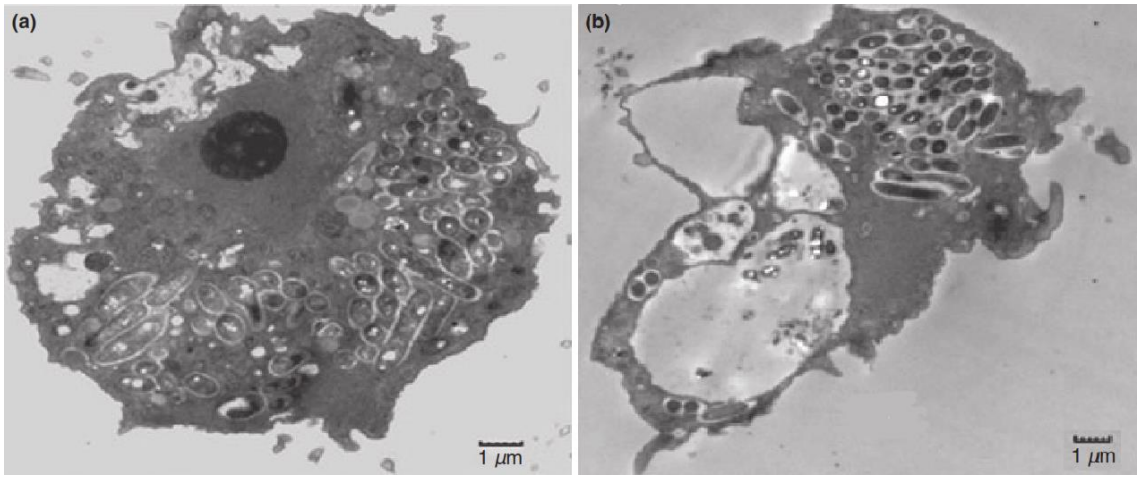

Figure 1 *A. polyphaga* infected with *L. pneumophila*. The multiplication of the latter was monitored by electron microscopy after 18 (a) and 48 hours (b), [Lau *et al*., 2009].

Protozoa help to protect *Legionella* spp. from the effects of biocides used to disinfect water and it has been postulated that this can be a mechanism by which *Legionella* spp. is able to survive to adverse environmental conditions (such as dehydration, high temperatures, osmolarity variations and pH) and persist in heat-treated water or subjected to disinfection systems.

Biofilm

In man-made aquatic environments *Legionella* spp. can be found associated with biofilms (Figure 2): microbial communities that lives in close association, immersed in a polymeric matrix produced by the same microorganisms. The biofilm facilitates nutrient and gaseous exchange and protects microorganisms not only from biocides but also from periodic increases in temperature and attempts at physical removal, especially in areas where surfaces are scaled or corroded. In these artificial water systems, microbial growth is detected almost exclusively in biofilm covering the interior of pipe walls.

To date, outbreak of legionellosis have never been associated with natural aquatic environments, rather with exposure to artificial water system, such as hot water and cooling towers in which the formation of biofilms contributes to the colonization and development of *Legionella* spp., protecting cells from the decontamination procedure applied [Lau *et al*., 2009].

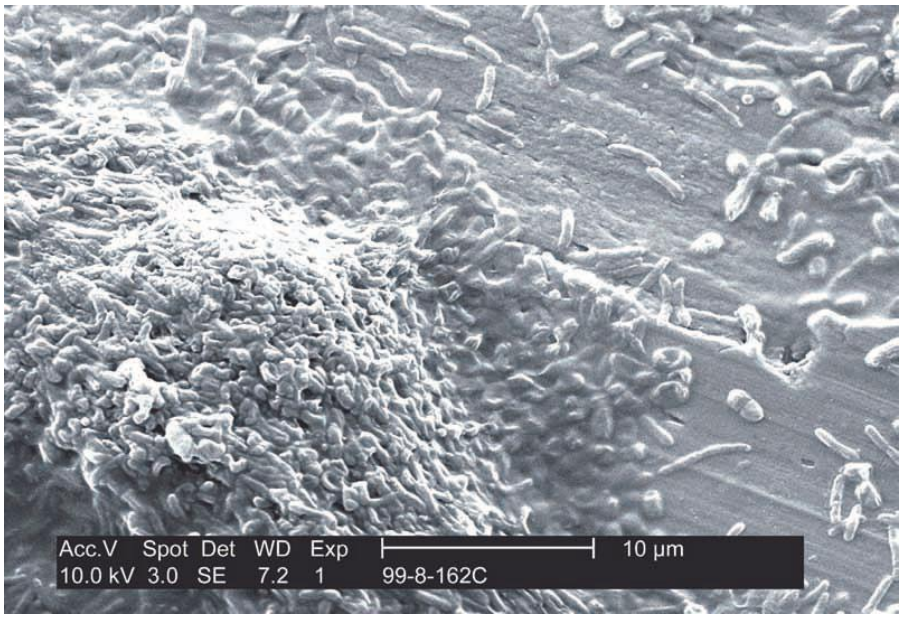

Figure 2: A scanning electron micrograph of *L. pneumophila* on potable water biofilms [HPSC, 2009].

1.3 Clinical presentation

Legionellosis classically presents as two distinct clinical entities, Legionnaires' disease, a severe multisystem disease involving atypical pneumonia, and Pontiac fever, a self-limited flu-like illness. Currently, there is no consensus as to why exposure to *L. pneumophila* may result in either Pontiac fever or Legionnaires' disease [Whiley *et al*., 2014].

Legionnaires' disease is the most severe, occurs after an incubation period ranging from 2 to 10 days with a mortality between 10-20% in healthy people and between 40-80% in hospitalized patients. It is not possible to clinically distinguish patients with Legionnaires' disease from patients with other types of pneumonia. Signs and symptoms include fever, non-productive cough, headache, myalgias, rigors, dyspnoea, diarrhoea and delirium. About half of patients develop pus-forming sputum, and about one third develop blood-streaked sputum or cough up blood (haemoptysis) [Fields *et al*., 2002]. Chest X-rays often show pneumonia with consolidation in the bottom portion of both lungs.

Some patient shows gastrointestinal disorders such as diarrhoea, nausea, vomiting and abdominal pain. Moreover, almost half of patients suffer from disorders related to the nervous system, such as confusion, delirium, depression, disorientation and hallucinations. These disorders may occur in the first week of the disease. If left untreated the disease gets

worse during the first week and can be fatal. The most frequent complications are respiratory collapse, shock, kidney failure and multi-organ dysfunction syndrome [WHO, 2007; Mekkour *et al*., 2013].

Pontiac fever is a flu-like syndrome without pneumonia, characterized by fever, asthenia, headache and myalgia and manifests after a short incubation period (5-40 hours). People generally recover spontaneously after 2-5 days.

The first outbreak of Pontiac fever was caused by *L. pneumophila* of serogroup 1, while subsequent epidemics were attributed to *L. feeleii, L. anisa* and *L. micdadei* [Guidelines, 2015].

It has been shown by autopsy that *L. pneumophila* can spread from the respiratory system to the body: extrapulmonary forms are rare but have a severe and highly lethal course. Legionellae have been found in the liver, spleen, kidneys, myocardium, bones, lymph nodes and digestive tract, sporadically spreading to the nervous system [WHO, 2007]. The most common site of extrapulmonary infection is the endocardium with consequent appearance of endocarditis initially reported in the literature only in subjects with prosthetic valves but later also in native [Samuel *et al*., 2011].

1.4 Risk factors and transmission

Legionellosis infections are attributed to inhalation of contaminated water aerosols produced by infected sources (e.g. faucets, showerhead, or cooling tower) or by aspiration of contaminated water into the lungs. The ability of *Legionella* to access the human respiratory tract depends on the size of the aerosol: droplets with a diameter less than 10 μm stop in the nose and throat, while drops of diameter between 2-5 μm are able to enter the respiratory tract (lungs) and finally those below 2 μm reach pulmonary alveoli [Torrisi *et al*., 2012].Variation in the size of aerosols also affects the infectivity, which makes it difficult to determine the infectious dose and what environmental concentrations are considered acceptable [Whiley *et al.*, 2014].

To date, the infectious dose for humans has not yet been determined, nor the reasons for which the different *Legionella* species have a variable virulence. However, this can be attributed to differences in surface hydrophobicity, aerosol stability and the ability to grow inside amoebas. The physiological state of *Legionella* spp. that causes the infection is not known either, but it can include both the stationary phase of growth and the logarithmic phase, as well as the so-called sporelike forms [Guidelines, 2015; Mekkour *et al*., 2013; WHO, 2007]. Infection may also occurs after inhalation of amoeba vacuoles containing *Legionella* spp. [Allegra *et al*., 2016].

Cases of legionellosis acquired by aspiration or micro-aspiration of contaminated water and cases of contagion through wound have also been reported in the literature mainly related to nosocomial Legionnaires' disease, while human to human transmission or cases due to *Legionella* ingestion have never been demonstrated.

Known host risk factors for community acquired or travel associated Legionnaires' disease (TAVLD) are smoking, chronic obstructive pulmonary disease, diabetes, alcohol abuse, older age (>50 years), and other immunosuppression. Susceptible patients for nosocomial Legionnaires' disease include transplant recipients, other immunosuppression, surgery, cancer, diabetes, treatment with respiratory devices, chronic heart or lung disease, smoking and alcohol abuse, which are associated with higher mortality rates [WHO 2007, Whiley *et al*., 2014]. Environmental risk factors associated with legionellosis outbreaks are travel, residence in a health care facility, and proximity to cooling towers, whirlpool spas and decorative fountains. Any system or equipment which contains, stores, or re-circulates non-

sterile water that can be aerosolized is a source of legionellosis: nebulizer and humidifiers are important sources of infection, moreover the majority of legionellosis outbreaks are related to cooling towers or hot water systems of large buildings as hotel and hospitals [Farnham *et al*., 2014].

Environmental risk factors associated with healthcare facilities

Approximately a quarter of all reported legionnaires' disease cases acquires their infection inside a hospital [HPSC; 2009]. Hospitals caring for immunocompromised patients such as organ or bone marrow transplant recipients are at increased risk of outbreaks of legionnaires' disease. Hospital size may also be an important risk factor.

In hospital hot and cold water systems are the main sources of infection, factors such as water temperature, configuration, age of the water distribution systems and plumbing material encourage legionellae growth. Old components of the pipeline system, area of stagnation or low flow, dead-legs and storage tanks allow *Legionella* spp. survival and development [Borella *et al*.; 2016].

In addition, in healthcare facilities the vulnerability of hospitalized patients and health practices concerning the airways (e.g., ventilation, aspiration, devices for artificial respiration and oxygen therapy, and dental tools) significantly increase the risk for *Legionella* transmission and severe outcomes [Montagna *et al.*, 2018]. In Figure 3 is shown the pathogenesis of nosocomial infection [HPSC; 2009].

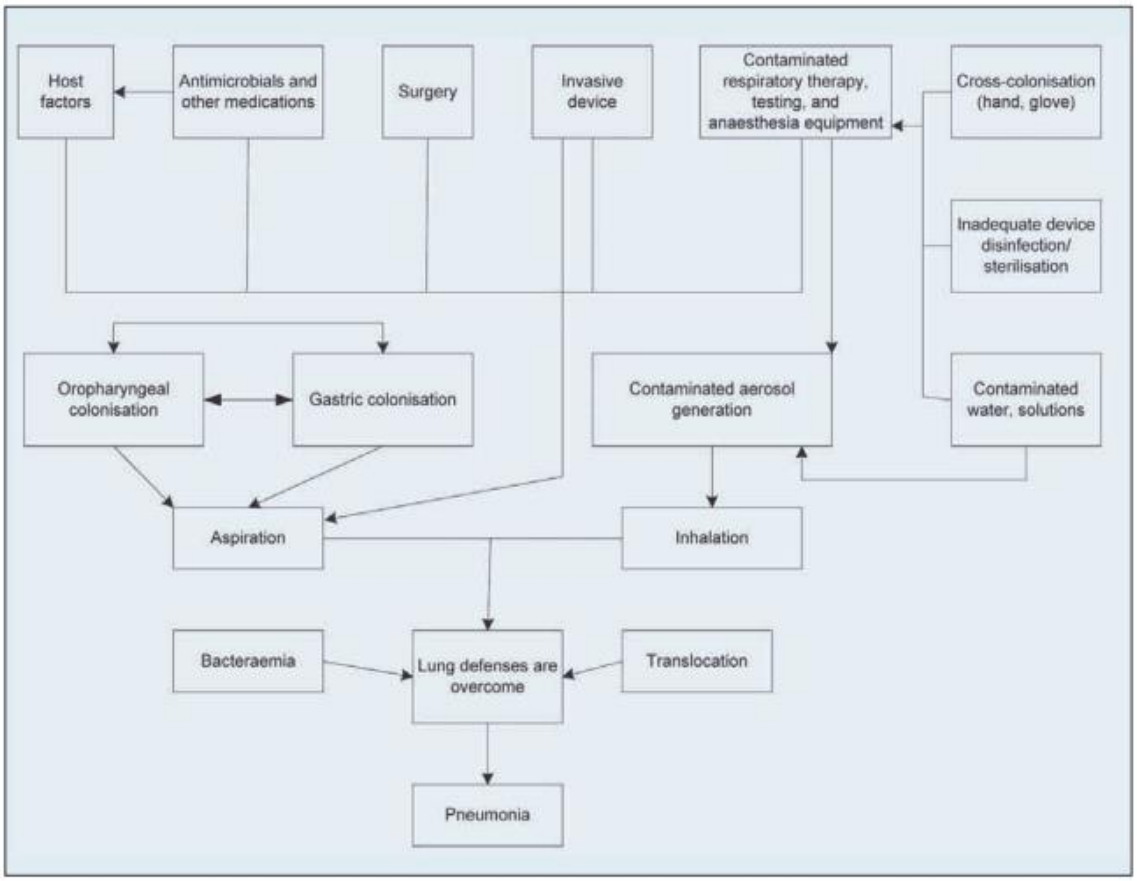

Figure 3: The pathogenesis of nosocomial bacterial pneumonia [HPSC; 2009].

Environmental risk factors associated with recreational water

Spa-pool systems and related recreational facilities are increasingly popular and frequented by people at higher risk of infection. Such systems pose a reasonably foreseeable risk as they are a recognised source of diseases caused by infectious agents including *Legionella* spp. Spa pools are designed to contain water that is vigorously agitated, which leads to the formation of aerosols that can be inhaled. The water is usually maintained within the temperature range where legionellae and other infectious microorganisms can rapidly grow (20–45°C) and the high organic content of spa-pool water makes it difficult to maintain effective disinfection [HSE; 2017].

A recent review published by Leoni and co-workers summarized outbreaks and *Legionella* spp. cases associated with recreational aquatic environments: from 1981 to 2015, 1 079 cases of legionellosis were reported with a fatality rate of 6.3%. The most important environmental risk factors reported were inadequate water treatment and residual disinfectant below the recommended levels (Figure 4) [Leoni *et al.*, 2018].

Therefore, spa-pool systems must be managed carefully to ensure a water quality level that does not encourage microbial growth and pose risks to users, people in the vicinity or passing near the spa pool. Other management strategies need to be implemented, which may include appropriate design and adequate disinfection residual and proper maintenance and cleaning of equipment as well as adequate ventilation. Features, such as water sprays, should be periodically cleaned and flushed with a level of disinfectant adequate to eliminate *Legionella* spp. [HSE; 2017].

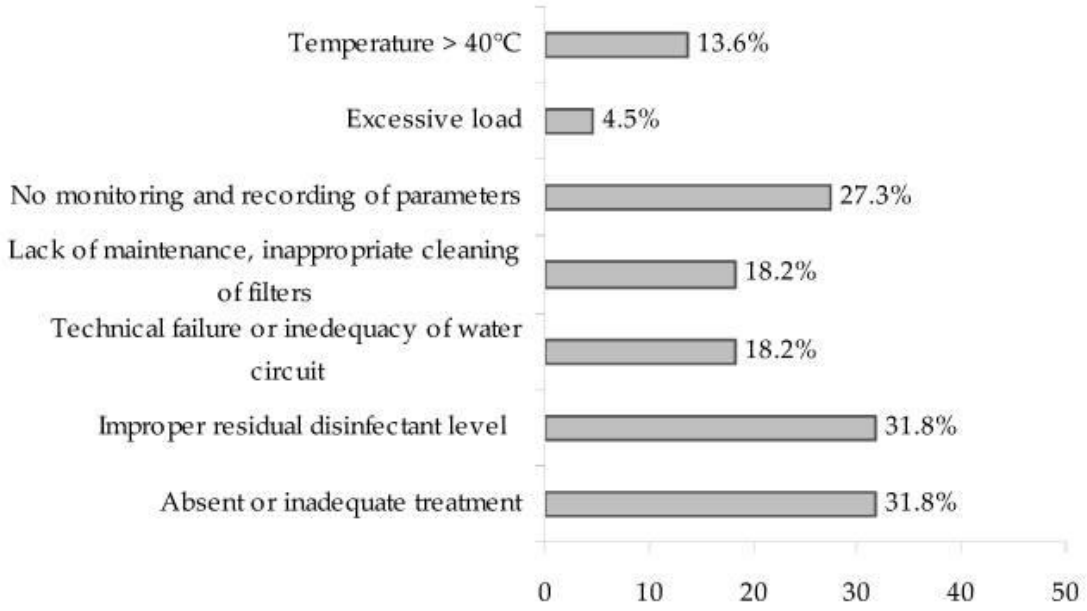

Figure 4: Distribution of environmental contributing factors in 22 recreational facilities as reported by Leoni *et al*., 2018.

1.5 Epidemiology of Legionnaires' disease

Legionnaire's disease remains an important cause of potentially preventable morbidity and mortality in Europe and there is no indication of decreasing burden.

Since 2010 the European surveillance of Legionnaires' disease has been carried out by ELDSNet and coordinated by the ECDC in Stockholm. The network aims to detect, control and prevent cases, clusters and outbreaks of Legionnaires' disease in EU/European Economic Area (EEA) countries, and assist with detection and response outside these countries. The network supports the Member States and other involved countries to share information and collaborate on response actions to provide better protection from travel-associated Legionnaires' disease, both domestically and abroad.

Data on epidemiological surveillance are collected through two schemes: the first studies all the cases reported by the 28 Member States, Iceland and Norway; the second scheme covers all travel associated cases of Legionnaires' disease including reports from countries outside Europe.

The surveillance of TALD cases is aimed at identifying clusters of cases that may not otherwise have been detected at national level, allowing a rapid adoption of corrective measures in the accommodation sites in order to prevent future infections.

Members of the ELDSNet network are appointed by their national Public Health authorities to act as national contact points for Legionnaires' disease surveillance under the scope of ELDSNet activities. Members usually have scientific knowledge about Legionnaires' disease or *Legionella* spp. bacteria and are involved in the microbiological diagnosis or epidemiological surveillance of Legionnaires' disease in their country.

At the country level, clinicians and microbiologists report individual TALD cases to their national surveillance scheme for Legionnaires' disease. The national contact point for ELDSNet reports these cases to ECDC using the EU case definition (Table 3). With complete and rapid reporting, ELDSNet can detect clusters of cases which have a history of travel to the same accommodation site. Receipt of the information leads to specific and timely action by members in order to protect EU/EEA residents travelling in and outside of Europe. A case of legionellosis is defined travel associated if it is associated with one or more overnight stays away from home, either in the country of residence or abroad, in the 10 days before onset of illness. A case is defined nosocomial (healthcare-acquired) if occurs

in a patient who was in a hospital or other healthcare institution during the 10 days before onset of symptoms [ECDC, 2017].

Table 3: The current European case definition for Legionnaires' disease stated in the Commission Implementing Decision of 8 August 2012 [European Commission, 2012].

Legionnaire's disease	Laboratory criteria	Clinical criteria
Confirmed cases	At least one of the following three:	
	• Isolation of *Legionella* spp. from respiratory secretions or any normally sterile site • Detection of *L. pneumophila* antigen in urine • Significant rise in specific antibody level to *L. pneumophila* serogroup 1 in paired serum samples	Any person with pneumonia
Probable case	At least one of the following four:	
	• Detection of *L. pneumophila* antigen in respiratory secretions or lung tissue e.g. by DFA staining using monoclonal-antibody derived reagents • Detection of *Legionella* spp. nucleic acid in respiratory secretions, lung tissue or any normally sterile site • Significant rise in specific antibody level to *L. pneumophila* other than serogroup 1 or other *Legionella* spp. in paired serum samples • Single high level of specific antibody to *L. pneumophila* serogroup 1 in serum	Any person with pneumonia

In 2017 the ECDC reported 9 238 cases of Legionnaires' disease, the 93% of which classified as confirmed, corresponding to 8 624 cases (Table 4). No large outbreaks contributed to the high number of reported cases: most were community acquired (69%), 21% were travel associated, 8% were associated with healthcare facilities and 2% with other settings.

L. pneumophila serogroup 1 was responsible for 801 of 1014 of culture confirmed cases (79%) [ECDC; 2019].

Table 4. Distribution of Legionnaires' disease cases and rates per 100 000 population by country and year, EU/EEA, 2014–2017.

	2014		2015		2016		2017		
Country	**Cases**	**Rate**	**Cases**	**Rate**	**Cases**	**Rate**	**Cases**	**Rate**	**Confirmed cases**
Austria	133	1.6	160	1.9	161	1.9	219	2.5	208
Belgium	101	0.9	118	1.1	157	1.4	235	2.1	183
Bulgaria	1	0.0	1	0.0	0	0.0	2	0.0	2
Croatia	26	0.6	48	1.1	31	0.7	33	0.8	33
Cyprus	6	0.7	2	0.2	3	0.4	1	0.1	1
Czech Republic	110	1.0	120	1.1	147	1.4	217	2.1	213
Denmark	158	2.8	185	3.3	170	3.0	278	4.8	216
Estonia	8	0.6	6	0.5	14	1.1	16	1.2	10
Finland	10	0.2	17	0.3	15	0.3	27	0.5	25
France	1 348	2.0	1 389	2.1	1 218	1.8	1 630	2.4	1 598
Germany	832	1.0	867	1.1	983	1.2	1280	1.6	1 043
Greece	27	0.2	29	0.3	31	0.3	43	0.4	43
Hungary	32	0.3	58	0.6	66	0.7	62	0.6	52
Iceland	4	1.2	1	0.3	3	0.9	3	0.9	3
Ireland	8	0.2	11	0.2	10	0.2	25	0.5	25
Italy	1 510	2.5	1 572	2.6	1 733	2.9	2 013	3.3	1 980
Latvia	38	1.9	22	1.1	24	1.2	31	1.6	24
Liechtenstein	.	-	.	-	.	-	.	-	.
Lithuania	8	0.3	7	0.2	11	0.4	14	0.5	11
Luxembourg	5	0.9	5	0.9	3	0.5	9	1.5	8
Malta	9	2.1	6	1.4	8	1.8	11	2.4	11
Netherlands	348	2.1	419	2.5	454	2.7	561	3.3	519
Norway	51	1.0	60	1.2	43	0.8	52	1.0	44
Poland	12	0.0	23	0.1	24	0.1	38	0.1	32
Portugal	588	5.6	145	1.4	197	1.9	232	2.3	228
Romania	1	0.0	3	0.0	2	0.0	19	0.1	15
Slovakia	14	0.3	14	0.3	14	0.3	14	0.3	12
Slovenia	59	2.9	106	5.1	93	4.5	117	5.7	117
Spain	925	2.0	1 024	2.2	951	2.0	1 363	2.9	1 349
Sweden	136	1.4	142	1.5	145	1.5	189	1.9	124
United Kingdom	370	0.6	412	0.6	383	0.6	504	0.8	331
EU/EEA	**6 878**	**1.3**	**6972**	**1.4**	**7 094**	**1.4**	**9 238**	**1.8**	**5 835**

The overall notification rate per 100 000 inhabitants was 1.8 and vary a lot between each reporting country (Figure 5), which likely represents underestimation of the real incidence in all countries. Overall the notification rate continues to increase over the 2013-2017 period from 1.2 to 1.8. Respect to the previous year, there was an increment of 30% in the number of reported cases, and of 58% over the 2013-2017 period. Four countries, France, Germany, Italy and Spain, accounted for 68% of all notified cases, although their combined populations only represented approximately 50% of the EU/EEA population. The disease mostly affected male 56 years old and the overall male-to-female ratio was 2.4:1.

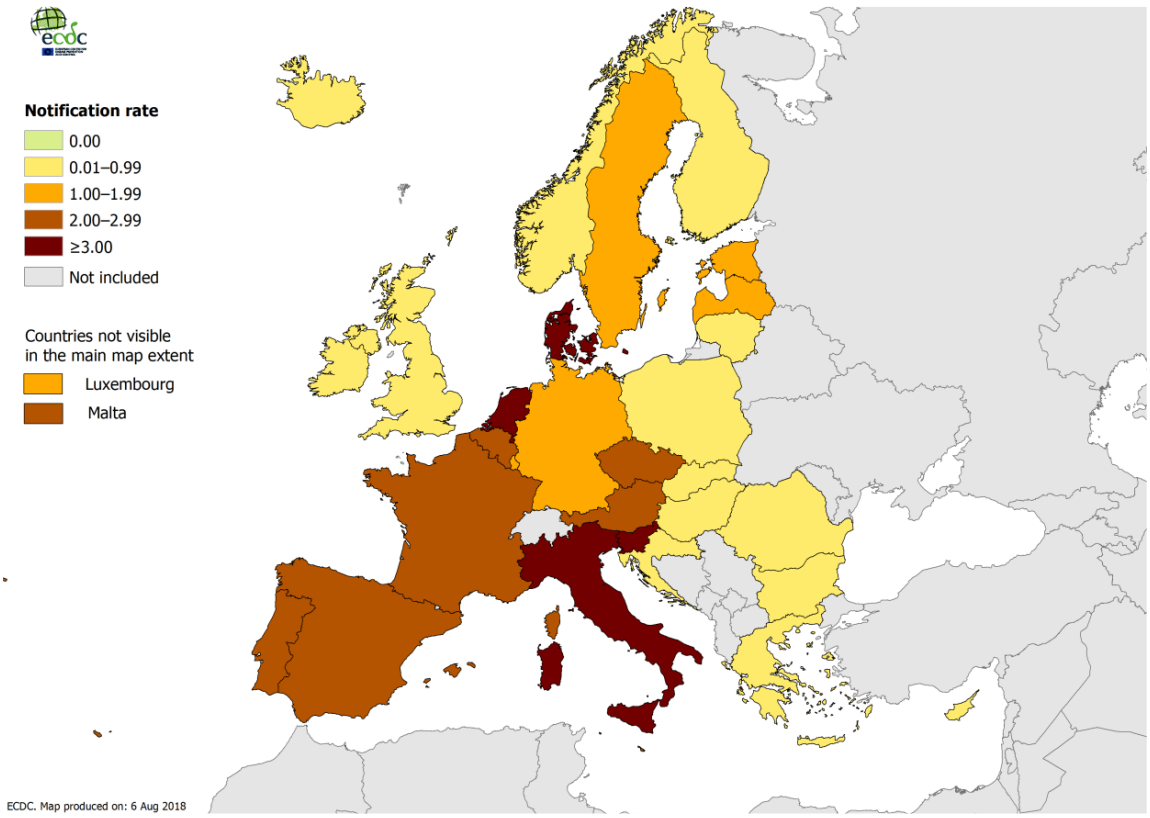

Figure 5: Distribution of Legionnaires' disease cases per 100 000 population.

In conclusion the European situation is therefore complex, with a broad range of notification rates across countries reflecting both the quality of the national surveillance system and the local risk for Legionnaires' disease. The increasing trend is probably due to several factors, including improved surveillance systems, population aging, travel pattern and changes in climate and weather factors. Temperature, humidity and rainfall have been associated with higher incidence of disease, probably due to an effect on the bacterial ecology and/or an increased use of aerosol-producing devices or installations in the environment, such as cooling towers [ECDC, 2019].

1.6 Control measures for water risk management

Control measures are activities or processes applied to a system to prevent the occurrence of a hazard. Such measures are applied at control points, which are steps at which control can be applied to prevent or eliminate a water safety hazard or reduce it to an acceptable level. The strategies for preventing microbial colonization of water systems are based on technical measures implemented to make the environmental conditions in the water network unsuitable for *Legionella* growth.

These procedures, although they do not guarantee the complete eradication of *Legionella* spp., contribute considerably to reduce the risk. First, *Legionella* spp. prevention starts from a correct design and construction of water networks in order to make the multiplication and colonization of the bacterium unlikely.

The WHO suggests developing a water safety plan (WSP) to manage specific risks of exposure to *Legionella* spp. from water samples. A preliminary stage in developing a WSP is to define a team of expert with a thorough knowledge of design and operating features of the water distribution systems in order to identify hazard and risks. Any WPS would be based on a combination of different control methods.

Preventing low flow rates and stagnation of water is an essential and the system should be designed to minimize areas of stagnation. Keeping water temperature outside the ideal range for legionellae is an effective control measure for both hot and cold-water systems. Guidelines recommend that hot water should be stored above 60°C and circulated at temperature of at least 50°C while the recommended temperature for cold water is below 20°C.

When temperature controls cannot be maintained, several different disinfection procedures that can be used alone or in combination are available. Each method has advantages and disadvantages related to the ease of implementation, cost, maintenance issue, and short- and long-term effectiveness.

Water treatments are divided between physical and chemical treatments and are summarized in Table 5 [WHO, 2007; Guidelines, 2015].

Table 5: Advantages and disadvantages of methods for controlling *Legionella* in water system

Treatment	**Type**	**Advantages**	**Disadvantages**
Thermal shock (70-80°C)	Physical	-Do not require special equipment -Can be implemented immediately	- Difficult to maintain temperatures in old systems -Long procedure -Short term effectiveness
Heat and flush (60°C)	Physical	- Simple to use	-Not completely effective -Risk of scalding
Point of use filter	Physical	-Simple to use -Effective	-Require frequent replacement of filter -Local disinfection systems
UV light irradiation	Physical	-Easy installation -no adverse effect on water or plumbing systems	-Effective only at point of application; no control downstream (no residual) -Not suitable for turbid waters -No effect on biofilm formation
Hyperclorination	Chemical	-Effective -Provides disinfection throughout the entire water distribution system -Effective on biofilm formation	-Highly corrosive -Prohibition of using water during treatment (produce potentially carcinogenic by products)
Dosing with chlorine Dioxide	Chemical	- Penetrates biofilm more effectively than chlorine - Less corrosive than chlorine - Wider pH range for activity than chlorine and Cu/Ag ionisation	-Conversion to potentially toxic chlorates and chlorites -Corrosion of pipelines at concentration >0.4 mg/L -Difficult to maintain the effective residual concentration
Ozone	Chemical	-Effective only at point of use; use can be limited to high risk areas or known contaminated taps.	-Temporary results -Low activity on biofilm formation -Corrosive to metals
Dosing with monochloramine	Chemical	- Provides a stable residual (more stable than chlorine) that penetrates biofilm	- Less active than chlorine. -Low activity against protozoa (and viruses)
Copper and silver ionization	Chemical	-Easy installation -Effectiveness at high water temperature -No adverse effect on water or plumbing systems	-Continuous monitoring of copper and silver ions in order to avoid that concentration exceed the limits
Dosing with hydrogenperoxide	Chemical	-No adverse effect on water or plumbing systems	-There are no exhaustive tests on dynamic behaviour over time -Not suitable for galvanized steel water networks

1.7 Regulations and guidelines for the control and prevention of legionellosis

In order to prevent the risk of Legionnaire's disease and ensure water safety, many international organizations have issued guidelines and/or regulations for controlling *Legionella* spp. in water networks which incorporate recommendations on *Legionella* primary prevention in the built environment, with detailed information on the appropriate prevention measures to be taken.

These organizations include both European agencies such as the WHO and the Health and Safety Executive (HSE), but also American organizations such as the Occupational Safety & Health Administration (OSHA), the Center for Disease Prevention and Control (CDC) and the Allegheny County Health Department (ACHD). There are also national guidelines in many countries that identify the necessary measures to prevent and control the risk of exposure to the *Legionella* spp. bacterium [HPSC, 2009; Guidelines, 2015].

Some guidelines can be applied to all types of water (HSE), while others are related to the control of legionellosis in healthcare facilities (CDC, OSHA) or to the prevention of Legionnaires' cases travel associated (EWGLI) [ACDH, 1997; WHO 2007; CDC 2003, HSE, 2000; EWGLI, 2011].

In Italy, on 7th May 2015, the State-Regions Conference sanctioned the Agreement between the Government, the Regions and the Autonomous Provinces of Trento and Bolzano, on the "Guidelines for the prevention and control of Legionnaire's disease" which brings together, updates and integrates in a single text all the indications reported in the previous national guidelines. In Italian Guidelines, it is foreseen for the first time the possibility to use a molecular method based on qPCR, developed and validated according to ISO/TS 12869, as a screening tool to quickly analyse environmental samples. qPCR can be used as a method for screening out negative samples in as quick as half a day after receipt of the sample in the laboratory, whereas positive samples must always be quantified according to culture method described in the ISO 11731:2017 [Anonymous, 2017]. The molecular approach provides information on the number of *Legionella* spp. genome units (GU) in the samples tested but equivalence with the number of colony forming units (CFU) has not yet been reliably established. Usually, the number of genome units is higher than the number of CFU, probably due to the presence of viable non-culturable and dead *Legionella* spp. cells in the samples tested [HPSC, 2009].

For these reasons, although the Guidelines recognize the importance of qPCR, the method cannot be considered validated. The Italian and European guidelines define the limit values for *Legionella* spp. concentrations in water system and suggest the corrective actions for water systems that must be immediately applied: action levels are expressed in CFU and there is no consensus on how the results obtained by one method can be compared with those obtained by the other [WHO, 2007; EWGLI, 2011; Guidelines, 2015]. Lee and co-workers conducted an international multicenter study on various types of water samples, in order to define the action thresholds of real-time PCR for the monitoring of legionellae and thereby to facilitate interpretation of environmental legionella monitoring results. They have thus proposed action and alert levels for *L. pneumophila* and *Legionella* spp. expressed as GU/L. However, the proposed levels are related to the assay used and the type of sample analysed in comparison with the culture method. As suggested by these authors, further studies are needed to derive guidelines that allow the use of qPCR in routine analyses, suggesting that in the future the cultivation method may no longer be considered the reference method [Lee *et al*., 2011].

1.8 Standard method to detect and quantify Legionellae in water

Cultivation dependent

The reference method for the detection and/or quantification of *Legionella* spp. in water sample is based on the cultivation of bacteria and is described in the ISO 11731-1 and ISO 11731-2 standards. The first was published in 1998 and can be apply to all types of environmental water samples, including drinking and industrial water; the ISO 11731-2 method can only be used for waters in which low bacterial contamination is suspected (eg. cold water). Following the standard method, 1000 mL of water is concentrated by filtration through 0.22 µm or 0.45 µm pore-size polycarbonate or nylon filters.

After filtration, membranes are placed into 10 mL of the original water samples and scraped to remove bacteria. Alternatively, 200 mL of samples is centrifuged, and the pellet is resuspended in 2-20 mL sterile diluent. Aliquots of the concentrate are treated with heat (30±2 min at 50±1°C) and/or acid (buffered 0.2 M HCl for 5 min) to reduce the background microflora.

The concentrate is spread on a Petri dish containing BCYE (Buffered Charcoal Yeast Extract with α-ketoglutarate, L-cysteine and ferric pyrophosphate) agar supplemented with vancomycin, polymyxin B, cycloheximide and glycine (GVPC medium).

After 7 to 10 days of incubation at 36±1°C a minimum of 5 presumptive colonies showing a greyish-white colour are streaked on both BCYE and BCYE agar without cysteine, and checked for growth after 2 days of incubation at 36±1°C. Alternative to BCYE agar without cysteine, blood agar or nutrient agar can be used.

Isolated colonies that grow only on a-BCYE agar with L-cysteine can be identified using several methods such as agglutination test or real-time PCR method complying with ISO/TS 12869 requirements. Results are expressed as CFU per litre of water samples analysed. A scheme of culture method is shown in Figure 6.

Due to the microbial complexity of environmental samples, isolating *Legionella* spp. by culture methods has a range of challenges: the presence of high level of contamination from background microflora can obscure the detection of *Legionella* spp., moreover the presence of VBNC cells can underestimate the real risk of infections. Another important shortcoming of this detection method is the long assay time that requires up to 14 days.

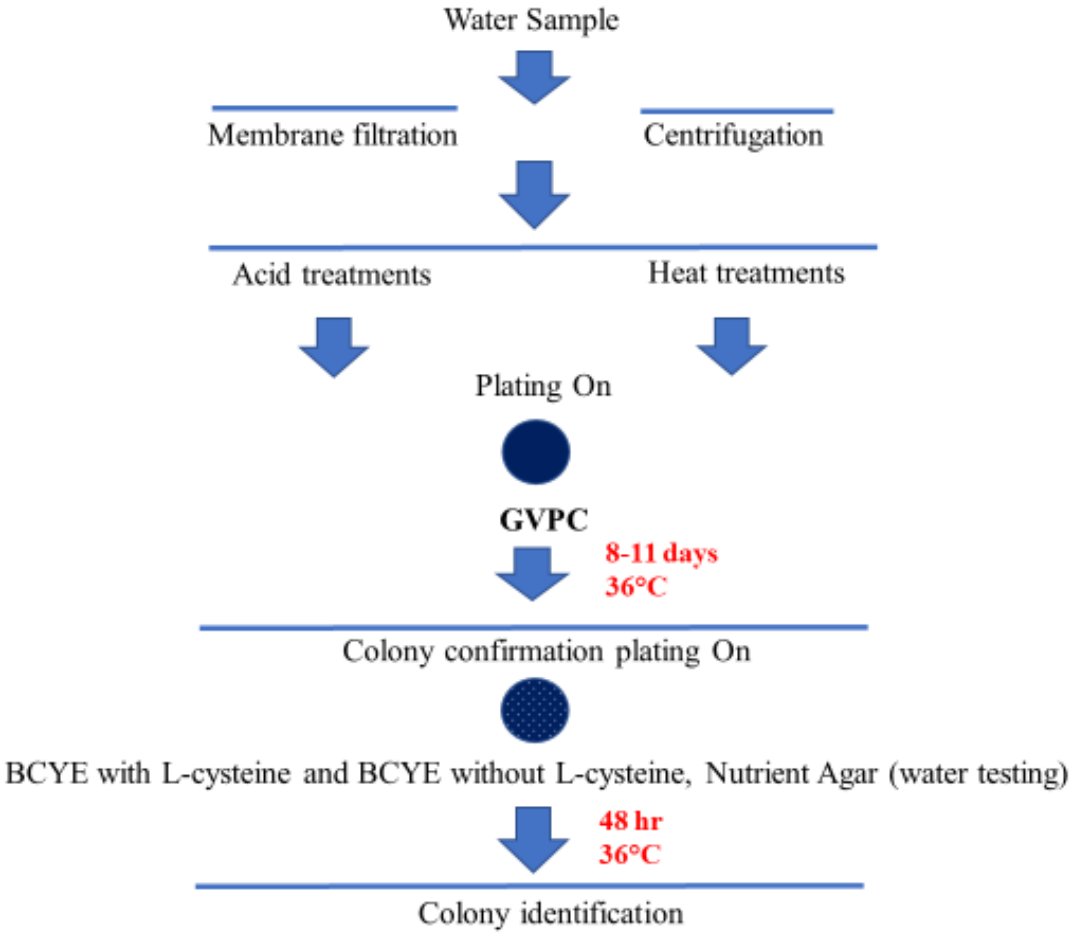

Figure 6: Scheme of culture method for the detection and quantification of *Legionella* spp.

Cultivation independent

qPCR is an alternative method for a rapid detection and quantification of *Legionella* spp. from water samples. This technology amplifies and quantify a target DNA sequence, giving the number of *Legionella* spp. (GU) per litre of water samples analysed.

Generally, the 5S and 16S genes are used as targets for the detection of all *Legionella* species, while for *L. pneumophila* the *mip* gene (macrophage infectivity potentiator) is commonly used [Delgado-Viscogliosi *et al*., 2005].

There are available several commercial kits on the market based on qPCR technology for both *Legionella* spp. and *L. pneumophila* that have been validated according to ISO/TS 12869: the iQ-Check™ Legionella kit (Bio-Rad, France), the mericon Quant Legionella kit (Qiagen, Germany), Aqua-Screen® L. Set Detection Kit (Minerva Biolabs, Germany), the GeneDisc® Legionella kit (Pall Corporations, France) and the *Legionella* spp. quantitative kit (Diatheva, Italy) [Anonymous, 2012]. The rapid turnaround time and the sensitivity of qPCR represent the main advantages when compared to culture method. However, this technology tends to overestimate due to the amplification of non-viable dead cells.

Recently Legioalert (IDEXX, US) was validated according to NF148 for the detection and enumeration of *L. pneumophila* in drinking water and industrial water [Anonymous, 2013]. The test is based on a bacterial enzyme detection technology that signals the presence of *L. pneumophila* through utilization of a substrate present in the Legiolert reagent. *L. pneumophila* cells grow rapidly and reproduce using the rich supply of amino acids, vitamins and other nutrients present in the Legiolert reagent. Actively growing strains of *L. pneumophila* use the added substrate to produce a brown colour indicator. Legiolert detects *L. pneumophila* at 1 organism in 100 mL within 7 days [https://www.idexx.com/en/water/water-products-services/legiolert/].

1.9 Methods to proof the viability of *Legionella* spp.

The ideal scenario in most applications of microbial diagnostics is that only viable cells are detected. Theoretically, cultivation-based approach represents an excellent method as it can detect legionellae that are able to proliferate, live and infectious to humans. However, it overlooks cells that are no more culturable after stress induced by unfavourable environmental conditions (chemical disinfection, heat-treatment, UV-disinfection, limitation in nutrients). A number of techniques have been proposed; these live/dead protocols typically address one of the three aspects of microbial viability: (1) the existence of an intact, functional cell membrane, (2) the presence of cellular metabolism or energy, or (3) the possession of self-replicating DNA that can be transcribed into RNA, which, if applicable, can subsequently be translated into protein [Emerson *et al*., 2017].

Cell-based approach

Membrane integrity is a biomarker for viable cells because cells with compromised membranes are dead (or near). One of the most commonly used fluorescent stains to determine viability by membrane integrity is propidium iodide (PI). PI is a hydrophilic cationic molecule that can cross the damaged cell membrane and then bind to the internal nucleic acids. Because of its fluorescent properties, PI can be used to detect membrane-compromised cells via epifluorescence microscopy and flow cytometry [Alleron *et al*., 2008; Keserue *et al*., 2012]. Flow cytometry is a fast, cost-effective and potentially automatable technology: a commercial kit is available from rqmicro (Switzerland) for the quantification of live *L. pneumophila* cells by flow cytometry. After concentrating the water samples by filtration, the filter is resuspended in a small volume of buffer, the sample is then incubated with magnetic particles that are bound to antibodies specific for *L. pneumophila* sg 1-15. The target cells are isolated by immunomagnetic separation and then sample can be analysed by flow cytometry. By adding PI it is possible to quantify viable cells population.

The LIVE/DEAD BacLight Bacterial Viability Kits (ThermoFisher, US) is another commercial kit that uses PI to stain membrane-compromised cells in combination with

SYTO 9 (a green fluorescent total nucleic acid stain) to distinguish between dead and viable *Legionella* spp. cells. This approach was tested on starved [Trigui *et al*., 2015], heat-inactivated [Allegra *et al*., 2008; Nocker *et al*., 2011] or chemically disinfected cells [Alleron *et al*., 2013], but it is not a suitable technology for water samples disinfected by UV, as this treatment does not harm the cellular integrity of the cell [Kirschner, 2016].
The ScanVit Legionella® (Vermicon, Germany) is a rapid commercial system based on the VIT® Vermicon Identification Technology which consists of fluorescent in situ hybridization (FISH): DNA gene probes labelled with a fluorophore bind specifically to the sequence target site on 16S rRNA. This system allows the detection of viable cells of *L. pneumophila* and *Legionella* spp., by detecting the 16S rRNA content. The analysis procedure consists of a water sample filtration followed by treatment with acid. The filter is then placed on a plate with a selective medium and incubated for 72 h. After incubation, the membrane is transferred to a support furnished with the kit (ScanVit Reactor; Vermicon), the detection of *Legionella* spp. takes place on a cultivated filter brought into contact with the gene probes marked with a dye. During the ScanVIT analysis, the marked gene probes enter the bacteria and bind to the matching signatures within the cells. The membrane is then transferred to a slide and examined under a fluorescence microscope [Ditommaso *et al*., 2010]. However, FISH technology after UV disinfection or other scenarios such as heat or chemical treatment, is not suitable alone for monitoring viability as rRNA can be intact but cells may have compromised cell membranes [Kirschner, 2016].

Nucleic acid-based approach

An alternative approach to detect viable cells by PCR is viability PCR: concentrated water samples are pre-treated prior to DNA extraction and qPCR amplification with a nucleic acid intercalating dye (EMA, PMA, PMAxx) that selectively enters cells with compromised cell membranes, whereas an intact cell membrane presents a barrier for this molecule. Once inside a (dead) cell, the dye intercalates into the cell's DNA to which it is believed to covalently crosslink after exposure to strong visible light due to the presence of an azide group. Photolysis converts the azide group into a highly reactive nitrene radical that can react with any organic molecule in its proximity. Reaction with DNA can be assumed to occur with a high probability considering the spatial proximity of the intercalated dye and

this modification strongly inhibits its amplification: the nitrene group cross-links with the DNA of the membrane-compromised cells, induces a structural change in the nucleotide angle, and because of this the DNA polymerase does not bind to the DNA which leads to signal reduction (Figure 7). The remaining unbound intercalating dye is inactivated by reaction with water molecules, forming hydroxylamine that is unable to further bind to DNA molecules [Fittipaldi *et al*., 2012 Kumar *et al*., 2019].

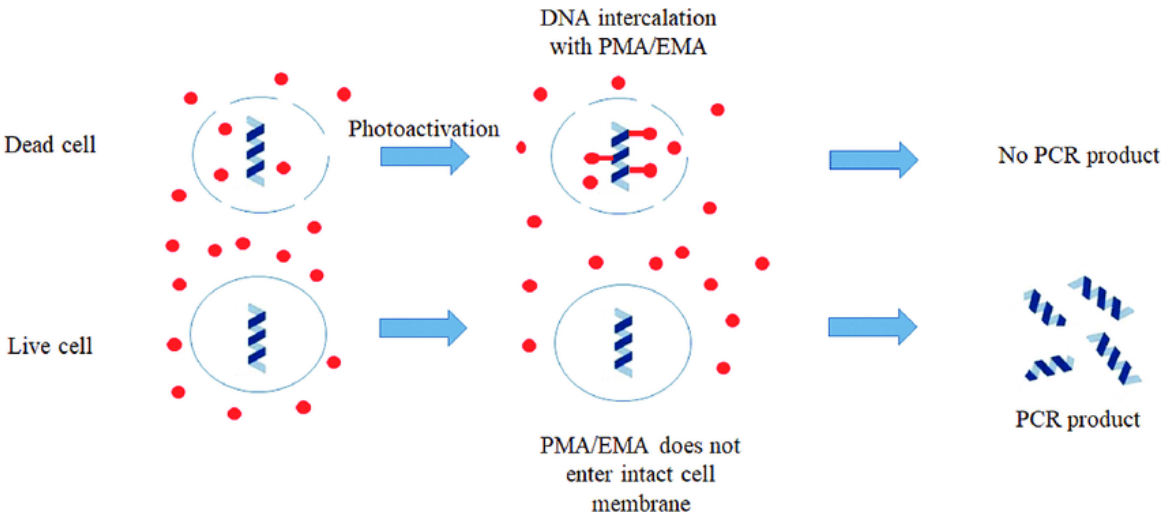

Figure 7: Viability PCR workflow (e.g., using EMA, PMA, or similar dyes). The dye enters compromised/dead cells, binds covalently to the DNA upon photoactivation and stops the amplification of DNA from dead cell [Kumar *et al*., 2019].

The first reagent that has been proposed in combination with PCR was EMA [Nogva *et al*., 2003], a molecule derived from ethidium bromide (EtBr), having the same basic structure as EtBr, with the addition of an azide group. In several studies EMA showed a higher cytotoxicity than PMA, due to its penetration into cells with intact membrane [Yanez *et al*., 2011]. This could be possible since EMA has only one positive charge and therefore more easily penetrates bacterial membranes than PMA, which has a double positive charge. However, this may also result in less efficient suppression of dead cell signals by PMA relative to EMA, because PMA may not as easily permeate cells with only slightly compromised membranes [Fittipaldi *et al*., 2012].

The PMA reagent is available commercially from several vendors, including Geniul (PhAST Blue), Qiagen (BLU-V PMA Viability Kit), and Biotium (PMA-Lite). In addition, a new variant on PMA, PMAxx (Biotium, Inc., Hayward, USA), has recently become

available, though the chemical composition of PMAxx and its relationship to PMA are proprietary [Emerson *et al*, 2017].

PMA has become the dye of choice as resulted to have the major specificity for dead cells [Nocker *et al*., 2006]: the PMA-qPCR has been applied for the quantification of viable *Legionella* cells from different authors [Scaturro *et al.*, 2016; Ditommaso *et al*., 2014]. However, the PMA-qPCR approach is not completely efficient in the suppression in the PCR signals of dead cell's DNA underling the need of further optimization to increase the efficacy of the protocol (Table 6) [Scaturro *et al*., 2016; Emerson *et al*.; 2017].

Table 6: Approaches to improve the efficiency of PMA-qPCR.

Parameter	Description	References
Amplification of longer sequences	Increasing the amplicon length increases the probability that at least one dye-binding event will have occurred, resulting in an increased suppression of signals from membrane-compromised cells	Ditommaso *et al.*, 2015
Multiple dye treatments	Repeated sample treatment with a viability dye (i.e., the addition of dye, followed by photoactivation, then additional rounds of dye addition and photoactivation) has been demonstrated that improve signal suppression	Kralik *et al*., 2010
Extending dye incubation time and concentration	Light exposure of dye treated samples is important for (i) activation of nucleic acid-bound dye and for (ii) inactivation of excess dye that has not entered cells and that could potentially bind to DNA from live cells during the DNA extraction procedure	Fittipaldi *et al*., 2012
Incubation in the presence of facilitating substances	Co-incubation of cells with PMA and the bile salt, deoxycholate, dimethylsulfoxide (DMSO) has been shown to improve PMA to affect dye permeability through membranes	Seidel *et al*., 2017

Differentiation between viable and nonviable cells based on membrane integrity alone is not always sufficient as some disinfection procedures cannot be monitored by vPCR using EMA or PMA, because the damage to the cells does not directly affect membrane permeability [Nocker *et al.*; 2007]. A new approach has been proposed by Codony and co-workers able to discriminate between cells with an intact cell membrane and the ability to actively maintain bacterial homeostasis and cells that have an intact membrane but are metabolically inactive, extending the concept of viability so that cells must not only have intact membranes, but they must also be functional and active [Codony *et al.*, 2015]. In this

case, “active” can be defined as capable of maintaining bacterial homeostasis using an active transport mechanism that requires ATP. Based on this approach the PEMAX dye was commercialized by Geniul (Spain): a double-dye reagent comprising a mix of photo-reactive azide forms of phenanthridium (Figure 8) [Codony *et al*., 2015; Augusti *et al.*; 2017].

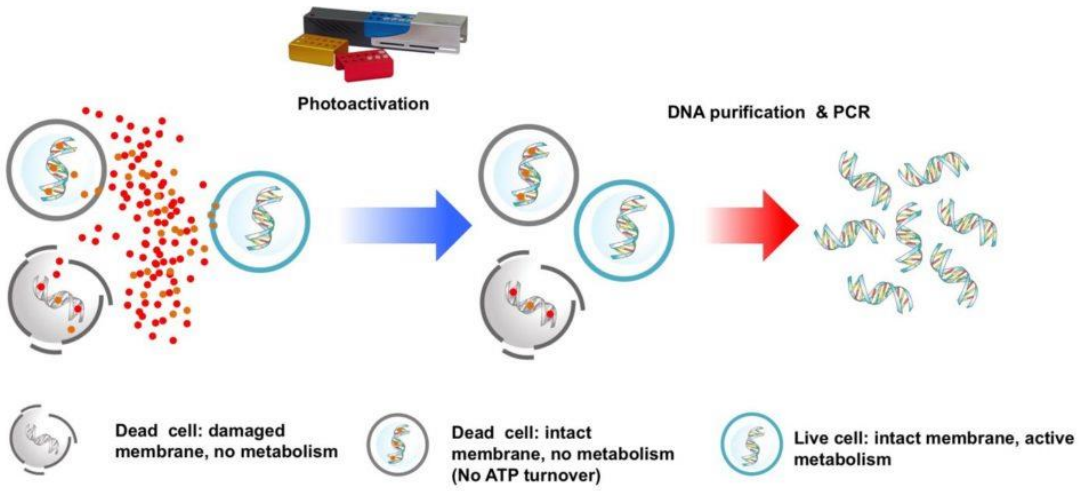

Figure 8: Mechanism involved in v-PCR when using PEMAX dye [www.geniul.com].

As alternative to v-PCR targeting DNA, RNA could be a possible target for qPCR. Pre-rRNA synthesis in response to nutritional stimulation is exploited in a method termed Molecular Viability Testing (MVT). Pre-rRNA stimulation is very rapid and requires exposure to nutrients for 1 to 2 generation times or less (1 to 3 h for most species).

All or nearly all bacteria synthesize pre-rRNA upon nutritional stimulation, allowing the successful application of MVT to multiple diverse species. Boss and co-workers have developed a MVT method for the detection of viable *L. pneumophila* cells in tap water in less than 8 hours, targeting the 16S rRNA. The method proved to have a sensitivity of 91% and specificity of 97%. This procedure has the disadvantage of higher manual workload and cost compared to the culture ISO method and further research are necessary to evaluate if VBNC cells have activated their RNA synthesis during nutritional stimulation [Boss *et al*., 2018].

Aim of the present study

The aim of the present research is to develop and optimize a molecular method for the detection and/or quantification of live *Legionella* spp. and *L. pneumophila* cells in sanitary and thermal water samples based on real-time PCR technology. The first step of the research activity was focused on the evaluation of four genomic DNA extraction methods, by comparing cell lysis efficiency and quality of DNA extract from complex water samples, such as *Legionella* DNA extraction kit, Bacterial DNA Isolation Single Step (Diatheva, Italy) and two-in house developed methods based on chelating resin and purification by two different types of columns for ultrafiltration. Owing to the presence of organic matter and contaminants in water that can cause partial or complete inhibition of qPCR, the DNA extraction method represents a fundamental step of the molecular method. The subsequent phase was the development and optimization of the v-PCR protocol using PEMAX dye, which selectively amplifies viable cells with both active metabolisms and intact cell membrane structure. This treatment was applied after water filtration, prior to the DNA extraction and, once optimized, was tested on field samples. Results obtained highlighted the need to apply further strategies to increase the effectiveness and the selectivity of the developed protocol in the exclusive detection of live cells. For this purpose, at first an immunomagnetic separation (IMS) of *L. pneumophila* cells was evaluated as a purification step before v-PCR to eliminate the competing microflora. A second method involved the use of Free DNA Removal Solution (FDRS) (Biorad, France) based on DNAse I and a mixture of CMIT/MIT that inhibits microbial growth to remove free DNA from water samples prior to qPCR analysis. A final approach followed in this study, called "nutritional stimulation", was based on the culture-enrichment of filtered water samples in *Legionella* specific liquid media to stimulate the growth of live *Legionella* cells, followed by DNA extraction and qPCR amplification. A shift between the cycle threshold (Ct) of an unstimulated and a stimulated aliquot of a sample (after incubation with liquid media) can be interpreted as the presence of viable legionella cells.

Chapter 2. Evaluation of robustness of DNA extraction methods for the recovery of *Legionella* spp. cells

2.1 Introduction

Environmental water samples are complex matrices that may contain PCR inhibitors that may antagonize the polymerase and decrease amplification efficiency. Moreover, PCR inhibitors present in environmental water samples may potentially lead to inaccurate target quantification or false negative results. Humic substances are the most commonly reported group of PCR inhibitors in the environmental samples and have been found to directly disturb the DNA polymerase and form colloids in water and complexes with iron ions, meaning that they could affect the ion content in PCR, probably by chelating magnesium ions. Other group of inhibitors include heavy metals, that can be present in water samples and for which the mechanisms of PCR inhibition are still not very well understood [Filion, 2012].

With the objective of applying the molecular method for quantification of *Legionella* spp. in water samples, the establishment of an optimal recovery is extremely important to ensure not only high sensitivity but also the consistency of results. At the same time, the quantification of the *Legionella* DNA must not be affected by the type and nature of water samples that are commonly analysed by laboratories. For these reasons, an accurate evaluation of DNA extraction methods was performed in order to select the best system able to recover a high amount of *Legionella* spp. DNA in compliance with the requirements of ISO/TS 12869.

Four DNA extraction systems were evaluated and tested for the determination of DNA extraction efficiency and the matrix effect (the possible interfering effect of some chemical components and PCR inhibitors present in water samples on DNA extraction, viability treatment or amplification) on a total of 76 water samples belonging to different water matrices (Table 7).

2.2. Material and Methods

2.2.1 Bacterial growth condition

L. pneumophila ATCC33152 supplied by the DSMZ (Braunschweig, Germany) were rehydrated, collected and maintained in culture as recommended by the Association Français de Normalisation (AFNOR, Paris, France) NFT90-471 and ISO/TS 12869:2012. This strain was cultured in Nutrient Broth and on Buffered Charcoal Yeast Agar supplemented with *Legionella* BCYE Growth Supplement (OXOID) at 37°C with 5% CO_2 for 24-48 h.

2.2.2 Types of water samples analysed

During a 14-month period, a total of 75 water samples (500 mL) from different water matrixes and sampling points were collected aseptically using sterile 1 L containers containing sodium thiosulphate 20 mg/L to neutralize chlorine and transported immediately at room-temperature. Samples were categorized into 4 groups:

1. 7 cooling towers and 19 sanitary water samples collected from hot water distribution system of a local Hospital (n=26)
2. 10 domestic hot water samples (n = 10 samples),
3. 8 water samples collected from thermal pools and 7 water samples of Tabiano water (sulphurous water) (n=15)
4. 12 distilled water samples and 12 mineral water samples (Levissima) (n= 24)

All water samples were previously tested by qPCR to evaluate a natural contamination by *Legionella* spp. using the home method protocol A as DNA extraction system.

Table 7: List of water matrices tested for the evaluation of the robustness of the DNA extraction methods.

	Legionella DNA extraction kit	Bacterial DNA Isolation Single Step	In house Method A	In house Method B
Distilled water	x	x	x	
Mineral water			x	X
Domestic hot water	x	x		X
Sanitary hot water	x	x	x	X
Water from cooling tower			x	X
Sulphurous water			x	
Thermal water pools		x	x	

2.2.3 DNA extraction methods and qPCR analysis

Four DNA extraction kits were evaluated: the *Legionella* DNA Extraction kit and Bacterial DNA Isolation Single Step, two commercial DNA extraction kits that do not allow to extract the *Legionella* spp. DNA directly from membrane filter, and two in house developed methods (protocol A and B) in which the DNA is extracted from filter as recommended by ISO/TS 12869.

In the commercially available kits, after water filtration using polycarbonate membrane filter with a porosity of 0.22 µm (Merck Millipore, US), the filters are put into sterile falcon tubes containing 1.5 mL of the original water sample. Samples were vortexed for 30 seconds and membranes scraped using sterile loops. The whole volume was then centrifuged for 5 minutes at 10 000 rpm and the resulting cell pellets extracted using the above-mentioned DNA extraction kits, following manufacturer's instructions.

Two in-house developed methods using the same lysis step based on chelating resin (Instagene, Biorad) and purification by two different purification columns (Protocols A - B) were evaluated directly extracting the DNA from membrane filters.

Water samples were filtered using 0.45 µm polycarbonate membrane filters (Merck Millipore, US); using sterile tweezers, membranes were folded to obtain a cone and transferred into tubes containing 2 mL of Instagene matrix (Biorad, France) made with a specially formulated 6% w/v Chelex resin. Samples were vortexed and incubated at 95±5°C for 15 minutes. After lysis step, 200 µL of the samples were purified by two different columns available from Merck Millipore that allow to purify the DNA by ultrafiltration.

For all protocols, 5 µL of the DNA extracted was quantified in duplicate with "*Legionella* spp. quantitative kit" (Diatheva) following manufacturer's instruction.

The *Legionella* spp. quantitative kit contains the Standard DNA for the preparation of calibration curve that is diluted to obtain 4 levels of *Legionella* spp. GU: 25 000, 2 500, 250 and 25 GU/5 µL. The detection limit of this qPCR method was 5 GU per well; while the detection limit of the entire method was 320 GU/L. The quantification limit was 25 GU/L corresponding to 1600 GU/L.

All qPCR amplifications were performed on Rotor-Gene Q (Qiagen) using software version 2.1.0.

2.2.4 Evaluation of robustness of DNA extraction methods according to ISO/TS 12869

The recovery of *Legionella* was evaluated according to ISO/TS 12869:2012 on artificially contaminated water samples prepared as follows: colonies of *L. pneumophila* ATCC33152, that are less than 72 h old, were inoculated in Tryptone salt (NaCl 8.5 g/L, Peptone 1 g/L) to obtain a mother suspension with an optical density (OD_{600nm}) of 0.5 corresponding to 10^9 CFU/mL. Serial 10-fold dilutions in the same medium were prepared and used for the artificial contamination of water samples (free of nucleic acids of *Legionella*) enabling to obtain the respective quantities of 10^5 and 10^3 CFU/mL.

L. pneumophila DNA from the mother suspension was isolated and amplified to estimate the GU number: three 100 mL-aliquots were subjected to DNA extraction according to the lysis protocol of each DNA extraction method without column purification and lysates diluted 1:100 before the amplification.

The mean value of bacterial concentration obtained for mother suspension was used as reference to calculate A expressed as decimal logarithm in the formula below, while the GU estimated for each spiked sample was used to calculate B. A scheme of one recovery experiment is shown in Figure 9.

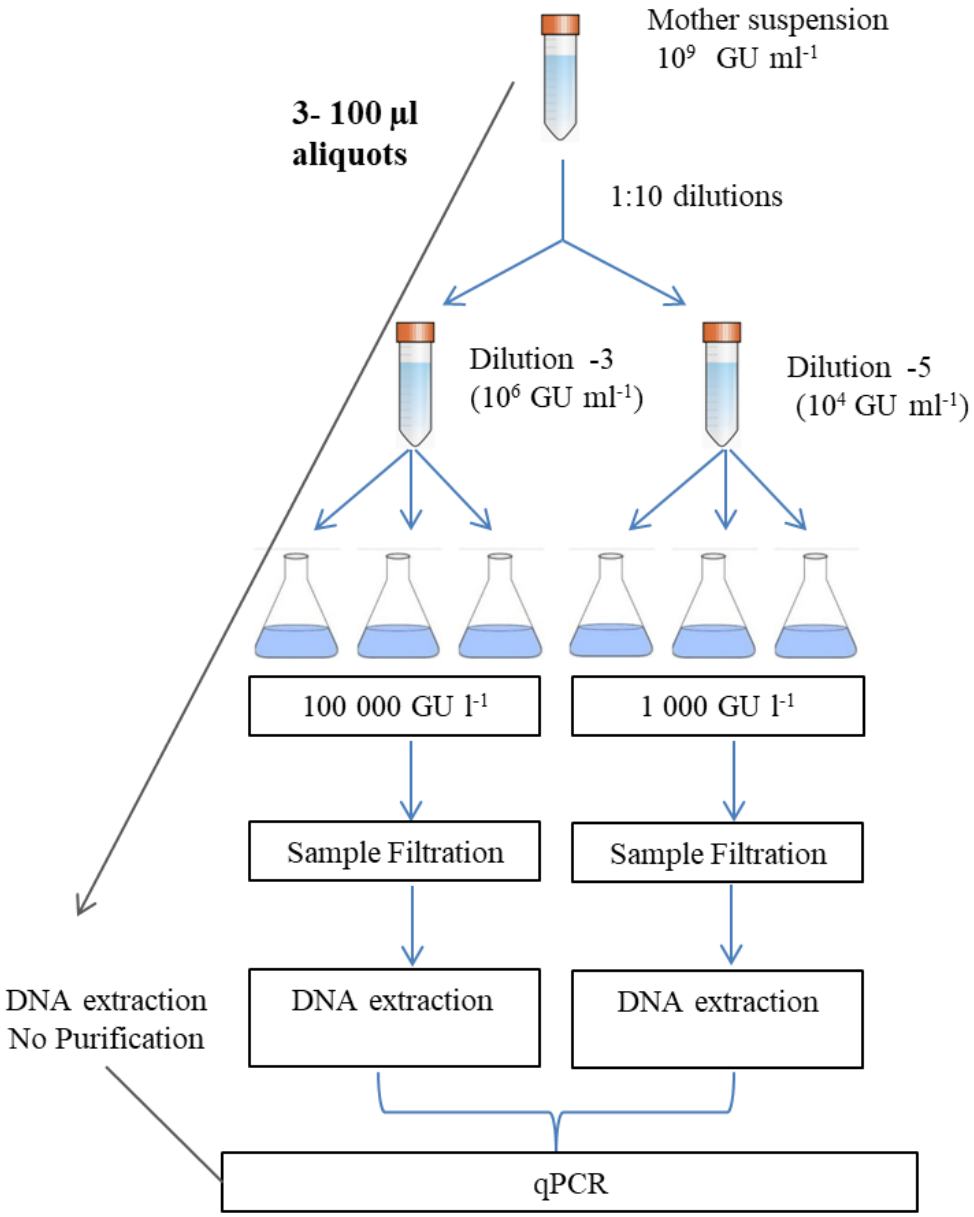

Figure 9: Example of a recovery study according to ISO/TS 12869.

The recovery for each sample was determined using the following formula:

$$\log_{10}\eta_x = B - A + D + \log_{10}1000/V_{pe}$$

where:

$Log_{10}\eta x$ is the decimal logarithm of recovery for sample x;

A is the reference value for the concentration of the mother suspension, expressed as a decimal logarithm of the number of genome units per milliliter;

V_{pe} is the volume of the spiking suspension, in microliters, μL;

B is the value measured from the spiked sample, expressed as a decimal logarithm of the number of genome units per sample;

D is the decimal logarithm of the dilution factor between the mother suspension and the spiked suspension, considering a value 3 for the 10 0000 GU level and 5 for the 1 000 GU level.

2.3 Results and discussion

Tests conducted for the calculation of the recovery and robustness of the DNA extraction methods evaluated were performed in order to verify the efficiency and the "matrix effect". Seventy-five water samples (500 mL) collected from different water sources were artificially contaminated with two different concentration levels under intermediate precision conditions (over several days, by several technicians). The calculated recovery values for both contamination levels are reported in Table 8-9-10-11.

Results were expressed as recovery rates, calculated as percentages of *Legionella* GU detected in the spiked samples respect to the contaminating suspension. The two commercial kits showed recovery rates <25% in almost all water matrices: the in-house method protocol A showed a mean recovery value of 26.5% while the protocol B showed the best results in terms of recovery rates corresponding to a mean value of 72%, in compliance with ISO/TS 12869 requirements (25-225%).

Table 8: Recovery calculated for *Legionella* DNA extraction kit for each sample tested.

Level	Recovery % for each matrix						Mean	s^2
	Distilled water	Domestic hot water		Sanitary hot water				
10^5 GU/L	56% 55%	5% 5% 5%	7% 7% 9%	5% 5% 8%	10% 12%		22%	0.26
10^3 GU/L	83% 56%	Not tested		Not tested				

Table 9: Recovery calculated for Bacterial DNA Isolation Single Step for each sample tested.

Level	Recovery % for each matrix				Mean	s^2
	Distilled water	Domestic hot water	Sanitary hot water	Thermal water		
10^5 GU/L	79% 65%	25% 38% 16%	22% 20% 32%	14% 8% 15% 16% 12% 11%	31%	0.22
10^3 GU/L	70% 26%	Not tested	Not tested	37% 60%		

Table 10: Recovery calculated for in house method protocol A for each sample tested.

Level	Recovery % for each matrix						Mean	s^2
	Distilled water	Mineral water	Cooling tower	Sanitary hot water	Thermal water	Sulphurous water		
10^5 GU/L	Not tested	26% 27% 24%	27% 27% 35% 28%	23% 26% 20% 32% 33% 31% 19%	Not tested	19% 20% 24% 21% 25%	26.5%	0.09
10^3 GU/L	53% 46% 45% 28%	13% 6% 18% 24% 26%	Not tested	Not tested	24% 26%	Not tested		

Table 11: Recovery calculated for in-house method protocol B for each sample tested.

Level	Recovery % for each matrix				Mean	s^2
10^5 GU/L	Domestic water	Mineral water	Cooling tower	Sanitary hot water	72%	0.36
	60%	56% 32%	90%	53% 59%		
10^3 GU/L	Not tested	50% 73%	163% 119%	54% 50%		

2.4 Conclusions

The recovery mean calculated for the in-house method protocol B corresponded to 72%, therefore this procedure represents a suitable method for the analysis of water from cooling towers and other complex water samples in which high concentrations of substances that can impact DNA extraction and inhibit PCR may be expected.

Chapter 3. Development and optimization of a viability PCR method

3.1 Introduction

Only the viable population of *Legionella* spp. represents a risk to public health. Among the molecular techniques available to proof the *Legionella* viability, the v-PCR represents a very promising approach. Up to date the v-PCR protocols proposed in the literature based on EMA and PMA dyes have never been completely efficient in the suppression of the dead cell signal and validated for routine use. Moreover, some water disinfection procedures (eg. UV exposure) commonly applied in water distribution system of Hospital or thermal water pool do not affect immediately the integrity of cell membrane.

The approach that has been undertaken in this study is therefore based on the new dye PEMAX able to enter in both membranes compromised and metabolically inactive and nonculturable cells with intact membranes [Seidel *et al.*; 2017].

Therefore, the second step of the present research concerned the development of viability PCR protocol using PEMAX dye and the alternative strategies to increase the efficiency of the treatment (IMS and treatment with FDRS). In a first instance we evaluated and optimized several parameters that influence the outcome of the results, such as dye concentration, buffers used, incubation conditions, contact mode with dye (cell suspension or cells on filters), light exposure, the presence of a high number of dead cells and the influence of the matrix on the protocol. In the second step of the present study we examined the suitability and the effectiveness of the developed method on field water samples collected from a local Hospital and a health spa, in which is more likely to find people at high risk of legionellosis. The availability of a rapid method for the detection of live *L. pneumophila* cells is fundamental for the prevention of infection in those locations.

3.2 Material and Methods

3.2.1 Bacterial strain and preparation of the viable and dead *L. pneumophila* cells

L. pneumophila ATCC33152 supplied by the DSMZ (Braunschweig, Germany) were rehydrated, collected and maintained in culture as recommended by NFT90-471 and ISO/TS 12869:2012. This strain was cultured in Nutrient Broth and on Buffered Charcoal Yeast Agar supplemented with *Legionella* BCYE Growth Supplement (Oxoid) at 37°C with 5% CO2 for 24-48 h. To obtain a bacterial suspension of live *L. pneumophila* in log phase, 2-3 colonies were inoculated on 50 mL of BYEα broth (1 g/L Yeast extract + *Legionella* BCYE growth supplement) (Oxoid) and incubated at 37°C with shaking at 120 rpm overnight. Five hundred µL of culture was inoculated into fresh 9.5 mL of BYEα broth and incubated at 37°C with shaking until the culture reached an OD_{650} of 0.1, approximately corresponding to 0.7 x 10^8 CFU/mL. Ten-fold dilutions were prepared in Ringer solution (1/40) in order to obtain the desired bacterial amount. This suspension was divided in two fractions, one of which was heat-treated at 85°C for 30 minutes to obtain the dead cell suspension, and the other (live fraction) immediately used for the experiments. Cell viability was checked by plating 100 µL in BCYE Agar plate incubated for 10 days at 37°C + 5% of CO_2.

3.2.2 Development and optimization of PEMAX dye pre-treatment

In order to evaluate the possible cytotoxic effect and select the best conditions for dye penetration without affecting live cells, preliminary tests were carried out using different concentrations of PEMAX dye and buffers to be used for sample incubation. The PEMAX dye was resuspended in 500 µL of PCR Grade Water (Thermofisher Scientific) to obtain a 2 000 µM stock solution and aliquots stored at -20°C protected from light.

Different amount of PEMAX dye were directly added to viable or dead *L. pneumophila* cultures prepared as described above (2.5 x 10^5 CFU/sample) to obtain PEMAX concentration of 12.5 µM and 25 µM in a final volume of 500 µL. Three different buffers were evaluated for each PEMAX dye concentration: BYEα broth, Standard buffer 1X

(Geniul) and a buffer composed of BYEα broth + 0.5% (v/v) DMSO (Sigma). Cells were exposed to PEMAX dye for 60 minutes at room temperature with occasional mixing, then photoactivated for 15 minutes by using Blu-V System (Qiagen) at 100% light intensity. To remove the unbound dye, the samples were centrifuged at 10 000 *xg* for 5 min and cell pellet washed in a solution of PBS 1X followed by centrifugation at 10 000 *xg* for 5 min prior to DNA extraction and qPCR analysis. In a second experiment, the incubation time with the dye was also evaluated by comparing 60 min to 20 min. Live and heat-treated cells suspensions not exposed to PEMAX treatments, directly extracted and analysed in qPCR, served as controls.

3.2.3 Evaluation of PEMAX pre-treatment directly on membrane filter or cell suspension on artificially contaminated mineral water samples

A comparison of PEMAX pre-treatment directly applied on membrane filter (Protocol α) and on cell suspension (Protocol β) was assessed on a total of 24 mineral water samples (free of nucleic acids of *L. pneumophila*) artificially contaminated with a suspension of live or heat-treated *L. pneumophila* ATCC33152 cells corresponding to a final concentration of 2.5 x 10^4 CFU/L. All water samples were filtered using 0.22 µM polycarbonate membrane filter (Merck Millipore). In the exposure protocol α, 1 mL of PEMAX solution 12.5 µM in Standard buffer 1X was directly applied to the filter, followed by incubation in the dark for 20 min at room temperature with occasional mixing and photoactivation using the PAUL (Photo Activation Universal Light) Photoactivation System (Geniul) for 15 minutes at 100% intensity. Filters were then overlaid and washed by filtration of 50 mL of PBS 1X. Genomic DNA was directly extracted from the filter and quantified in qPCR.

In exposure protocol β, after water filtration the membranes were placed in a sterile 50 mL tube containing 1.5 mL of Ringer Solution (1/40), scraped using a sterile loop and vortexed for 30 seconds. The resulting cell suspensions were centrifuged at 10 000 *xg* for 10 minutes. Finally, the cells pellets were washed in a solution of PBS 1X followed by centrifugation at 10 000 *xg* for 5 min prior to PEMAX dye pre-treatment, DNA extraction and qPCR analysis, like for samples processed with protocol α. Four samples contaminated with live and heat-treated cells suspensions but not exposed to PEMAX treatments directly extracted and analysed in qPCR served as controls.

3.2.4 Selectivity of the PEMAX-qPCR method in the quantification of live *L. pneumophila* cells

The optimized PEMAX-qPCR protocol was applied on pure cultures of mixed viable and heat-treated cells to evaluate if the PEMAX-qPCR based quantification of viable *L. pneumophila* could be affected by the presence of a background of dead cells of the same species.

Pure cultures containing serial dilutions of dead *L. pneumophila* cells from 3.1×10^6 to 3.1×10^4 CFU/sample were mixed with a constant number (3.1×10^4 CFU/sample) of live *L. pneumophila* cells. These suspensions corresponded to ratios live:dead bacteria of 1:1, 1:10 and 1:100. As controls, separate samples containing 3.1×10^4 CFU/sample of only live or dead cells were also extracted and analysed in qPCR. The experiment was conducted in duplicate.

3.2.5 Robustness of the PEMAX-qPCR method in the quantification of live *L. pneumophila* cells in artificially contaminated cooling tower water

The optimized PEMAX-qPCR method was evaluated on ten cooling tower water samples artificially contaminated with a suspension of live or heat-treated *L. pneumophila* ATCC33152 cells corresponding to a concentration of 2.5×10^4 CFU/200 mL samples. As controls, four water samples were analysed without viability treatment.

3.2.6 DNA extraction and qPCR enumeration of *Legionella* spp. genome units

Genomic DNA was extracted directly from the filter using the in-house method protocol B developed in previous Chapter of the present document.

DNA extracted were analysed by qPCR using *Legionella* spp. quantitative kit and DI-Check *Legionella pneumophila* kit (Diatheva) following the manufacturer's instructions. The last kit meets the specification of ISO/TS 12869:2012, the detection limit was 5 GU

per well and the detection limit of the whole method was 320 GU when 1 liter of water sample is filtered. The quantification limit was 25 GU/PCR well corresponding to 1 600 GU/L.
All PCR amplification were conducted on QuantStudio 3 and QuantStudio 5 (Thermofisher Scientific) using the provided analysis software version 1.4.3.

3.2.7 Application of the developed method on field samples

Seven cooling tower water samples and five thermal water samples were collected respectively from a local Hospital and from thermal pools of a spa. Water samples were collected aseptically using sterile 1 L containers containing sodium thiosulphate 20 mg/L to neutralize chlorine and transported immediately at room-temperature. Samples were received in the laboratory within 12 h and immediately used for the analysis. One 1 L-aliquot was examined according to culture method ISO 11731:2017, and the other aliquots of 500 mL each were tested by qPCR with and without viability pre-treatment. For each method the samples were tested in duplicate.

3.2.8 Evaluation of alternative strategies to increase the efficacy of the developed protocol

Immunomagnetic separation step

In a first instance, to increase the efficiency of the developed protocol, an IMS of *L. pneumophila* serogroup 1 cells using *Legionella pneumophila* SG1 kit (rqmicro) was tested prior to apply the PEMAX dye treatment (IMS-vPCR). Twelve field sanitary water samples collected from hot water distribution systems of a local Hospital were tested in parallel with four methods: IMS-qPCR, IMS-v-PCR, v-PCR and 1-liter aliquot with culture method ISO 11731.
For all protocols with IMS, water samples (100 mL) were filtered using 0.22 µm polycarbonate membrane filter, the membrane was then transferred into 50 mL tube

containing buffer 1 provided in the kit and vortexed for 60 seconds in a horizontal position. Prior to IMS step, the whole volume was passed through cell Strainer. In the IMS-vPCR protocol, the PEMAX pre-treatment was performed at the end of the immunomagnetic separation step, resuspending the purified magnetic beads in 500 µL of the PEMAX dye solution. The IMS step was performed following the manufacturer's instruction (Figure 10).

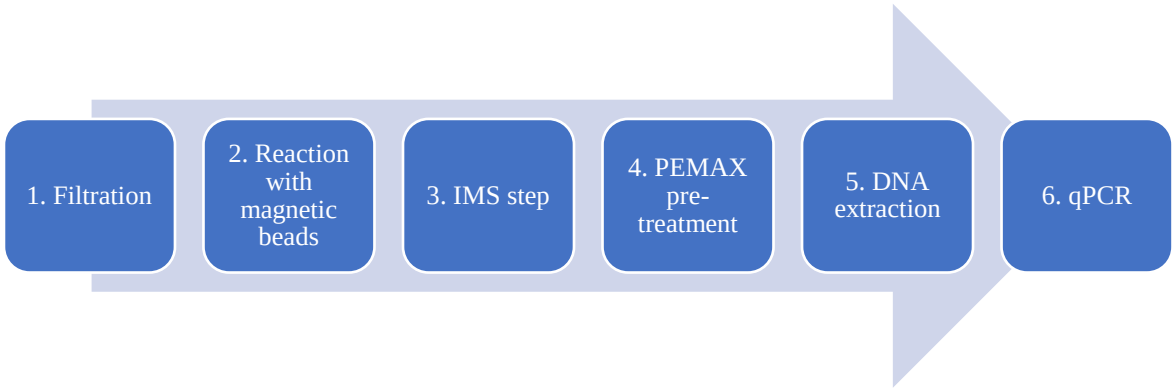

Figure 10: Workflow of IMS-vPCR protocol.

FDSR - Removal of free genomic DNA

To investigate the possible influence of free DNA in the efficacy of v-PCR protocol, an alternative strategy not based on v-PCR was assessed. The FDRS (Biorad) was previously evaluated on ten mineral water samples artificially contaminated with suspensions of live and heat-treated *L. pneumophila* ($1x10^5$ CFU/L) and five mineral water samples contaminated with a suspension of purified *L. pneumophila* genomic DNA ($1x10^5$ GU/L). Samples were analysed in parallel with by FDSR method and qPCR.

Briefly, after water filtration using 0.45 µm polycarbonate membrane filter, the filter was folded to form a cone and transferred into 1.5 mL tube containing 450 µL of *Legionella* DNA free water and 40 µL of FDRS. The tube was then inverted to mix several times and incubated at 37°C for 30 minutes. To inactivate the solution, the sample was then lysed according to Aquadien protocol (Biorad) and 5 µL analysed by qPCR in duplicate using DI-Check Legionella pneumophila (Diatheva) (Figure 11). The protocol was then tested on four field cooling towers water samples and five sanitary water analysed in parallel with FDRS method, qPCR and culture method.

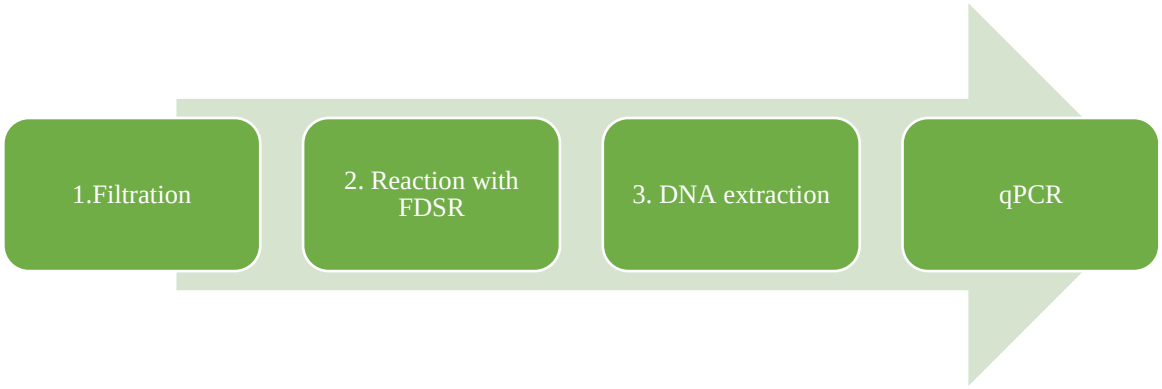

Figure 11: Workflow of FDRS method.

3.3 Results and discussion

3.3.1 Optimization of PEMAX-qPCR treatment

A critical step in the v-PCR procedure is the condition used for sample incubation with the dye: during this phase it is important to maximize the reagent diffusion through dead cells and at the same time maintain the optimum conditions such as nutrient, pH and ionic strength to prevent viable cells damage [Codony *et al*., 2015].

Aliquots of pure cultures of live and heat-treated *L. pneumophila* ATCC33152 cells were exposed to different concentration of PEMAX dye in three buffers: BYEα broth, Standard buffer 1X (Geniul) and a buffer composed of BYEα broth + 0.5% (v/v) DMSO. DMSO is well known to affect permeability of cell membranes and, as demonstrated by Seidel and co-workers, may improve the efficiency of dye treatment [Seidel *et al*., 2017].

Like for all subsequent experiments, the inhibitory effect of PEMAX dye on DNA amplification has been expressed as $\Delta GU(Log_{10})$ calculated by subtraction of GU (log10) quantified by PEMAX-qPCR from GU of qPCR w/o treatment in parallel examined samples (Figure 12).

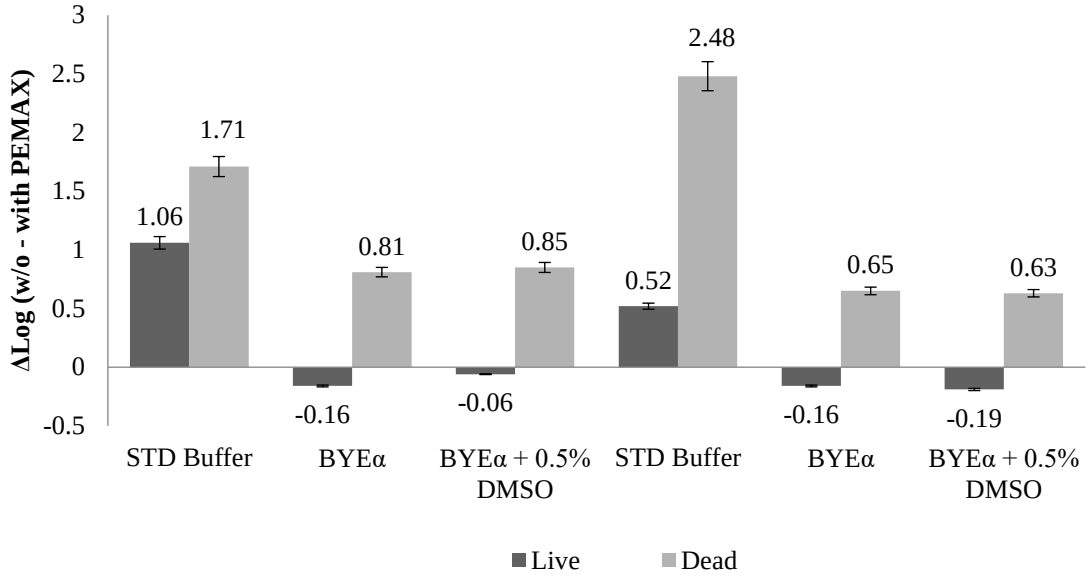

Figure 12: Results obtained from optimization experiments of PEMAX concentration and buffer used for sample incubation on pure culture of *L. pneumophila* live and heat-treated (2.5 x 10^5 CFU/sample). Error bars represents standard deviation.

In all samples contaminated with heat-treated bacteria, pre-treatment with PEMAX dye was able to inhibit DNA amplification, as expected. The presence of DMSO did not determine a significant difference in samples incubated in BYEα. The highest inhibition, instead, was observed in samples incubated in Standard buffer. However, in these conditions a signal inhibition proportional to dye concentration (25 vs 12.5 μM) has been noticed in both dead and living bacteria, and a possible unspecific entry of the dye also in living cells should be hypothesised as reported by other authors for PMA [Nkuipou-Kenfack *et al.*, 2013] and EMA [Nocker *et al.*, 2006].

Nevertheless, the reduction of incubation time at 20 min, instead of 60, was helpful in limiting the unspecific inhibition in living cells, and in the meantime increased amplification reduction in dead bacteria (Figure 13).

Therefore, the following conditions have been used for all subsequent experiments: 12.5 μM PEMAX dye in Standard buffer 1X solution incubated for 20 min at room temperature.

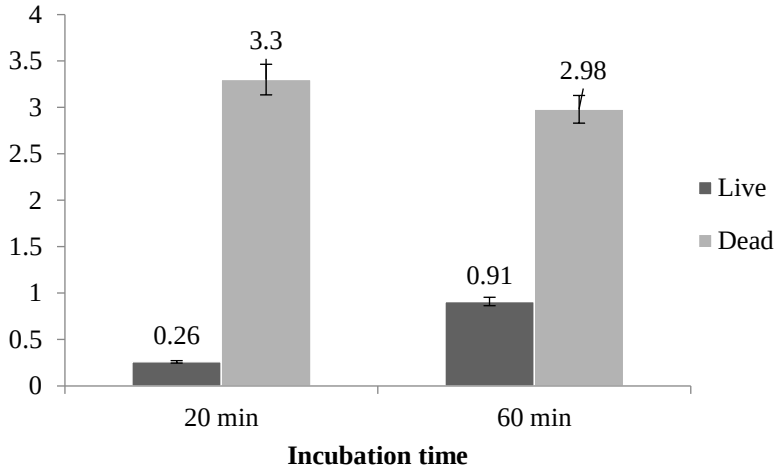

Figure 13: Results obtained from optimization experiments of incubation time on pure culture of *L. pneumophila* live and heat-treated (2.5 x 10^5 CFU/sample). Error bars represents standard deviation.

3.3.2 Evaluation of PEMAX pre-treatment directly on membrane filter

A second series of experiments was conducted with the aim to evaluate if the treatment with PEMAX dye was compatible with an initial filtration step, which is always the first phase in standardised ISO protocols for water analysis. The objective was to assess if the exposition to the dye and the subsequent photoactivation could be performed directly on the membrane filter, without bacterial detachment. Indeed, water filtration is an important way to concentrate samples and to increase detection sensitivity, and a method permitting the viability dye treatment directly on the membrane may be very useful to avoid bacterial loss, maximise recovery and reduce sample processing time. Two exposure protocols were evaluated: in protocol α PEMAX dye solution was directly on filter membranes, while on exposure protocol β the PEMAX-qPCR protocol was applied on cell suspensions.

These tests were made on 24 mineral water samples artificially contaminated with live or heat-treated (12 samples each) *L. pneumophila* cells. Mineral water was chosen for these experiments, although different in its composition from real samples of sanitary and thermal water used in this study for final method assessment, since reasonably supposed free from *L. pneumophila*, thus ideal matrix for artificial contamination.

The *L. pneumophila* GU per sample calculated by each exposure protocol are summarized in Table 11.

Table 11: Results of experiments with mineral water artificially contaminated with 25 000 CFU/L of *L. pneumophila* (4.4 $\log_{10}$/L) and PEMAX dye sample treatment on filter membrane (Protocol α) or in cell suspension (Protocol β).

Sample #	***L. pneumophila* suspension**	**Protocol α GU ($\log_{10}$)/L**	**ΔGU ($\log_{10}$)**	**Protocol β GU ($\log_{10}$)/L**	**ΔGU ($\log_{10}$)**
1	Live cells	3.74	1.68	4.40	1.15
2		3.84	1.58	4.31	1.24
3		3.83	1.59	4.24	1.31
4		4.75	0.67	4.38	1.17
5		4.54	0.88	4.43	1.12
6		3.46	1.96	4.38	1.17
Average ± SD		4.03 ± 0.50		4.36 ± 0.07	
7	Heat treated cells	2.74	2.65	nd	5.55
8		2.92	2.47	nd	5.55
9		2.65	2.74	nd	5.55
10		2.51	2.88	nd	5.55
11		2.98	2.41	nd	5.55
12		2.71	2.67	nd	5.55
Average ± SD		2.75 ± 0.17			

When the PEMAX dye was applied directly on membrane filters, in live cells (samples 1-6) the treatment gave not reproducible results and was more toxic, with a $\Delta\log_{10}$ value of 1.39 respect of when applied on cell suspensions ($\Delta\log_{10}$ value of 1.2). Moreover, with dead cell samples (samples 7-12) the efficiency of exposure protocol α was lower compared with that of exposure protocol β, that showed an efficiency of 100% in signal reduction of dead cells.

Considering the above results, working with samples in cell suspension was chosen as the most suitable condition for use.

3.3.3 Effect of PEMAX on defined ratio of viable and dead cells and on artificially contaminated water samples

The ratio between dead and viable cells is an important parameter to consider because it can affect the efficiency of the protocol by lowering the concentration of available dye

molecules per cell (Fittipaldi *et al*., 2012). The influence of dead *L. pneumophila* cells in the quantification of viable *L. pneumophila* by PEMAX-qPCR was assessed.

Increasing numbers of heat-killed *L. pneumophila* cells (3×10^4 to 3×10^6 CFU/sample) were mixed in defined ratios with a constant number of live *L. pneumophila* cells (3.1×10^4 CFU/sample).

In the presence of different amounts of dead cells (1:1, 1:10, 1:100 live:dead cell ratio), the PEMAX-qPCR method was able to quantify approximately the same amount of live cells (Figure 14), showing no interference by dead cell DNA.

These results underlined that the amplification signal (and the resulting quantitative data) derived almost uniquely from DNA of living cells, and the contribution of dead cell DNA was minimal.

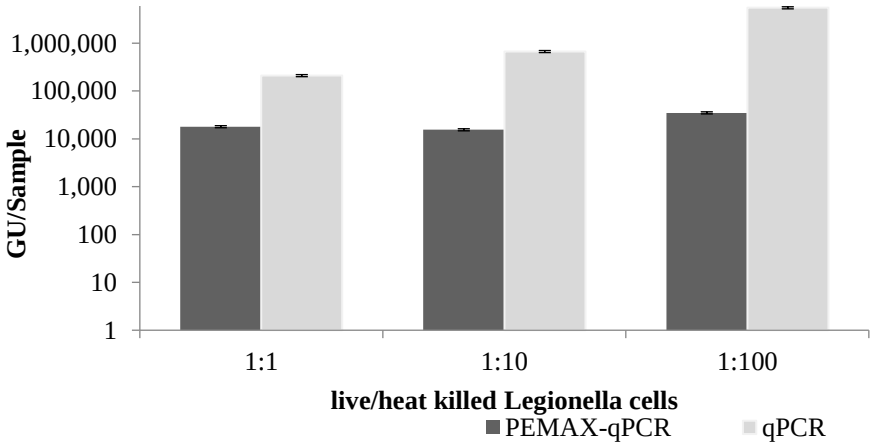

Figure 14: Influence of dead *L. pneumophila* on the quantification of the viable count of *L. pneumophila* by PEMAX-qPCR. Mixtures of a fixed number of viable *L. pneumophila* with increasing numbers of heat-killed *L. pneumophila*

3.3.4 Robustness of the PEMAX-qPCR method in the quantification of live *L. pneumophila* cells in artificially contaminated cooling tower water

Complex matrices as commonly found in environmental samples, food, or clinical samples can negatively influence the efficiency of EMA/PMA treatment (Kramer *et al*., 2009). Apart from lowering the effective dye concentration by chemical adsorption, organic and inorganic compounds can interfere with photoactivation. Different factors including turbidity, pH, salt, have the potential to interfere with v-PCR results. For this reason the developed protocol was tested on artificially contaminated cooling tower water to study the effect of the matrix of the efficiency of the protocol [Fittipaldi *et al*., 2012].

Cooling tower water samples were artificially contaminated with variable concentrations of live and dead *L. pneumophila* cells. Results in Table 12 indicate that, although the PEMAX-qPCR method was able to inhibit the amplification of DNA of dead cells, with log reduction similar to those obtained in experiments with bacterial cultures (Figures 9 and 10) it also determined a moderate inhibitory effect on the amplification of DNA of living bacteria. This result could be ascribed to a possible unspecific entry of the dye in some of these cells, or otherwise could be explained with the presence of a small proportion of bacteria with damaged membrane/cell wall.

Table 12: Results obtained on artificially contaminated water samples.

Sample type		**Average Ct ± SD**	**Log_{10} GU/sample**
Live cells	qPCR	28.70 ± 0.04	4.74 ± 0.06
	PEMAX qPCR	35.17 ± 0.20	2.90 ± 0.15
	Δ Log (w/o – with PEMAX)		1.84
Dead cells	qPCR	28.17 ± 0.07	4.93 ± 0.01
	PEMAX qPCR	38.26 ± 0.70	2.22 ± 0.37
	Δ Log (w/o – with PEMAX)		2.71

3.3.5 Results of the application of PEMAX-qPCR and alternative strategies on field samples and comparison with qPCR and culture

Water from air cooling towers and thermal water pools (n=12) were analysed using either the standard culture method ISO 11731, qPCR and PEMAX-qPCR. Bacteriological analysis was not able to detect *L. pneumophila* in all sample tested (<100 CFU/L). However, the PEMAX-qPCR method quantified a high contamination level corresponding to 5 x 10^5 GU/L for water pools and 4.4 x 10^7 GU/L without any signal reduction respect to qPCR controls.

The PEMAX-qPCR method showed good results in inhibiting DNA amplification of dead bacteria in pure culture and artificially contaminated water samples, however on field samples in which *Legionella* spp. is not grown under laboratory condition the method was not able to quantify only the live cell fractions.

The discrepancies between culture and PEMAX-qPCR could be due to a number of reasonable explanations: (1) culture method may underestimate bacterial number due to the presence of VBNC cell (2) the presence of a high amount of free-DNA and/or a background microflora that affect the accessibility of the cells to the dye.

To exclude the influence of a background microflora, a purification step by IMS was applied prior to PEMAX-qPCR protocol: on culture positive water samples (200 CFU/L) the IMS-qPCR quantified 1.9 x 10^4 GU/L with a slightly decrease respect to qPCR control, helping in the reduction of dead cells signal. However, when the protocol was tested on culture negative samples, the IMS-vPCR quantified a high amount (10^4 GU/L) of cells without any signal reduction respect to qPCR controls. This strategy was not more undertaken as it not offers significant advantages, the protocol was labour-intensive and time-consuming.

Free DNA derived from *Legionella* spp. or other microbial species potentially existing in water sample may interfere with the PEMAX-qPCR protocol as even if not clear it appears plausible that high numbers of dead cells with a high capacity of taking up dye would lower the concentration of available dye molecules per cell [Fittipaldi *et al*.; 2012].

The final attempt was the application of FDRS prior to DNA extraction and qPCR amplification to remove free DNA potentially present in field water samples previously analysed with PEMAX-qPCR.

The FDRS method was initially assessed on artificially contaminated water samples: comparing with qPCR controls, the FDSR protocol achieved a strong signal reduction when tested on water samples contaminated with genomic DNA (100% of suppression).
On the contrary, in sample contaminated only with heat-treated *L. pneumophila* cells, the *Legionella* DNA was quantified with negligible reduction (0.8 log) respect to qPCR controls. This result suggests that the killing method at 85°C for 30 minutes does not completely release DNA from cells, resulting the FDRS protocol unhelpful. On samples contaminated with live cells, a 0.5 log difference respect to qPCR controls was evidenced, showing a modest toxicity for cells probably due to the presence of CMIT/MIT that affect microbial's growth.
On field sanitary water samples resulted culture positive (2.5×10^4 CFU/L), the FDRS protocol may have helped to eliminate dead cell-free DNA, resulting in 1 log reduction respect to qPCR control. No difference was observed between FDRS protocol and qPCR, in cooling tower water samples resulted culture negative, allowing to exclude also the influence of free DNA in the quantification of live cell by v-PCR in field samples.

3.4 Conclusion

Environmental monitoring represents a fundamental tool for the control of *Legionella* spp. infections and the availability of rapid method such as qPCR could be of great value for the rapid identification of contaminated water sources. The possibility of live/dead differentiation may lead to a better estimate of sanitary risk and is therefore an advantage for implementing qPCR in routine analysis. Overall, simply applying a dye pre-treatment to PCR from our study was not possible to quantify reliably viable *Legionella* spp. cells in environmental water samples and new approaches were evaluated.

Chapter 4. Development and optimization of a nutritional stimulation method

4.1 Introduction

Assessing bacterial contamination in environmental samples is critical in determining threats to public health. Growth and division are a widespread and accepted parameter for the detection of bacterial viability. The Molecular Viability Testing (MVT) uses the ability to synthesize macromolecule in the presence of nutrition by bacterial cells. Detection of RNA and especially of the highly unstable mRNA thus tends to indicate the presence of live cells as messenger RNA (mRNA) is only produced by metabolically active cells, making mRNA suitable to specifically detect living microorganisms [Bleve *et al*., 2003]. However, due to its shorter average half-life and unstable nature it is a challenge to use mRNA as a marker to determine viability. rRNA precursors called pre-rRNA has a half-life of days, which is much longer than mRNA and accounts for 90% of the total cellular RNA. However, rRNA has been reported to persist in dead bacterial cells [Cangelosi *et a*l., 2010; Weigel *et al*., 2017] and Boss and co-workers demonstrated that the DNA can be quantified by this procedure leading to overestimation of cells counts [Boss *et al*., 2018]. Moreover, the RNA extraction implicates a higher manual workload, degradation can occur by inadequate sample processing and storage or as a result of sample contamination with RNA-degrading enzymes. For all these reasons, RNA may not be the appropriate macromolecule to detect for a PCR based method aimed to the detection of pathogen for routine base testing [Fittipaldi *et al*., 2012].

The approach evaluated in the present study exploited the detection of *Legionella* spp. and *L. pneumophila* DNA after a microbial enrichment of filtered water samples. This procedure called "nutritional stimulation" consists in six steps: (1) water samples filtration, (2) bacterial detachment from membrane filter and preparation of non-stimulated aliquot (-STIM) (3) enrichment in specific liquid media for *Legionella* spp. (4) sampling of

stimulated aliquot (+STM) (5) DNA extraction (6) qualitative real-time PCR amplification. *Legionella* genomic DNA was detected in samples after the enrichment step and a shift between the cycle threshold (Ct) of an unstimulated and a stimulated aliquot of a sample interpreted as the presence of viable *Legionella* spp. cells. A scheme of the method is shown is Figure 15.

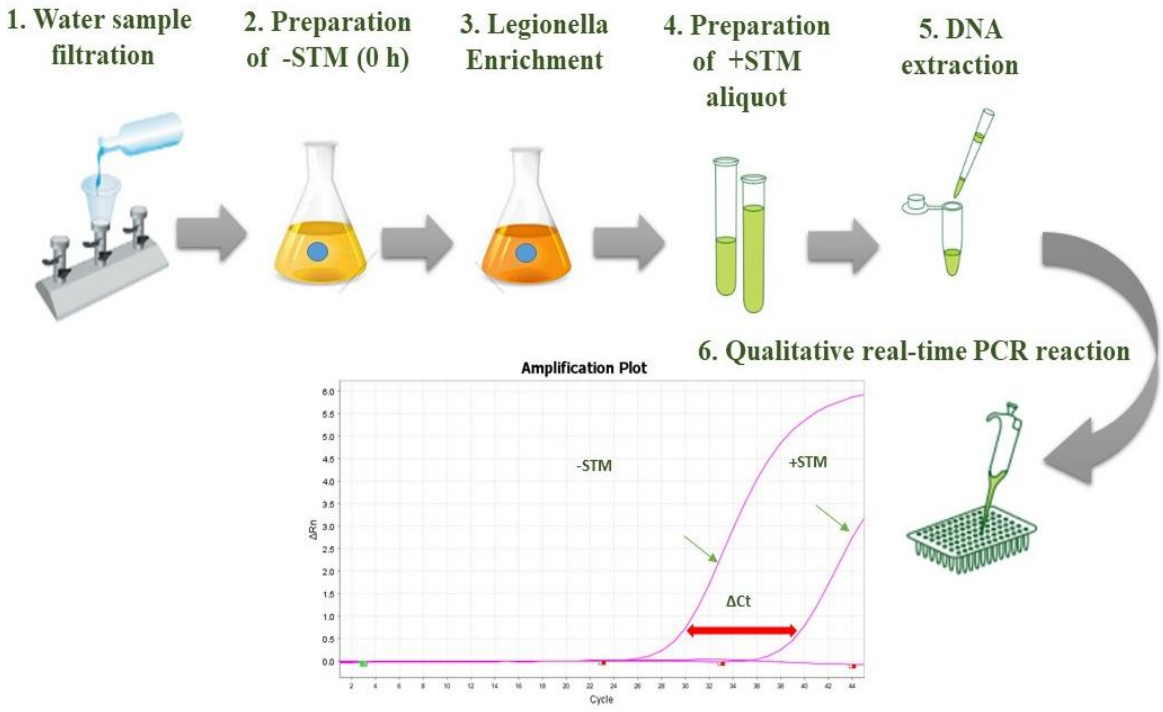

Figure 15: Scheme of the nutritional stimulation method for the qualitative detection of live *Legionella* spp. DNA from water samples.

4.2 Material and methods

4.2.1 Types of water samples analysed

Water samples (500 mL) were collected from domestic hot water system and from sanitary water samples into sterile 1 L containers containing sodium thiosulphate 20 mg/L to neutralize chlorine and transported immediately at room-temperature.

4.2.2 Bacteriological detection of *Legionella* spp.

Water samples were analyzed with the reference method ISO 11731. One liter of water was filtered through a 0.22 µm polycarbonate membrane filter and the bacterial cells was then resuspended by vortexing for 60 seconds, 100 µl aliquot of the samples was plated on BCYE agar and incubated for 10 days at 37°C + 5% of CO_2. Plates were evaluated three times during the incubation period. For colony confirmation, DI-check *Legionella pneumophila* kit or *Legionella* spp. quantitative kit was used analysing 2 µl of DNA directly extracted from isolated colony.

4.2.3 Detection of living *Legionella* spp. and *L. pneumophila* by DNA stimulation method

Since only living bacteria are able to multiply in culture and to detectably increase their concentration inducing their DNA synthesis, the "nutritional stimulation method" was developed for a qualitative detection of live *Legionella* cells.
Optimization experiment were performed on field sanitary and domestic water samples analysed in parallel with culture method.

The following parameters were optimized: two different liquid media (BYEα broth and GVPC broth), incubation times (24, 48, 72 h) and the introduction of a pre-treatment step at 50°C prior to the nutritional stimulation step.

Concentration. 500 mL of water samples were filtered through a 0.22 µm polycarbonate membrane. The membrane was then transferred into a 175 mL sterile flask containing 30 mL on pre-warmed broth and then vigorously mixed to detach cells. This concentrate was used to prepare the -STM aliquot (0 h): 1 mL aliquot in duplicate was transferred into a 1.5 mL and centrifuged at 10 000 *xg* for 5 minutes. The cells pellet was stored at -20°C until the molecular analysis.

Stimulation. For the preparation of stimulated aliquots, the sample was incubated at 37°C under shaking (120 rpm) for 72 h. During the incubation +STM aliquots were prepared in duplicate after 24, 48 and 72 h as for -STM aliquots.

Molecular detection of Legionella spp. *and L. pneumophila*. Cell pellets of -STM and +STM aliquots were resuspended in 500 µL of Instagene matrix (Biorad) and then genomic DNA extracted following the in-house method protocol B developed in Chapter 2 of the present document. Five µl of the DNA extracted were analysed by qPCR using *Legionella* spp. quantitative kit and DI-Check *Legionella pneumophila* kit (Diatheva) following the manufacturer's instructions in duplicated. All PCR amplification were conducted on QuantStudio 3 and QuantStudio 5 (Thermofisher Scientific) using the provided analysis software version 1.4.3.

4.3 Results and discussion

As starting point, BYEα broth was tested on sanitary water samples resulted naturally contaminated with *L. pneumophila* cells to a concentration of 300 CFU/L.
The genomic DNA were extracted from +STM and -STM aliquots and the DNA was analysed in real-time PCR with two PCR assays targeting a gene common for all *Legionella*

spp. (using *Legionella* spp. quantitative kit- Diatheva) or species-specific for *L. pneumophila* (DI Check *Legionella pneumophila* kit).

Results obtained from both assays suggested that 48 h incubation at 37°C (shaking at 120 rpm), allows to obtain a ΔCt (Ct of -STM– Ct +STM) between 3.23 and 2.23 (Figure 16) for *Legionella* spp. gene and a ΔCt between 9.82 and 7.28 (Figure 17) for *L. pneumophila*, respect the unstimulated aliquots (time 0). Furthermore, on culture negative sanitary water samples, no decrease of Ct values were detected in stimulated aliquots (ΔCt mean at 48 h - 0.86 ± 0.56) respect to unstimulated ones.

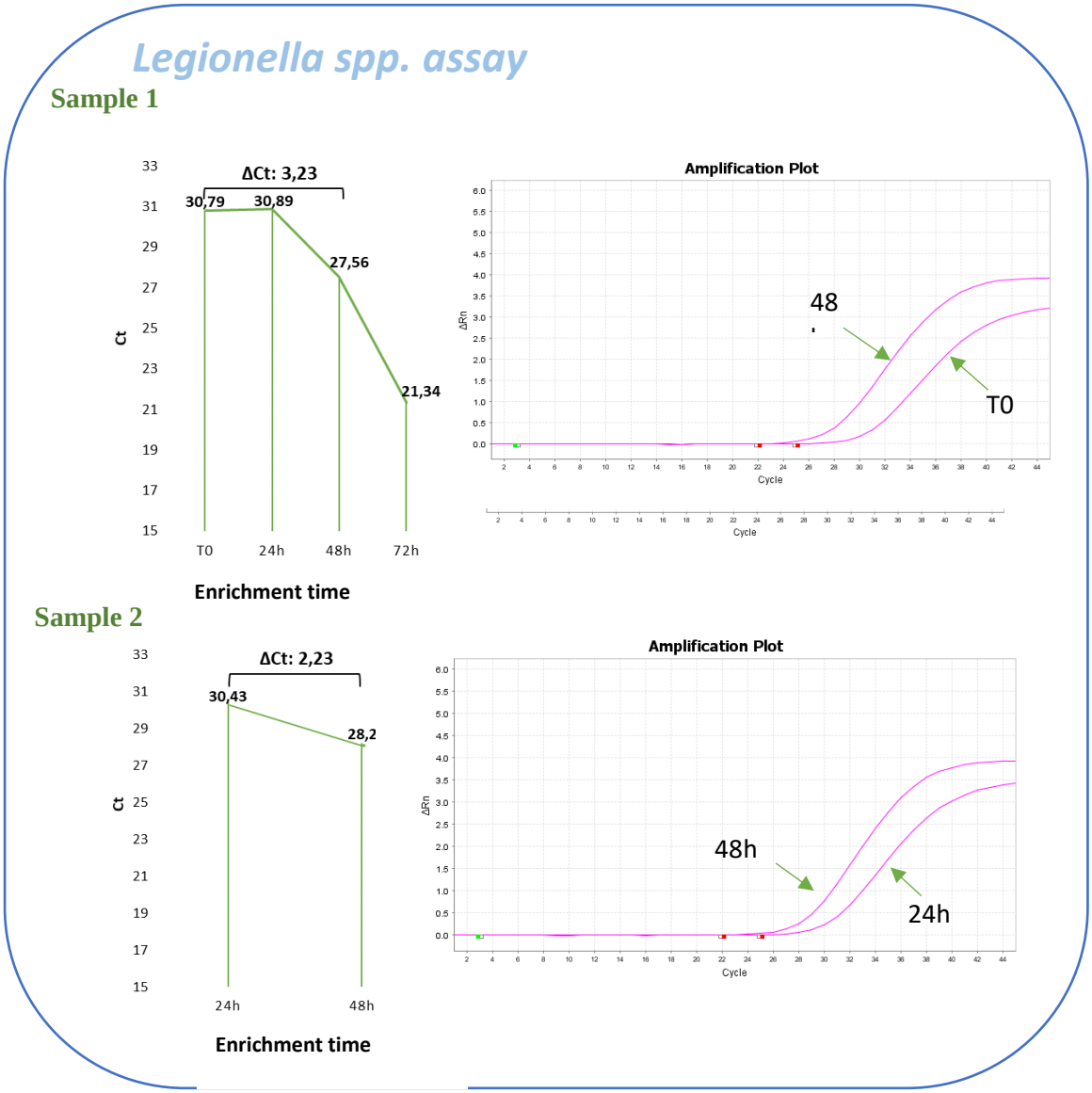

Figure 16: Results of nutritional stimulation method on *L. pneumophila* naturally contaminated samples (300 CFU/L) tested with *Legionella* spp. assay.

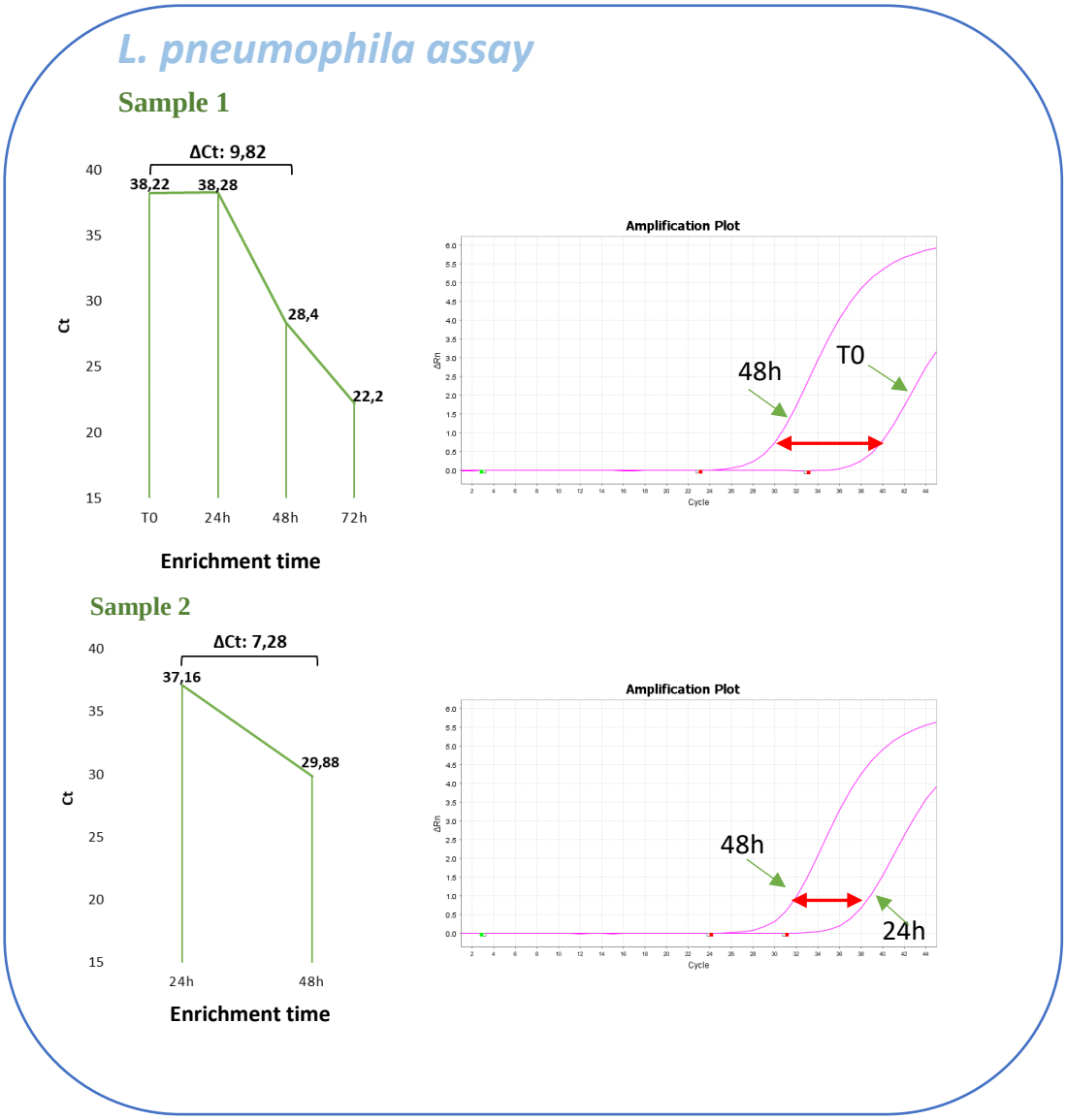

Figure 17: Results of nutritional stimulation method on *L. pneumophila* contaminated samples (300 CFU/L) by amplifying the DNA using *L. pneumophila* detection kit.

When the nutritional method was tested on 6 domestic water samples (500 mL) highly contaminated (10^4 CFU/L) with *L. pneumophila*, the stimulation for 48 h in BYEα broth resulted ineffective as no decrease of Ct were detected respect unstimulated aliquots. Moreover, the internal amplification control of the PCR reaction resulted strongly inhibited in most of the samples and 1:10 dilution of DNA extract was performed to obtain an amplification in the target channel. We hypothesize that this failure of the nutritional stimulation method could be due to a high level of microflora present in water samples.

Two different conditions were evaluated with the aim to inhibit contaminant microflora: (1) a pre-treatment of sample at 50±1°C for 30±2 minutes as recommended by ISO 11731 method, and (2) the use of GVPC supplement, a selective supplement that contains

antibiotic and antifungal substances. Comparing these protocols on a second sampling of the same domestic water samples culture positive for *L. pneumophila* (10^4 CFU/L) the pre-treatment at 50°C resulted ineffective. The results obtained by applying these two treatments are shown in Figures 18 and 19.

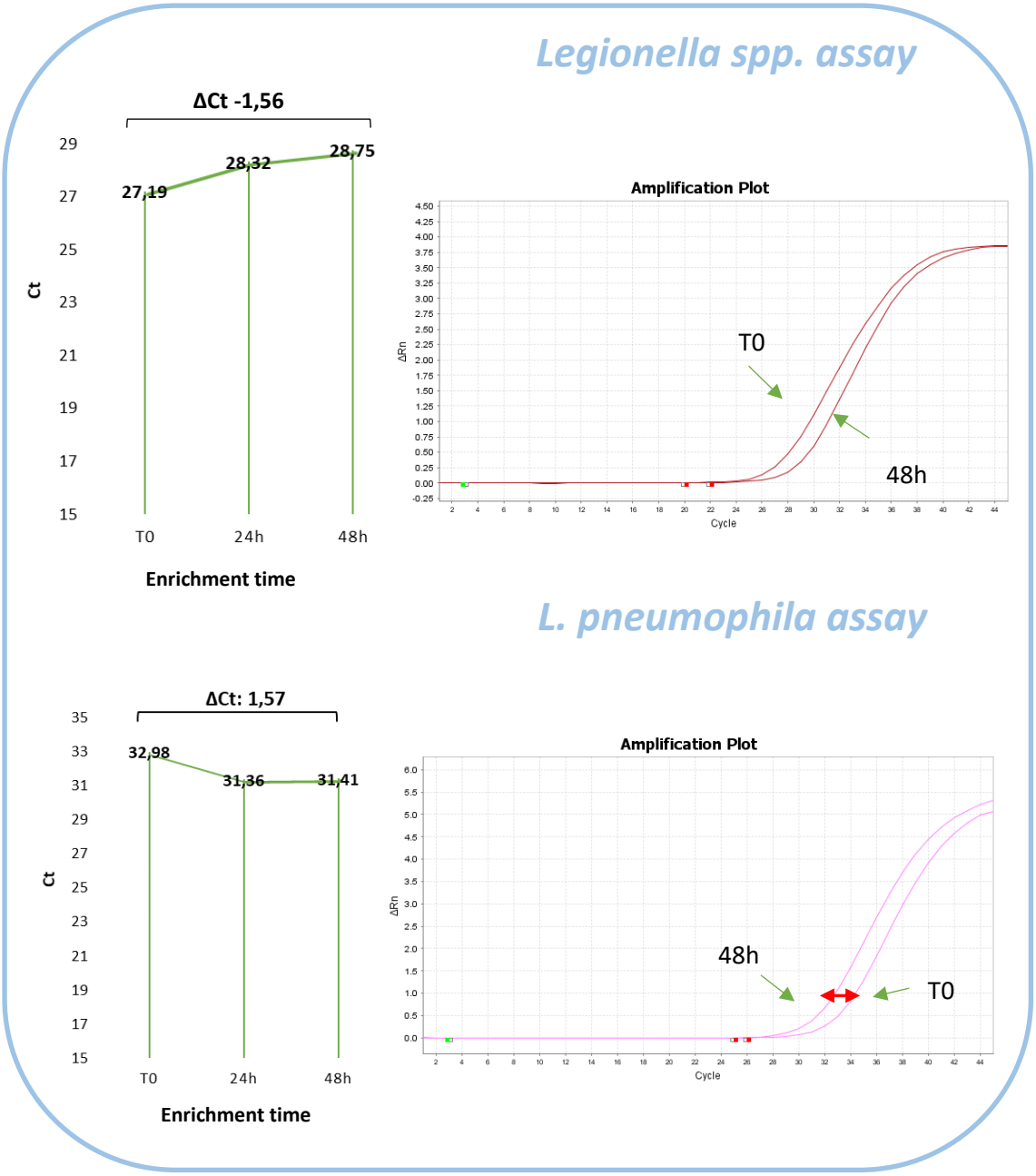

Figure 18: Results of pre-treatment at 50°C on domestic hot water sample contaminated with *L. pneumophila* (10^4 GU/L).

Using an enrichment broth supplemented with GVPC the results obtained with nutritional stimulation method and culture were in concordance, confirming the presence of viable *L.*

pneumophila cells. A ΔCt of 4.9 was detected when samples were amplified with the assay for *L. pneumophila*. Contrarily, a minimum ΔCt (1.33) was detected by amplifying samples with the *Legionella* spp. assay (Figure 19): a possible interference in the amplification by a large amount of dead cell DNA from non-*L. pneumophila* species that compete with *L. pneumophila* DNA may have impacted on amplification.

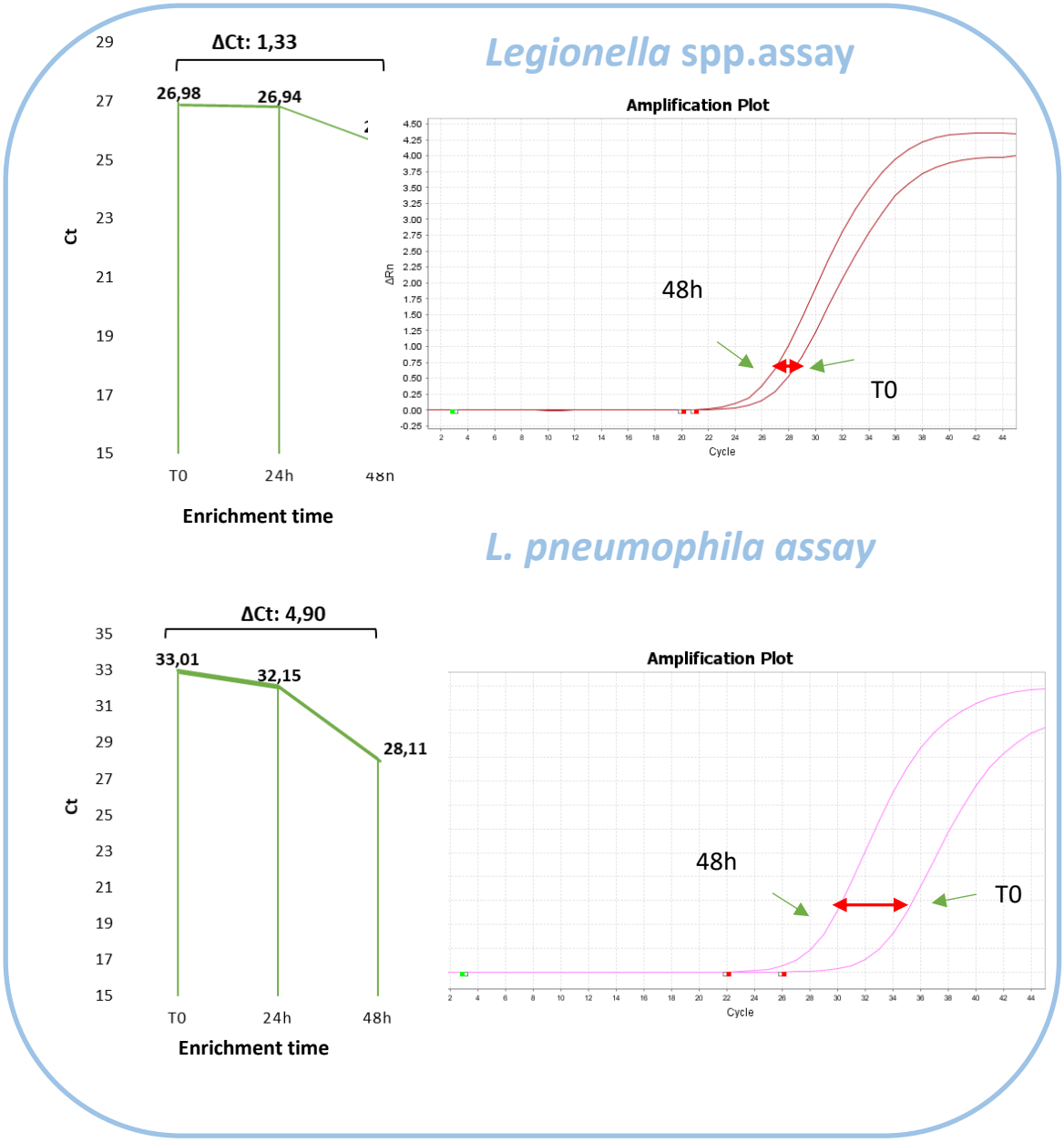

Figure 19: Results of nutritional stimulation with BYEα broth supplemented with GVPC on domestic hot water sample contaminated with *L. pneumophila* (10^4 GU/L).

Conclusion and future directions

Legionnaires' disease is an important cause of community-acquired and hospital-acquired pneumonia with outbreaks of public health significance being reported globally. The disease is caused by any species of the Gram-negative aerobic bacteria belonging to the genus *Legionella*, but *L. pneumophila* serogroup 1 is the causative agent of most cases in Europe.

Legionella spp. is found ubiquitously throughout aquatic environment. From its natural reservoir (e.g. lakes, rivers, thermal springs) Legionellae can reach and colonise man-made water supply systems responsible for transmission to humans. Although a direct correlation between *Legionella* spp. load and legionellosis risk has not been demonstrated, national and international guidelines recommend risk control and intervention based on the detected *Legionella* spp. load [Ditommaso *et al*., 2015].

Obviously, culture-based methods cannot match the challenges that *Legionella* spp. pose to public health. Standard cultivation method ISO 11731 require more than 10 days before results can be scored, moreover often fails despite the presence of viable cells [Kirsher *et al.*, 2012]. In four of the ten largest Legionnaires' disease clusters in Europe occurred in 2015 no environmental sources were identified and in 57% of 624 environmental investigation no source of *Legionella* could be found. Several reasons may explain that: (1) some environmental sources are not considered as a potential as they are during an epidemiological investigation for example because are far away from the site of infection (like cooling towers) or they are not recognised as a source because of missing knowledge; (2) a heterogeneous population of *Legionella* is present and the causative strain is not detected (3) the amount of *Legionella* bacteria is too low and under the detection limit of culture method but the strain is highly virulent (4) competitive background microflora that inhibit growth of *Legionella* (5) the presence of VBNC cell [ECDC, 2015; Kirshner; 2016].

The advantage of PCR method for detection of *Legionella* spp. in environmental samples are well known and have been widely demonstrated [Tronel H. and Hartemann; 2009]. However, the great challenge of replacement of culture by PCR is still ongoing because

PCR can overestimate *Legionella* by the detection of dead cell DNA. For these reasons, qPCR is recognized mainly as a screening tool and is unsuitable for risk evaluation [Scaturro *et al.*, 2016].

In this study a method for the detection of *Legionella* spp. and *L. pneumophila* was developed including an efficient DNA purification step that allows a high recovery of cells suitable for the subsequent real-time PCR reaction. Moreover, a viability PCR protocol based on PEMAX dye was developed and other strategies were evaluated for the selective quantification of live *Legionella* cells in water samples. On cooling tower water samples artificially contaminated with suspensions of live and heat-treated *Legionella* cells the PEMAX qPCR method developed showed a good efficiency in the suppression of dead cell DNA ($\Delta\log_{10}$ of 2.71) and unspecific effect also on live *L. pneumophila* cells ($\Delta\log_{10}$ of 1.84) was detected.

However, when the protocol was applied on field thermal pool samples disinfected by sand filtration, UV light and a non-chlorine shocking agent and on cooling towers water samples treated with a corrosion inhibitor and a biocide (based on 5-chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-4-isothiazolin-3-one) resulted culture negative, the PEMAX-qPCR method was ineffective. This discrepancy between culture and v-PCR is hard to explain: we can hypothesise that in field samples dead *Legionella* cells are not accessible to the dye, for example can be associated in biofilm or inside amoeba. The presence of VBNC cells was not evaluated in this study.

Several authors reported that in cooling towers the high background microbiota can cause problems in the interpretation of results [Ditommaso *et al.*, 2015], for this reason we decided to introduce an IMS step to purify *L. pneumophila* cells from the background microflora prior to viability treatment, but this strategy did not improve the efficacy of the treatment.

Although protocols of viability PCR have been presented in literature for many years, overall, we conclude from our study that PEMAX dye combined with qPCR cannot be reliably used to quantify viable *Legionella* in environmental samples.

A very promising approach was developed based on nutritional stimulation of filtered water sample in BYEα and BYEα broth supplemented with GVPC for 48 h at 37°C, as it is easy to perform and in 51 hours from sampling provides information about the presence of viable *Legionella* spp. cells by simply evaluating a shift in the Ct values between +STM and -STM aliquot of the same sample. Preliminary satisfactory results were obtained for nutritional stimulation method and further tests are required, especially to evaluate the

efficacy on water samples contaminated by other *Legionella* species not only *L. pneumophila* and testing different type of water samples. During our experiments on sanitary water samples contaminated by *L. pneumophila* at low levels (300 CFU/L) the use of BYEα broth for 48 h was sufficient to obtain a ΔCt of 9.82-7.28, while in domestic hot water samples collected from a local building with old plumbing systems the addition of GVPC supplement was necessary to reach a ΔCt of 4.3. A wider range of different water matrices will need to be tested prior to choose the best conditions for nutritional stimulation. Respect to qPCR the nutritional stimulation method evaluated was able to distinguish between viable and non-viable cells. This method implicates higher manual workload and costs compared to ISO culture method, however the possibility to provide in more or less 2 days an indication about the presence of live cells could have a great impact on the monitoring of legionellosis in nosocomial or recreational facilities where people at higher risk of legionellosis could be protected promptly from the exposure.

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Annex 1: Publications produced during the PhD

E. Carloni, G. Amagliani, E. Omiccioli, **V. Ceppetelli**, M. Del Mastro, L. Rotundo, G. Brandi, M. Magnani, (2017).
Validation and application of a quantitative real-time PCR assay to detect common wheat adulteration of durum wheat for pasta production. *Food chemistry*, *224*, 86-91.

UNDER REVISION
- Assessment of hygienic conditions of recreational facility restrooms: an integrated approach.
G.F. Schiavano; G. Baldelli; V. Ceppetelli; G. Brandi; G. Amagliani. International Journal Of Environmental Health Research

IN PREPARATION
- Detection and quantification of viable *Legionella pneumoph*ila cells in sanitary and thermal water samples: development and application of a method of viability qPCR.
V. Ceppetelli, G. Amagliani, E. Omiccioli, E. Carloni, A. Grottoli, M.Magnani.

Accepted abstract

-Validation according to OIE criteria of the *Salmonella* Abortusovis Test.
S. Dominici, M. E. Laguardia, F. Collacchi, A. Grottoli, V. Ceppetelli, M. Tittarelli, A.C. Ferreira, P.Themudo, I. Cruz F. Perletta, G. Addis, M. Liciardi, E. Omiccioli
Presented as oral Presentation at ISWAVLD 2019, Chiang Mai 19-22 June 2019.

-Research of viable *Legionella pneumophila* cells in sanitary water samples: development and application of a viability qPCR method.
V. Ceppetelli, E. Omiccioli, G. Amagliani, A. Grottoli, E. Carloni, P. Barbadoro, S. Savini, E. Ponzio, G. Brandi, M. Magnani, M. D'Errico.
Presented as a poster at 29th ECCMID, the European Congress of Clinical Microbiology and Infectious Disease Amsterdam, 13 – 16 April 2019.

-Validation of HIV-1 DNA qPCR assay and comparison with digital PCR.
E. Carloni, E. Omiccioli, C. Orlandi, L. Pucci, V. Ceppetelli, B. Canovari, Z. Pasquini, F. Barchiesi, M. Menotta, A. Casabianca, M. Magnani.
Presented as a poster at the 29th ECCMID, the European Congress of Clinical Microbiology and Infectious Disease Amsterdam, 13 – 16 April 2019.

-HIV-1 DNA test: a reliable method for the management of HIV infection.
E. Carloni, E. Omiccioli, C. Orlandi, L. Pucci, V. Ceppetelli, I. Bon, A. Bertoldi, B. Canovari, Z. Pasquini, F. Barchiesi, M. Menotta, A. Casabianca, M. Magnani.
Presented as a poster at the Italian Conference on AIDS and Antiviral research (ICAR). 5-7 June 2019, Milan.

-HIV-1 DNA qPCR assay: validation and comparison with the digital PCR.
E. Carloni, E. Omiccioli, C. Orlandi, L. Pucci, V. Ceppetelli, B. Canovari, Z. Pasquini, F. Barchiesi, M. Menotta, A. Casabianca, M. Magnani.
Presented as a Poster at the 2nd National Congress of the Italian Society for Virology. Rome, 28-30 November 2018.

-Validation according to OIE criteria of the Salmonella AbortusovisTest.
S. Dominici, M.E. Laguardia, F. Collacchi, A. Grottoli, V. Ceppetelli, M. Tittarelli, A.C. Ferreira, P. Themudo, I. Cruz, F. Perletta, G. Addis, M. Liciardi, E. Omiccioli.
Presented as a poster at the 5th European Association of Veterinary laboratory Diagnosticians,Bruxelles 14-17 Ottobre 2018.

-Ricerca di cellule vitali di *Legionella pneumophila* in campioni di acqua sanitaria e termale: sviluppo e applicazione di un metodo di viability qPCR.

Ceppetelli V, Omiccioli E, Amagliani G, Grottoli A, Barbadoro P, Brandi G, D'Errico M, Magnani M, Legio-Printage WCG.
Presented as a poster at the 51° Congresso della Società Italiana di Igiene, Riva Del Garda 17-20 October 2018.

-Quantification of *Legionella* in sanitary water sample by a qPCR method.
V.Ceppetelli, E. Omiccioli, G. Amagliani, A. Grottoli, P. Barbadoro, E. Ponzio, L. Napolitano, S. Savini, M. Magnani, G. Brandi, M D'Errico.
Presented as a poster at the 9th International Conference on Legionella, Rome 26-30 September 2017.