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Analysis of *Legionella*'s Presence and Concentration in Water Systems Control

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Relatório de Estágio apresentado à
Faculdade de Ciências da Universidade do Porto
em Tecnologia e Ciência Alimentar

2020

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Mestrado em Tecnologia e Ciência Alimentar

Departamento de Química e Bioquímica

2020

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Agradecimentos

Em primeiro lugar gostaria de agradecer à Faculdade de Ciências da Universidade do Porto e ao Instituto Nacional de Saúde Doutor Ricardo Jorge pela oportunidade e disponibilidade na realização deste estágio.

Ao meu orientador, Professor Doutor Fernando Tavares, que já há muito me inspirava pela maneira eficaz e dinâmica de resolver e criar novos desafios, um muito obrigado. Apesar de este ano não ter sido particularmente fácil, o Professor nunca deixou que isso me afetasse ou demovesse.

À minha supervisora local, Dra. Cristina Pizarro, o meu agradecimento pela disponibilidade, acessibilidade e determinação. Um ano que penso ter sido de grande partilha. A todo o Departamento de Saúde Ambiental, do Centro de Saúde Pública Doutor Gonçalves Ferreira, principalmente ao Laboratório de Microbiologia, o meu muito obrigada. Poder observar mulheres na linha da frente da ciência em Portugal foi extremamente inspirador e motivacional.

Aos meus amigos de faculdade, obrigada por me acompanharem nesta bonita jornada, sempre com palavras assertivas, sorrisos largos e mão estendida. Tenho a certeza de que a vida nos juntou para que me pudesse inspirar em vocês e, quem sabe, vocês em mim. Um obrigado especial às minhas duas companheiras nesta aventura, Catarina e Teresa, que fizeram com que estes dois últimos anos fossem especialmente divertidos.

Às minhas pessoas, os meus amigos do secundário, o meu muito obrigada pela constante transmissão de confiança, boa energia e alegria durante, não só este ano, mas todos os que vivemos juntos até agora. A nossa amizade pode ter arrancado à semelhança das minhas corridas à volta do estádio do Maia, mas acabou por ter um crescimento diretamente proporcional ao aumento de concentração de adrenalina durante um daqueles testes de Matemática A no 11º ano.

Aos meus pais, não existem palavras que consigam expressar o meu agradecimento, não só pelas oportunidades criadas, mas também, e principalmente, pela educação, paciência e amor incondicional.

Finalmente, aos meus avós, a quem dedico este trabalho, agradeço a sabedoria transmitida e o amor pela natureza, e sobretudo o amor pela Biologia, incutidos na minha infância.

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Abstract

Legionella spp. is ubiquitous in nature, with the aquatic environment acting as its natural habitat. Nonetheless, these bacteria can find alternative niche in many human-made systems characterized by a relative high humidity, such as cooling towers related to air conditioning systems, hot and cold water distribution systems, evaporative condensers, humidifiers, hot water systems, among others. Such systems can have implications in human health since these display the capacity of creating small water droplets, named aerosols, that can contain *Legionella*, and, when breathed or aspirated by humans, may result in infection with a clinical condition's severity ranging from a mild, febrile illness, called the Pontiac fever, to a rapid and possibly fatal pneumonia, the Legionnaires' disease.

In Portugal, the Legionnaires' disease was first detected in 1979, but only 35 years later the country has experienced a major outbreak. This originated in Vila Franca de Xira and resulted in 377 confirmed cases and 14 deaths. Recognizing the urgency to control and understand how the situation regarding water systems, possible contaminated by *Legionella* spp., including *Legionella pneumophila*, is found in Portugal, the work here described aimed not only to organize two *Instituto Nacional de Saúde Doutor Ricardo Jorge* (INSA) databases related to the presence and quantification of *Legionella* in these systems, but also to understand and promote the improvement of the surveillance of this bacterium's dissemination. With this purpose, the data from the two referred databases was selected and organized in a single database, thus, gathering dispersed information. In some cases, data about the treatments used in the water systems was also added, when provided.

During this process, several weaknesses and inconsistencies in the data provided became clear, such as poorly explicit collection points, uncertainty about the provenance of samples, lack of measurement of physical and chemical parameters, ignorance of the equipment's description and record where collection is carried out, and no information about the sanitation treatments carried out in the systems. Alterations and uniformizations on the current treatment of *Legionella* analysis information revealed to be necessary, mainly because of their importance concerning epidemiological assessments. This reality fosters the need to design and propose a framework for a new database for *Legionella* surveillance, capable of providing a set of metadata important to

implement assertive corrective measures when needed, and to make accurate risk assessment analysis for the occurrence of *Legionella* spp.

Key words: *Legionella*, *Legionella pneumophila*, Legionnaires' Disease, Water Systems, Data Base, Framework

Resumo

A *Legionella* spp. é ubíqua na natureza, com o ambiente aquático a atuar como o seu habitat natural. No entanto, estas bactérias podem encontrar nichos alternativos em muitos sistemas fabricados pelo Homem, caracterizados por uma humidade relativamente elevada, tais como torres de arrefecimento relacionadas com sistemas de ar condicionado, sistemas de distribuição de água quente e fria, condensadores evaporativos, humidificadores, sistemas de água quente, entre outros. Estes sistemas podem ter implicações na saúde humana, uma vez que apresentam a capacidade de criar pequenas gotículas de água, denominados aerossóis, que muitas vezes contêm *Legionella*, e, quando respirados ou aspirados pelo homem, podem resultar em infeção com uma gravidade clínica que vai desde uma doença leve e febril, denominada Febre de Pontiac, até uma pneumonia rápida e possivelmente fatal, a Doença dos Legionários.

Em Portugal, a Doença dos Legionários foi detetada pela primeira vez em 1979, mas apenas 35 anos mais tarde o país experienciou um surto importante. Este surto teve origem em Vila Franca de Xira e resultou em 377 casos confirmados e 14 mortes. Reconhecendo a urgência de controlar e compreender como se encontra a situação dos sistemas hídricos em Portugal, possivelmente contaminados pela *Legionella* spp., incluindo *Legionella pneumophila*, o trabalho aqui descrito visava não só organizar duas bases de dados do Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA) relacionadas com a presença e quantificação da *Legionella* nestes sistemas, mas também compreender e promover a melhoria da vigilância de disseminação desta bactéria. Com esta finalidade, os dados das duas referidas bases de dados foram selecionados e organizados numa única base de dados, reunindo, assim, informação dispersa. Em alguns casos, foram também acrescentados dados sobre os tratamentos utilizados nos sistemas de água, quando fornecidos.

Durante este processo, várias fraquezas e inconsistências nos dados fornecidos tornaram-se claras, tais como pontos de colheita pouco explícitos, incerteza quanto à proveniência das amostras, ausência de medições de parâmetros físicos e químicos, desconhecimento da descrição e registo do equipamento onde a colheita é efetuada, e nenhuma informação sobre os tratamentos sanitários efetuados nos sistemas. Alterações e uniformizações no atual tratamento da informação de análises da *Legionella* revelaram-se imprescindíveis, principalmente devido à sua importância no que diz respeito às avaliações epidemiológicas. Esta realidade fomenta a necessidade de conceber e propor uma framework para uma nova base de dados de vigilância da *Legionella*, capaz de fornecer um conjunto de metadados importantes para implementar medidas corretivas assertivas, quando necessário, e de fazer uma análise precisa da avaliação de risco para a ocorrência de *Legionella* spp.

Palavras chave: *Legionella*, *Legionella pneumophila*, Doença dos Legionários, Sistemas de Água, Base de Dados, Framework

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List of Abbreviations

AFLP - Amplified Fragment Length Polymorphism
BCYE - Buffered Charcoal Yeast Extract
CDC - Centers for Disease Control and Prevention
CFU - Colony Forming Unit
DGS - *Direção-Geral da Saúde*
DL - Detection Limit
DNA - Deoxyribonucleic Acid
ECDC - European Center for Disease Prevention and Control
EEA - European Economic Area
EU - European Union
EWGLI - European Group for the Study of *Legionella* Infections
GU - Genome Unit
GVPC - Glycine Vancomycin Polymyxin Cycloheximide
IC - Internal Control
IDE - Industrial Diagnostic Edition
INSA - *Instituto Nacional de Saúde Doutor Ricardo Jorge*
IPQ - *Instituto Português da Qualidade*
ISO - International Organization for Standardization
LD - Legionnaires' disease
Lp1 - *Legionella pneumophila* serogroup 1
MAb - Monoclonal Antibody
MLST - Multilocus Sequence Typing
PCR - Polymerase Chain Reaction
QL - Quantification Limit
RFLP - Restriction Fragment Length Polymorphism
RNA - Ribonucleic Acid
SBT - Sequence-based Typing
SEM - Scanning Electron Microscopic
SINAVE - *Sistema Nacional de Vigilância Epidemiológica*
TEM - Transmission Electron Microscopic
TS - Technical Specifications
USA - United States of America
UV - Ultraviolet
WHO - World Health Organization

bp - base pairs

dotA - defective organelle trafficking

gyrB - gyrase subunit β

mip - macrophage infectivity potentiator

qPCR - quantitative Polymerase Chain Reaction

rpoB - ribonucleic acid polymerase β-subunit

rRNA - ribosomal Ribonucleic Acid

Introduction

In July of 1976, a massive pneumonia outbreak named the Legionnaires' disease (LD), occurred among individuals attending an American Legion convention [1, 2], being, at the time, characterized as an exceptional event due to its unknown character, magnitude, the particular affected group, and severity [3]. Two hundred twenty-one individuals, mainly men, were infected, and thirty-four died. This case led to the identification of a new bacterium species [1, 2], further named as *Legionella pneumophila* [4]. However, this was not the first time that strains from this bacterium species were isolated [5]. In 1947, Jackson isolated an unrecognized gram-negative rod that was cryopreserved [6] and that only thirty years later it was confirmed to be a *L. pneumophila* strain [7].

The 1976's outbreak was related to a poorly maintained cooling tower reservoir, which created an optimal condition for the proliferation of the pathogen, its subsequent aerosolization and spread throughout the convention building [8], emphasizing the need for attentive maintenance and surveillance of these ventilation systems.

Legionella spp. Group Description

Etymologically, the substantive *Legionella* is composed by the nouns legio and ella, meaning small legion or army [5], and, posteriorly to the Legionnaires' disease being recognized by the clinicians and infectiologists, different *Legionella* strains were isolated, identified and characterized. As a consequence, the family *Legionellaceae* was established, comprising a single genus, *Legionella* [4, 9].

Bacteria belonging to the *Legionella* genus are characterized by a 2–20 μm long and 0.3–0.9 μm wide dimension cell, depending on the culture age (Figure 1). In fact, while in fresh cultures, it is possible to observe 2–6 μm long coccobacilli, whereas older cultures may produce filamentous forms up to 20 μm long, as mentioned by Harrison & Taylor, in 1988 [10]. In terms of motility, the cell possesses one or two polar flagella, which expression depends on temperature [11]. Despite the bacteria's thin cell wall [10], Gram staining is frequently inconclusive, particularly if neutral red or safranin is applied as the counterstain. Nonetheless, there are a set of other characteristics relevant to the microorganism's identification: the fact that legionellae are nutritionally fastidious bacteria requiring iron salts and L-cysteine for growth; generally aerobic; urease

negative; non-reducers of nitrates; oxidase negative or weakly positive; incapable of ferment or oxidize carbohydrates; chemoorganotrophic; most species have the capacity of liquefying gelatine; and the mol% G+C of deoxyribonucleic acid (DNA) is very variable, ranging from 38 to 52 [5]. The World Health Organization acknowledges legionellae as facultative intracellular parasites, which means that these bacteria are able to grow in different environmental conditions, for example, in the presence or absence of specific environmental factors, such as oxygen [10].

Currently, the genus *Legionella* includes around 65 species [12], namely the *Legionella pneumophila*, the first species to be described and the type species [4]. Other species that have been associated with diseases are, for example, the *Legionella longbeachae*, *L. micdadei*, *L. bozemanii*, *L. macaechernii* and *L. sainthelensi* [13].

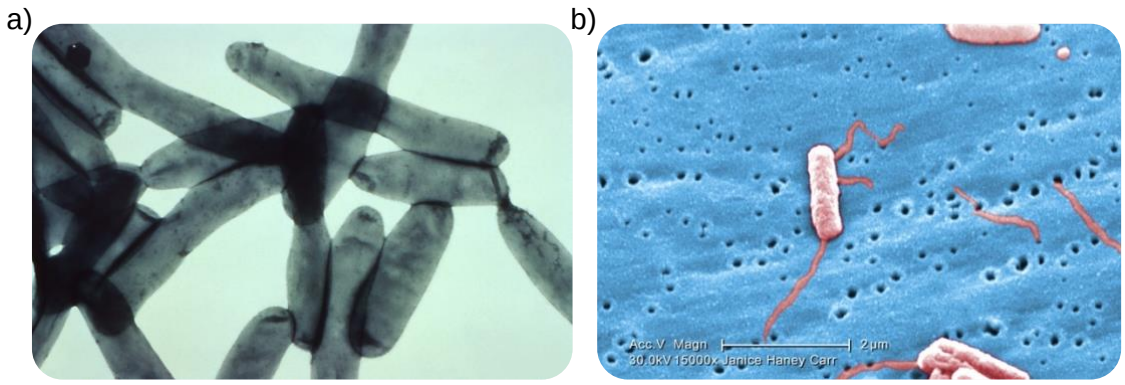

Figure 1 - Morphology of *Legionella pneumophila*. a) Image obtained from a transmission electron microscope (TEM), under a magnification of 43,700X, created from a whole preparation of the bacteria, which had been negatively stained using uranyl acetate, and grown; b) Digitally colorized scanning electron microscopic (SEM) image, magnified 15000X depicted a small grouping of the bacteria. These bacteria originated on a 1-week-old culture plate (+/- 1 day), forming a single colony, at 37ºC, on a buffered charcoal yeast extract (BCYE) medium with no antibiotics. The original sample was acid-treated for 15 min, to minimize fungal impurities, which would have inhibited the visualization of these organisms. Adapted from IPQ and EPAL [14].

Legionella pneumophila

The term "pneumophila" means "*lung-loving*" [10], attributed to the fact that these bacteria were firstly isolated from human lung tissue by McDade and Weaver in 1977 [4]. *Legionella pneumophila* has typically limited motility and some strains are even completely non-motile [10].

Brenner *et al.*, in 1988, subdivided this species into three subspecies: *L. pneumophila* subsp. *pneumophila*; *L. pneumophila* subsp. *fraseri*; *L. pneumophila* subsp. *pascullei* [15]. Nonetheless, these subdivisions are rarely reported because the tools to distinguish them are not available in routine laboratories [3]. Currently, this species is subdivided into 16 serogroups, yet *Legionella pneumophila* serogroup 1 (Lp1) has been responsible for the majority of culture-confirmed LD cases occurring worldwide (84% worldwide, 80% in Europe) [16-18].

Infection Process

The development of the disease caused by *Legionella* spp. generally implicates three premises: a host, usually compromised by chronic cardiopulmonary disease or immunosuppression; a vehicle that generates aerosols; and, finally but equally important, the bacteria inoculum and virulence [5].

The reservoir

Nowadays is known that legionellae are ubiquitous in nature [8, 19] with the aquatic environment corresponding to its primary container [20], the natural habitat for this microorganism [21]. These bacteria can be found in natural reservoirs, such as streams, ponds, lakes and compost, and even in deep terrestrial subsurface environments [8]. The bacteria spread via soil is also possible, but not so frequent, and the transmissions mode can be a complicated matter in this case [10]. Furthermore, legionellae can even find alternative niche in many human-made systems [8, 19] characterized by a relative humidity over 60% [14], namely natural thermal springs [22, 23], misting devices associated with food displays [24], domestic plumbing systems [25] and thermal spas [26-28]. Typically, the transmission of the pathogen between people does not occur [29].

Although the high humidity and water are well known environmental features associated with *Legionella pneumophila* presence, the biotic reservoirs are essential for this bacterium species to multiply, once water alone is insufficient to allow this bacterium species to proliferate [10]. In fact, naturally occurring *L. pneumophila* persists and multiplies in non-sterile tap water due to other microorganisms that help the bacteria to proliferate [30]. Also, this species grows when fed solely with filtered and sterilized drinking water for prolonged periods, in a continuous-culture model system, that is

seeded with a mixed microflora from a potable water system [31, 32]. However, has been shown that *L. pneumophila* is capable of survive, but not multiply, in the long term in sterile distilled water and sterile tap water [33, 34]. This information suggests that *Legionella* requires nutrients present in the tap water [10]. Other bacteria species, or other associated microorganisms, may supply these substances, directly or indirectly, in the form of dissolved organic constituents. This event can occur through the microorganisms' decay or due to the excess production of organic nutrients [30, 35, 36].

Legionella spp. is thermotolerant, capable to grow in the water at temperatures ranging from 20°C to 50°C, achieving its optimal growth at temperatures between 35°C and 46°C [21] (Figure 2). Even though a reasonable number of heterotrophic bacteria that support the development of *Legionella* spp. start to die above 40ºC, there are other helpers, such as protozoa amoeba, that are still capable of providing favourable conditions for rapid multiplication. Furthermore, it is known that many countries cannot achieve incoming cold-water temperatures below 20ºC, a fact that is relevant for the risk's assessment, since the potential of infection increases as the growth rate follows the increase of the temperature [37]. In hot water heaters, the bacterium can be eliminated by raising the temperature over 50ºC in outlets and over 60ºC near the heating element, if combined with regular flushing [20].

Concerning the pH, another environmental variable particularly important for bacterial growth, *Legionella pneumophila* is acid-tolerant, being able to withstand exposures to pH 2.0 for short periods [10]. Actually, legionellae have been isolated from environmental sources ranging from 2.7 to 8.3 pH [38, 39], proving their capacity of surviving in a range of environmental conditions [40], from groundwaters and plants in rainforests [41, 42] to seawater [43]. Nonetheless, the ideal range of pH for this microorganism is found between 5 and 8 [14].

Figure 2 - The effect of temperature on the growth of *Legionella pneumophila*. Adapted from Gerba [20].

Legionella sources in the natural and human-made environment

Even though drinking-water supplies that feed buildings water systems such as cooling towers have been reported with small numbers of *Legionella* spp. [25, 44], the water present in natural reservoirs has the potential to introduce legionellae into storage tanks and systems (Figure 3), where the physical and chemical conditions support their multiplication [10]. When talking about the human-made environment, some water systems may contain *Legionella*, including mainly cooling towers related to air conditioning systems [45], hot and cold water distribution systems [37], evaporative condensers, humidifiers, hot water systems, including showers and faucets [45], among others (Figure 3). Legionnaires' disease has a history of being related to sizeable complex water systems present in buildings. Nursing homes, hospitals, hotels, automobile manufacturers, and plastic injection moulding manufacturers are some workplaces that have been reporting outbreaks [46]. Regarding this topic, structures like air-conditioning, cooling towers, hot water systems, evaporative condensers, retail store misters and decorative fountain are very prone to initiate the outbreaks [20], since they can disseminate contaminated aerosols over vast distances [47], causing large community outbreaks with significant morbidity and mortality and in a short time [10].

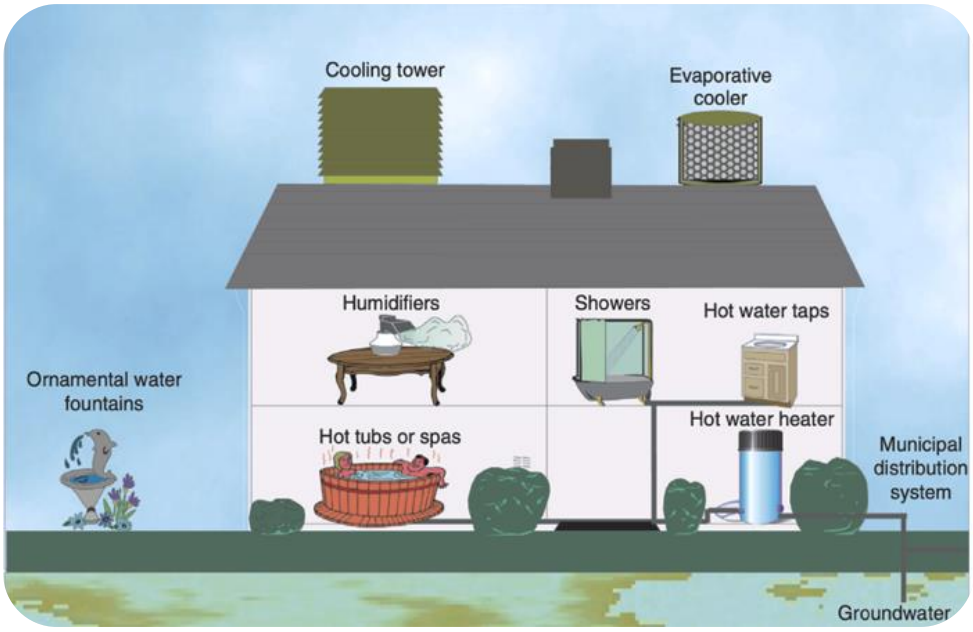

Figure 3 - *Legionella* spp. sources in the natural and human-made environment. Retrieved from Gerba [20].

Biofilm composition and formation

In 1901, Whipple observed how waterborne microorganisms' adherence to surfaces improved their bacterial activity [10]. Later, in 1980, Timothy Rowbotham described the *Legionella pneumophila* capacity to multiply inside protozoa [48], a phenomenon that mimics what occurs in human infections, as will be explained further ahead. Even though a diversity of environmental cofactors have been implicated, the essential factor is the legionellae association with free-living protozoa [5].

Currently, it is known that biofilms are structures formed by microorganisms, like *Legionella pneumophila*, to withstand adverse conditions, such as limited nutrients or extremes temperatures. Microbial biofilms may be compounded by algae, bacteria and grazing protozoa, and are heterogeneous ecosystems tremendously complex. Poorly managed cooling towers or buildings' surfaces are naturally more prone to develop protozoa and legionellae biofilms. The process behind the biofilm formation initiates with a given surface being conditioned with nonspecific binding, and, after that, pioneering microorganisms colonizing it and multiplying to form microcolonies or stacks. Microorganisms end up involved in an extracellular matrix, with the pH, oxygen, nutrients, structure, stability and protection needed to survive from severe conditions. These severe conditions include temperature increases, biocides, possible toxic effects of the substrate upon which the biofilm developed, and physical remove [10]. *Legionella*

developed in biofilms is more resistant than the same bacterial species in the water phase of the systems [49-52]. Biofilms and *Legionella* spp. that grow inside protozoa are more tolerant to chlorine and other antimicrobial agents at concentrations exceeding those commonly used to disinfect water supplies and with lethal manifest under laboratory conditions [49]. Additionally, it is important to underline that during biofilm's construction, if shear stress caused by water movements are observed, portions can be sloughed off [53, 54] and resuspend microorganisms [48], that with the right conditions, may colonize other regions of the system [10] (Figure 4). The biofilm amount, after a certain period, in a given system depends on the balance between bacterial accession from the planktonic phase, bacterial proliferation within the biofilm and dynamic detachment from the surface [55].

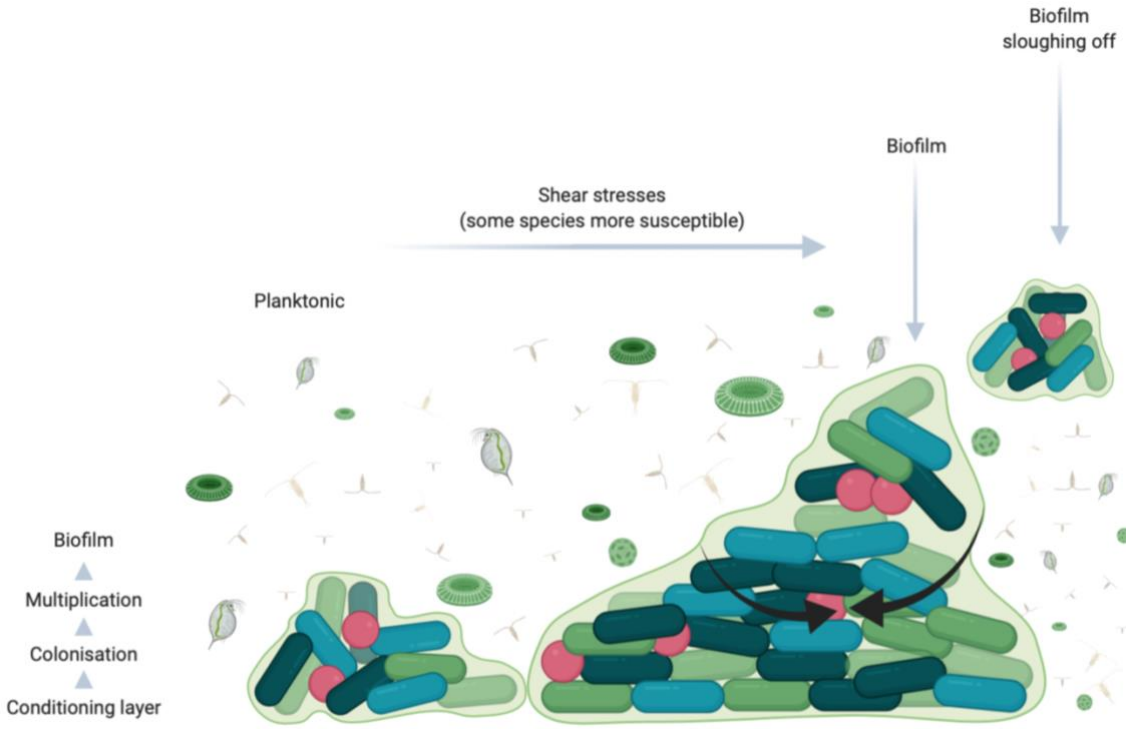

Figure 4 - Biofilm formation. Adapted from WHO [10].

For controlling the legionellae proliferation within systems, biofilm prevention is a fundamental measure, because, once developed, is very difficult to remove. These particular ecosystems are more likely to be found in regions where water can stagnate or in areas with low water flow, scale, corrosion, warm water temperatures and nutrients presence (in the systems materials or in the water source). Moreover, besides the standard interfaces, especially between solid surfaces and water, biofilms can be found on oil-water interfaces [10].

The bacterium entry in the organism

The exposure to *Legionella* is frequent, 1 to 20% of the population carries antibodies to *Legionella pneumophila*, as it is shown by serologic assays [20]. Breathing in or aspirating small water droplets containing a type of pathogenic *Legionella* are considered the primary transmission route [20, 45] (Figure 5).

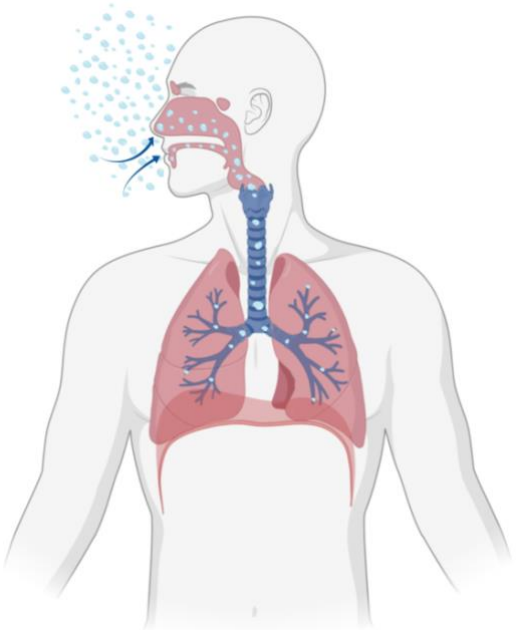

Figure 5 - Illustrative example of the intake of small water droplets (aerosols), possible containing pathogenic *Legionella* spp.

These droplets suspended in the air are called aerosols [8], and if such airborne particles come from a biological origin (e.g. fungal spores, pollen, bacteria, viruses, fungi and their fragments, including various antigens), these are named bioaerosols [56, 57]. The bioaerosols size fluctuate considerably, ranging from 0.02 to 100 µm in diameter (Figure 6), as well as in the composition, dependent on factors like the associated particles type (e.g. dust or mist), the gases that suspend the aerosol and the type of toxins or microorganisms that these contain [8].

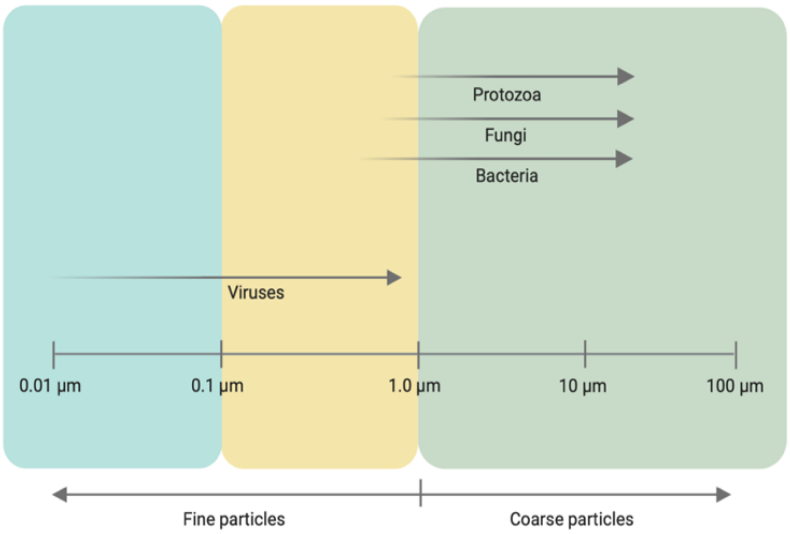

Figure 6 - Diagrammatic representation of the relative sizes of bioaerosols. The depictions of the various kinds of organisms are indicative of their potential sizes when associated with airborne particles (rafts). Adapted from Pepper and Gerba [8].

It is important to note, however, that the smaller the aerosol is, the further it travels into the respiratory system, causing more health problems than larger particles [8]. Additionally, dust or soil particles can act as a "raft" for bioaerosols [41] and the most virulent *Legionella* strains survive longer than the less virulent ones, which makes the virulence factor a significant one to the *Legionella* spp. survival in aerosols [58].

The *Legionella* Life Cycle

There exist several studies that describe how *Legionella* spp. ecology and pathogenesis are inherently related [10]. The process by which *Legionella* infect protozoa is strikingly similar to the one by which the bacterium infects mammalian phagocytic cells [59-61], using common genes and gene products. Transposing this to an infected person, as soon as *Legionella* enters the lung, alveolar macrophages phagocyte virulent and non-virulent strains, that persist intact inside the phagocytes [10]. After this stage, only virulent strains are capable of multiplying on the phagocytes and inhibit the fusion of phagosomes with lysosomes [62]. After the macrophage death and the release of many bacteria, these infect other macrophages, increasing the bacterial density in the lungs (Figure 7). The crucial step for the bacteria success corresponds to its adherence to the host cell, requiring the presence of a flagellum, pili, and bacterial surface proteins. Although there is a similarity between the *Legionella* spp. life cycles on protozoa and mammalian phagocytic cells, the mechanisms utilized to enter and exit the respective host cells are different [10].

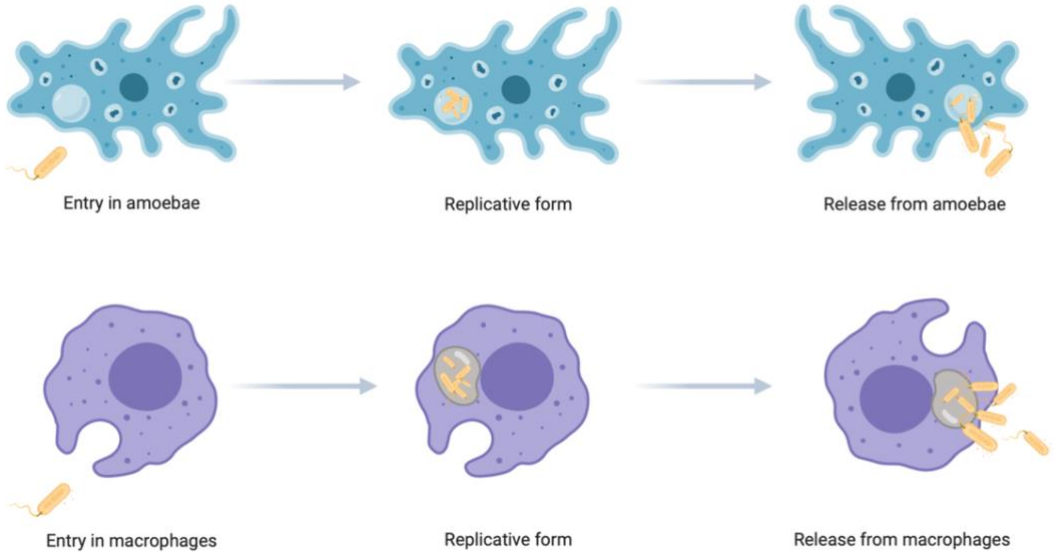

Figure 7 - Life cycle of *Legionella pneumophila* in protozoa and human macrophages. Adapted from WHO [10].

As already mentioned, virulence is a critical factor on which the lifecycle depends. This criterium is influenced by the surrounding environment such as nutrients, temperature and sodium concentration [63, 64]. Nonetheless, the infection depends not only on the *Legionella* virulence but also in the host susceptibility [10].

Legionella Infection Clinical Condition

When *Legionella* spp. causes infection, the generic term "legionellosis" is used [10]. The severity range of the infection can go from a mild, febrile illness, called Pontiac fever [65] to rapid and possibly fatal pneumonia, the Legionnaires' disease [1, 10]. The case-fatality rate is defined by diverse risk factors, such as the disease severity, the acquirement way, the timely infection determination, the proper antimicrobial treatment, among others [10, 66-69]. This infections collection that emerged in the second half of the 20th century tends to affect persons aged 50 or more years old; smokers; males; and people with chronic lung disease, hematologic malignancies, end-stage renal disease, lung cancer, immune suppression, or diabetes history [17]. However, severe legionellosis has also occurred among healthy people, such as young persons without underlying disease, and those free of another known risk characteristic [70].

Legionellosis can be domestically acquired, community-acquired, nosocomial (or "health-care acquired") or associated with traveling. In Europe, about 20% of the legionellosis cases are related to the later. Such acquirement forms constitute a problem due to the difficulties associated with identifying the infection sources [10]. In the case of community-acquired, the case-fatality rate corresponds to 10%, and each year several deaths are recorded in healthy persons without known underlying risk factors, despite the appropriate antibiotic treatment availability [37]. In Table 1 lies more information about the different categories.

In terms of attempts to calculate infectious doses, these are very complicated due to, not only the illness disparity as a consequence of the exposure to *Legionella* [71], but also due to the *Legionella* virulence variations based on environmental conditions [72], lifecycle [73] and strain type [74, 75]. Despite the scarce information about the infective dose in humans, it has been reported infection after short and long distances (i.e. over 3 km) exposure from the point source outbreak, suggesting that the infection dose in humans is low, at least for more susceptible people [76].

Table 1 - Risk factors for *Legionella* infection by category. Adapted from WHO [10].

	Community-acquired	Travel-Associated	Nosocomial
Examples of *Legionella* Reservoirs	Industrial sites, shopping centres, clubs, leisure centres, sports clubs, private residences, etc.	Hotels, cruise ships, campsites, shopping centres, clubs, leisure centres, sports clubs, etc.	Hospital, medical equipment, etc.
Environmental Risk Factors	Proximity to transmission sources, cooling water systems poor design or poor maintenance, inadequate staff training	Stay in accommodation designed for short stays and seasonal use; intermittent room occupancy and water use; intermittent water supply and fluctuating water temperature control; complex water systems; lack of trained staff to manage water systems	Complex water distribution system, long pipe runs, poor water temperature control, low water flow rates
Personal Risk Factors	Age > 40 years; male; underlying disease such as diabetes; chronic heart disease; smoking; immunosuppression; structural pulmonary comorbidity; chronic renal failure; recent travel; haematological malignancy; iron overload; other immunosuppression	Age > 40 years; male; heavy smoking, alcohol abuse; change in lifestyle; underlying disease such as diabetes; chronic heart disease, other immunosuppression	Age > 25 years; transplant patient; other immunosuppression: surgery, especially head and neck; cancer, including leukaemias/lymphomas; diabetes; treatment with respiratory devices; chronic heart/lung disease; smoking, alcohol abuse

Legionnaires' disease

The Legionnaires' disease takes 2 to 10 days to incubate after the presumed airborne exposure [8], although it may extend to an even larger period [10]. This disease reaches 5% of those exposed [8] and has a fatality rate of 15% in hospitalized cases [20]. This disease does not have exclusive characteristic signs or symptoms, and not everyone exposed will develop symptoms [77-83]. Nevertheless, a variety of clinical signs is associated with LD rather than with other pneumonia causes. About half of patients manifest nervous system disorders, including delirium, depression, confusion, hallucinations and disorientation, that may occur during the first symptomatic week. A more detailed examination may reveal the absence of deep tendon reflexes, extremities fine or coarse tremors, hyperactive reflexes and cerebral dysfunction signs. However, in immunocompromised patients the syndrome may be more subtle. When talking about severe legionellosis, poor memory, weakness, and fatigue are many times secondary symptoms that can last months [10]. With this degree of severity, different neurological problems can appear [84, 85]. Nonetheless, retrograde amnesia is the more frequently deficit reported. If untreated, the most frequent complications are shock, multi-organ failure, acute renal failure and respiratory failure. Even with appropriate early treatment, that usually leads to full recovery and sequelae may occur, such as pulmonary scars and restrictive pulmonary disease [10]. The LD main characteristics are presented in Supplemental Table 1.

Pontiac fever

The Pontiac fever is an epidemic disease with a short incubation period of hours to several days [5]. Is not associated with pneumonia, and patients recover spontaneously within 2 to 5 days [20]. In contrast to LD, Pontiac fever has a high attack rate, affecting up to 100% of those exposed, even though it is not generally associated with mortality [8]. Complications are rare to occur, and treatment serves as a support and intend to relieve symptoms [10]. Several *Legionella* species have been linked to this disease, such as *L. pneumophila* [65], *L. feeleii* [86], *L. micdadei* [87], and *L. anisa* [88]. The Pontiac fever main characteristics are described in Supplemental Table 1.

Extrapulmonary syndromes

Extrapulmonary clinical manifestations of *Legionella* infections are many times dramatic and the capacity of *Legionella pneumophila* spreading throughout the respiratory system to the entire body can be observed in the autopsy [10]. This bacterium has been implicated in cellulitis, peritonitis, pancreatitis, sinusitis and pyelonephritis cases [89, 90]. Moreover, legionellae have been reported in the liver, myocardium, spleen, kidney, joints, bone and bone marrow, digestive tract, inguinal and intrathoracic lymph nodes [91]. Of these sites, the most commonly affected is the heart [90].

Epidemiological Data

Worldwide

In 2017, the European Union (EU)/European Economic Area (EEA) countries notified the highest LD number ever observed, 1.8 per 100 000 inhabitants, remaining a primary sporadic and uncommon respiratory infection [92]. The sporadicity are likely related to identical exposures; however, the investigation to identify the sources becomes more difficult [5]. In the same year, these same disease notifications rates, ranged from less than 1.0 per 100 000 inhabitants in Bulgaria, Croatia, Cyprus, Finland, Greece, Hungary, Iceland, Ireland, Lithuania, Poland, Romania, Slovakia and the United Kingdom, to more than 3.0 per 100 000 inhabitants in Denmark, Italy, the Netherlands and Slovenia, as can be seen in Figure 8. Some countries have a notification rate below 0.5, and several even below 0.2 cases per 100 000 population, which probably represents the underestimation incidence in these regions and leads to the prioritization of these cases in the combat against European LD morbidity and mortality, requiring a diagnosis and report improvement [92].

Between 2013 and 2017, the annual report rate increased continuously, from 1.2 to 1.8 per 100 000 (58%). And in relation to 2016, 2017 displayed a notable increase in the cases number, 30% (1.4 to 1.8 per 100 000 population). A variety of elements can be responsible for this trend, such as travel patterns, improved surveillance, ageing population, and climate and weather changes. In the case of weather changes, the higher LD incidence has been linked to temperature, rainfall and humidity, most likely due to amplified utilization of aerosol producing devices or installations [92].

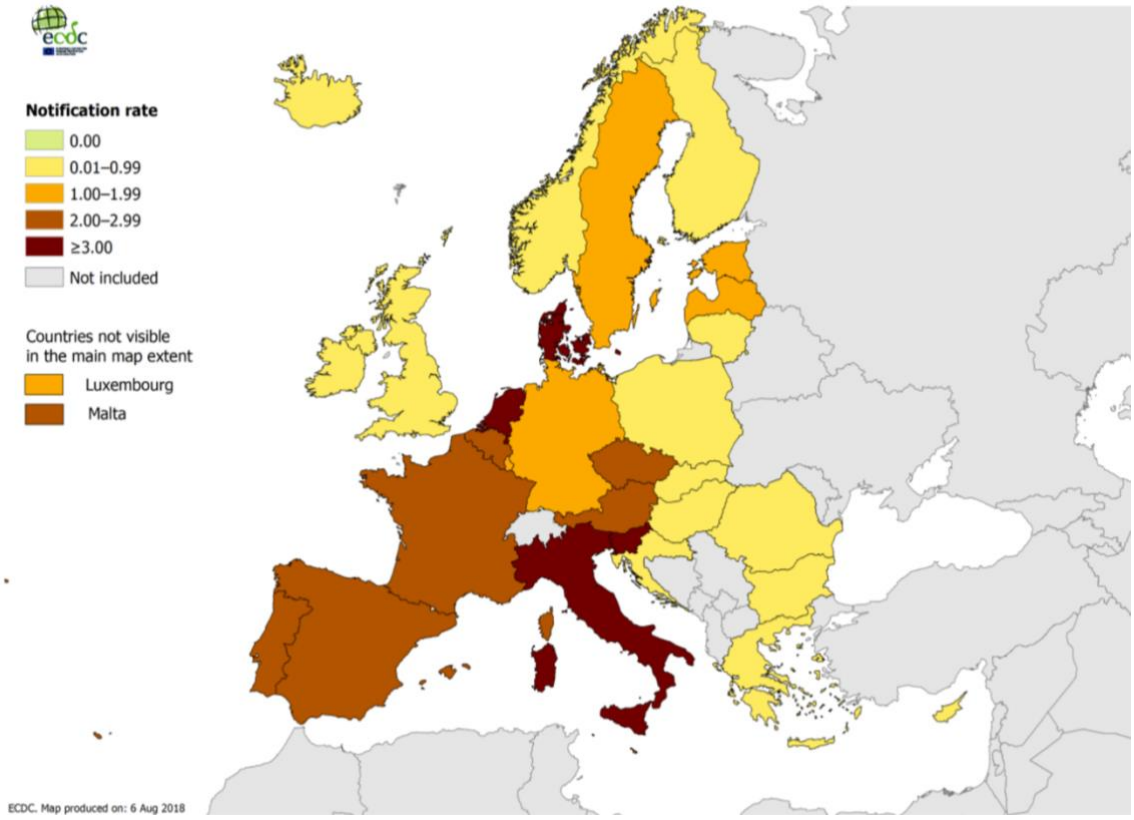

Figure 8 - Distribution of Legionnaires' disease cases per 100 000 population by country, EU/EEA, 2017. Retrieved from ECDC [92].

The majority of cases are reported between June and October, and in 2017 September was the month with the highest monthly number recorded to date under EU/EEA surveillance. However, it was not reported any massive outbreak that could justify this exceptional increase [92].

The infections male-to-female ratio in 2017 corresponded to 2.4:1. Furthermore, people who were 45 years and older accounted for 91% of the cases with known age, with the disease mostly affecting males with 65 years old and above, as can be seen in Figure 9 [92].

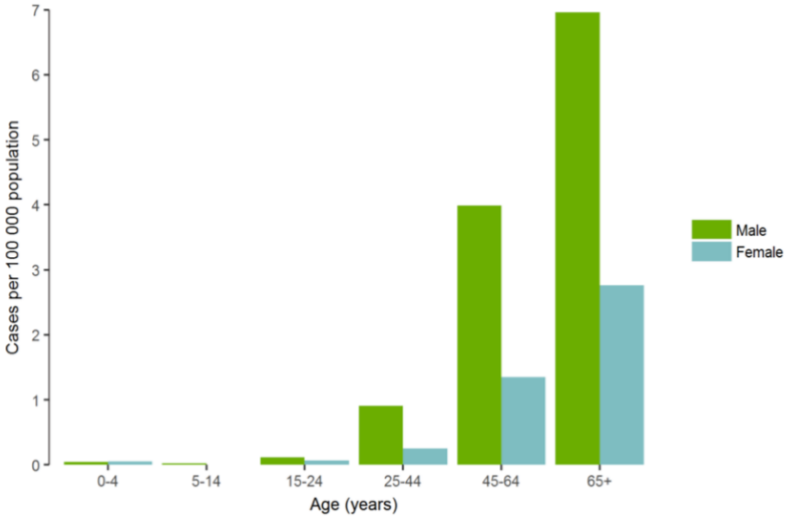

Figure 9 - Legionnaires' disease cases distribution per 100 000 population by age and gender, EU/EEA, 2017. Retrieved from ECDC [92].

In the same year, Lp1 was the pathogen associated with more cases (79%). And, similarly to previous data, 69% of the cases are associated with community acquirement, 21% with travelling, 8% with healthcare facilities and 2% with other settings. In that year, 13 EU/EEA countries divulged no outbreaks, and 9 countries reported 28 community- or hospital-acquired outbreaks, varying from 1 to 7 per country [92].

Portugal

In Portugal, the LD was first detected in 1979 [14]. More recently, on 7 November 2014, this country experienced a major outbreak of the disease. In that day, a local hospital laboratory informed the Directorate-General of Health in Portugal that about 18 patients were diagnosed with LD. All of them were originated from Vila Franca de Xira, Lisbon. As time passed, the number of cases increased, and the disease spread. It was later concluded that the outbreak started on 14 October, from 1 November occurred a substantial increase in cases, peaking on 6 November, with the outbreak being declared on 7 November (Figure 10). Only on 21 November the outbreak was considered controlled. By 2 December 2014, a total of 377 cases were confirmed, for which 66% corresponded to males, with a median age of 59 years old. The case fatality was 3.5%, dying 14 people. 208 of the total confirmed cases reported increased disease susceptibility due to the existence of chronic cardiorespiratory disease and smoking [93].

The microbiological analysis results suggested a relation between the isolates from the patients' clinical specimens and the isolate from a wet cooling system, a phenomenon already experienced with large community outbreaks previously described in Spain [67] and the United Kingdom [94, 95].

Later in 2016, George *et al.* affirmed that a probable cause of the outbreak was the cross-contamination of four closely located towers [93]. Furthermore, the bacteria proliferation in cooling tower systems was favoured by the unseasonably warm temperatures recorded during October of that year [96] and the cases spread was influenced by the high relative humidity, the NE wind, the thermal inversion [93], and by the heightened virulence [97] and longevity [98] of a bacterium that possesses the epitope monoclonal antibody (MAb)3/1 [93].

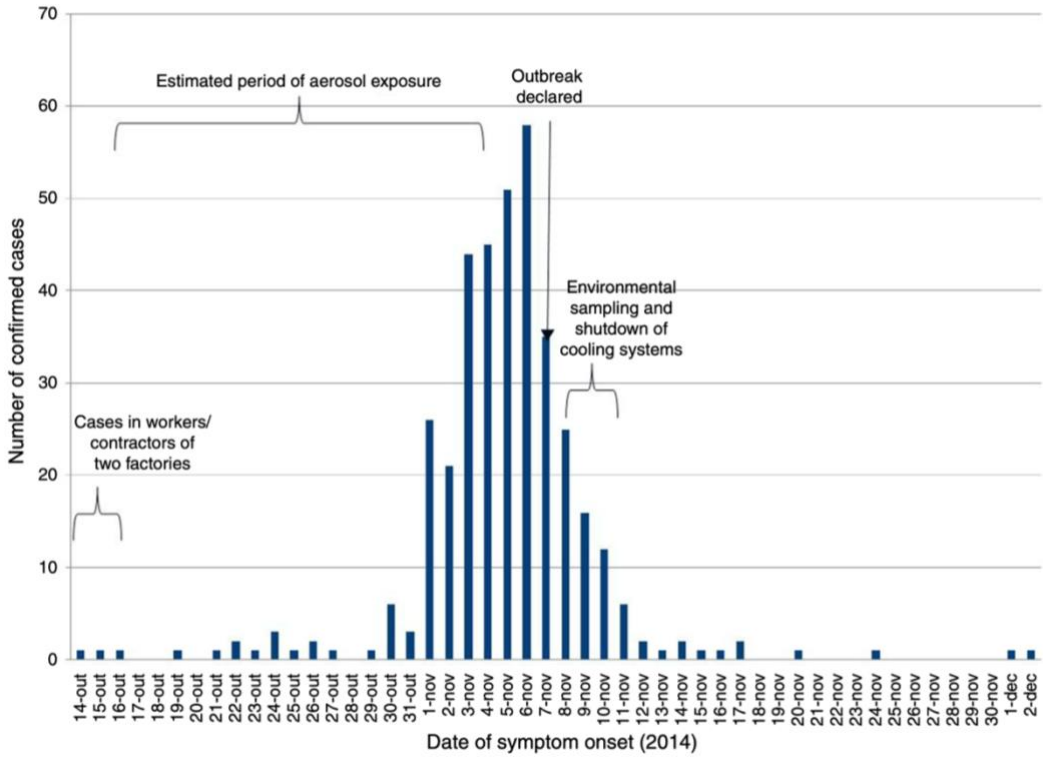

Figure 10 - Legionnaires' disease confirmed cases by date of symptom onset, Vila Franca de Xira, Portugal, 14 October – 2 December 2014 (n= 377). Retrieved from George *et al*. [93].

Some actions were taken in the municipality while the investigations were ongoing. On 8 November, the public water authority raised chlorine levels and public pools, spas and fountains were shut down and disinfected after sampling, and, on 9 November, the Environmental Inspectorate shut down and ordered the disinfection of all wet cooling systems operating in the municipality [95]. The observed case fatality rate (3.5% of all cases) was inferior to the 6% usually observed in outbreaks linked with cooling towers and systems [99], likely explained by the early diagnosis with appropriate and timely treatment. Whether the unique traits of the outbreak-associated strain underlined important characteristics of the lethality factor remains, however, under study [93].

This outbreak was the second largest LD outbreak recorded to date and emphasized the impact of bacterial phenotypes in cooling tower and climatic conditions on community outbreaks of LD [93]. Before that, between 2000 and the end of 2015, the notified cases were frequently associated with accommodation in hotel units [14]. From 2008 to 2012, the annual number of reported cases ranged between 88 and 140 cases, being the majority from sporadic nature [100]. Meanwhile, the distribution of LD cases

keeps increasing [92], as can be seen in Table 2. In 2017, the majority of cases recorded were from male's gender; residents in Lisbon Metropolitan Area; and persons with more than 75 years old [101].

Table 2 - Distribution of Legionnaires' disease cases and rates per 100 000 population in Portugal, EU/EEA, 2013–2017. Adapted from ECDC [92].

	2013	**2014**	**2015**	**2016**	**2017**
Reported Cases	94	588	145	197	232
Rate	0.94	5.88	1.45	1.97	2.32
Confirmed Cases					228

Legislation Related to *Legionella* in Water Systems

Worldwide

Even though *Legionella* proliferation and infection constitutes a worldwide concern, depending on the country, the regulatory framework has variants, such as the dangerous *Legionella* concentration levels and the target group. Nevertheless, there exists broad common aspects in the fundamental principles, such as avoiding water stagnation, keeping sufficient raised water temperature (above 60°C, below 25°C) and avoiding and monitoring critical spots. The critical points of the different regulations are summarized in Table 3, with the authority (continent, country or organization) listed in the first column and the next five columns clarifying if *Legionella* is a reportable disease, the existence of any testing guidelines, the action levels for sampling results, the maintenance strategies and the mitigation plans [102].

The regulations in several European countries are summarized in Supplemental Table 2. The first column lists the country, the object of the regulations is listed in the second column, and it is marked with green when applied and with red when not applied,

in the third column, it is mentioned if the allowed critical concentration levels are deviant from the critical concentrations set by European Group for the Study of *Legionella* Infections (EWGLI), by country, the fourth column lists the context of the regulations origin, and the last column mentions the document name with some additional information, if pertinent [102].

Portugal

Since 1986 that Portugal belongs to the EWGLI to ensure the surveillance of Legionnaires' Disease in Europe. In 2010, all EWGLI competencies were transferred to the European Center for Disease Prevention and Control (ECDC), and, meanwhile, the Integrated Epidemiological Surveillance of Legionnaires' Disease Program was implemented in Portugal, according to the *Circulares Normativas* of the *Direção-Geral da Saúde* (DGS) n˚05/DEP (Clinical and Laboratory Case Notification) and n˚06/DT (Epidemiological Research) of 2004, under the requirements of the *Sistema Nacional de Vigilância Epidemiológica* (SINAVE) according to Despacho n˚5855/2014, from *Diretor-Geral da Saúde* published in the *Diário da República* 2nd series, n˚85 of May 5, 2014 [14].

Portaria **Nº 1220/2000 of 29 December**

Portaria Nº 1220/2000 defines the conditions to which natural mineral waters used in thermal establishments must obey to be considered bacteriologically proper. Among other criteria, the 1L of the analysed sample has to be free of *Legionella pneumophila* and respect the reference value for the total number of *Legionella* spp. non-*pneumophila*, 100 CFU/L [103]. Under *Decreto-Lei* Nº 142/2004 of 11 June, every year the DGS establishes the analytical programme for natural mineral waters used in thermal establishments and the assessment of *Legionella* spp. and *Legionella pneumophila* is mandatory [14].

Circular Normativa **Nº 05/DEP of 22/04/04**

This *Circular Normativa* presents the Program for Integrated Epidemiological Surveillance of Legionnaires' Disease [104].

Table 3 - Key factors of regulations. Adapted from Van Kenhove *et al.* [102].

	Reportable disease	Presence of testing guidelines	Actions levels for sampling results	Preventive maintenance strategies	Mitigation plans
WHO	Reportable in some countries	Testing is recommended; requirements and frequencies are included	Only for cooling water systems	Temperature (recommended temperature for storage and distribution of cold water is below 25ºC, and ideally below 20ºC. For hot water, they only state that hot water temperature should be maintained)	Disinfection, cleaning, monitoring, and regular service and maintenance
EWGLI	Reportable in some countries (e.g. United Kingdom), reporting of travel-associated cases	Testing is recommended; requirements and frequencies are included	Different actions required between 1,000 and 10,000 and above 10,000 CFU/L*	Temperature (total volume of the tank needs to be heated up to 60ºC for at least an hour a day or a week, depending on the risk; cold water below 25ºC), chlorination in Italy	Risk assessment and management plan in combination with regular measurements
USA	Reportable	Recommendations for *Legionella* testing	Not included	Temperature (hot water heater outlet temperature should be at or above 60ºC at the coldest point in the hot water heater, storage tank or distribution system at or above 51ºC, and cold-water temperature in any part of the system at or below 25ºC), standard chlorination	Creation and implementation of a risk management process and *Legionella* water management program
Asia	Only reportable in Hong Kong	Not included	Not included, only target levels in Russia	Temperature (hot water production above 60ºC)	Not included
Other countries					
New Zealand	Not reportable	Some recommendations	Not included	Temperature	Not included
Africa	Not reportable	Not included	Not included	Temperature	Not included
South America	Only reportable in Brazil	Not included	Not included	Temperature	Not included

EWGLI, European Working Group for *Legionella* Infections; USA, United States of America; WHO, World Health Organization.
*It is not clear whether these values are referent to *Legionella pneumophila* or *Legionella* spp.

***Circular Normativa* Nº 06/DT of 22/04/04**

In this document are described guidelines for the epidemiological investigation that must follow the notification of a case [105].

***Circular Normativa* Nº 14/DA of 21/08/2009**

The current legislation for recreational waters is described in *Circular Normativa* Nº 14 DA of 21/08/2009. The results for *Legionella* in hydromassage tanks have to be expressed in Nº/1000mL, with the limit value of $<10^2$ for *Legionella* spp. It is also delineated the interpretation of the results related to the research of *Legionella* spp. in hydromassage tanks and several measures to be developed. If *L. pneumophila* is identified, it should be adopted the procedure described for *Legionella* spp. $>10^3$. To the analysis, the method used is International Organization for Standardization (ISO) 11731:1998 [106].

***Portaria* Nº 353-A/2013 of 4 December**

This *portaria* establishes not only the minimum values of fresh air flow per space but also the protection thresholds and reference conditions for indoor air pollutants in new buildings of commercial and service, that were subject to significant intervention and individual evaluation. Regarding the reference conditions for microbiological pollutants, the ordinance mentions the concentration below to 100 CFU/L for *Legionella* spp. in water, except for the case of research in cooling tower tanks where a concentration of less than 1000 CFU/L should be verified. *L. pneumophila* has to be absent [107].

***Orientação* Nº: 20/2017**

In this document, it is presented some considerations for health system professionals about laboratory diagnosis of Legionnaires' Disease and *Legionella* research in environmental samples [108] .

***Orientação* Nº: 021/2017**

Legionnaires' Disease surveillance and epidemiological research techniques are developed in this guideline for health system professionals [109].

***Orientação* Nº: 024/2017**

This document grouped in a single document multiple scattered technical documents for actions on the health care institutions regarding the prevention and environmental control of *Legionella* bacteria to facilitate their approach in the units providing health care system [110].

***Norma Portuguesa* 4542:2017**

This document describes the characteristics that public use swimming pools' water composition must comply with, in order to ensure that its physical, chemical and microbiological composition is compatible with the intended uses without endangering human health [111].

***Lei* Nº 52/2018 of 20 August**

This law establishes the LD prevention and control regime, describing the equipment registration procedure, prevention and control plan, water monitoring and treatment program, audits, at-risk procedure, procedure in cluster or outbreak situations, and LD prevention and control strategy [112].

In relation with the Article 9 "At-risk procedure" of this document, when is mentioned "according to the classification to be fixed in an ordinance by the Government member responsible for the health area", is essential underline that after two years this ordinance still does not exist, what makes very difficult characterized the risk situations cited [112].

Microbiological Water Analysis

Methods involving *Legionella* spp. need to be done taking into account some precautions. They should be accomplished by experienced microbiologists in a conventional microbiology laboratory in conformity to containment level 2 [113]. Before any conclusion regarding microbiological water analysis, it is fundamental to understand that a sample is merely a minor proportion of the total system volume, what means that a negative *Legionella* result does not guarantee that the entire system is safe and under control. This aspect is very important because in areas with reduced flow and stagnation

or where controls are not adequately maintained, microorganisms will not be uniformly distributed throughout the water system at all the time [37].

Sampling

For choosing sampling sites, it is fundamental to take into consideration if the sampling aims to investigate an outbreak or simply to constitute a routine monitoring. Moreover, the type and number of sites must be determined on an individual system basis due to the diversity of ventilation, plumbing, heating and air-conditioning systems in the diverse institutions [10]. In typical situations, water samples should be taken for routine sampling since the results are comparable throughout time, making them very useful for trend analysis [37].

Culture method

The culture method is the most common technique used for environmental surveillance of *Legionella*. This methodology is essential not only for identifying but also for typing *Legionella* strains despite the long incubation time required, which goes up to 10 days [98]. Results from the cultural method, together with the percentage of samples containing *Legionella*, gives useful information about the degree of this bacterium amplification in a system. However, a culture result from a water sample cannot determinate the public health significance, since the result is not directly related to exposure concentration, the infectious dose of the microorganism, virulence or the survival of the microorganism in an aerosol [10], the water systems installation, operation, design, commissioning, maintenance and management [37] and the metals concentration in those systems [20]. The host susceptibility level can be, as well, considered a variable that World Health Organization (WHO), the Occupational Safety and Health Administration and the Centers for Disease Control and Prevention (CDC) recognize to the likelihood of infection [10, 114, 115]. It is very dangerous to assume that it is safe when the *Legionella* levels are below the acceptable limit, since the concentration can increase rapidly if a system is not adequately controlled [10]. Additionally, environmental conditions have the potential to modulate the virulence of individual strains [63], and the culture technique does not differentiate between virulent and non-virulent strains. Another issue is the existence of a bias towards the species that are currently recognized to be associated with the disease, especially *Legionella pneumophila*, what may lead to the non-detection of all legionellae present in the

environment. Laboratories should have a consolidated experience in identifying various species of *Legionella* from the many bacteria species that exist in an aquatic environment. Since *Legionella* is usually present in a minor concentration of the total bacterial community in environmental samples, it is generally necessary to firstly concentrate the microflora, by centrifugation, membrane filtration, or both [10]. If a high number of colonies is visible, it can presumably indicate the presence of biofilm [116].

For the confirmation of presumptive colonies of *Legionella,* it should be used a cysteine-free agar to show dependency on L-cysteine [85]. The confirmation of Lp1, other serogroups and some other pathogenic species is vital for epidemiological investigations. For this, a range of antibody reactions can be used like direct or indirect immunofluorescence, crossed immunoelectrophoresis, immunodiffusion and slide agglutination. Preliminary identification of *Legionella* spp. can be done with commercially available latex agglutination kits where suspect colonies are simply emulsified and mixed with each latex reagent independently on a disposable reaction card. The reagents are synthesized with antibodies specific to *Legionella.* When in the presence of homologous antigens, a visibly positive reaction of the latex particles' agglutination occurs for some minutes [10] (Figure 11). Another interesting fact, that can help in the recognition of the colonies present in the medium, is that colonies of *L. pneumophila* do not exhibit autofluorescence when exposed to long-wave ultraviolet (UV) light [117].

Isolation of some strains may be only successfully done resorting of coculture with amoebae, both for human [118] and environmental [119] samples.

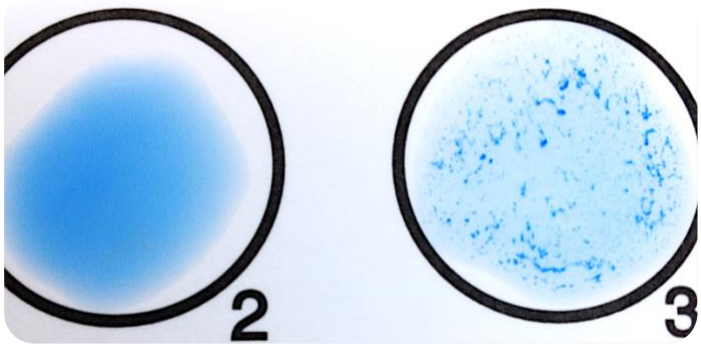

Figure 11 - Example of the two possible results from the latex agglutination kits. In contrast to the first case, where the agglutination does not occur, the second reaction presents agglutination.

Real-time PCR method

In complementation to the previous method, molecular methods, like quantitative polymerase chain reaction (qPCR), can be used for a same-day result, a significant advantage for investigations, particularly for excluding potential sources [37]. There exists a significant discrepancy between the polymerase chain reaction (PCR) results and the cultural ones when talking about detection of legionellae. This fact can be due

to the presence of viable but non-culturable *Legionella*, non-viable or injured organisms, the presence of new species of *Legionella*, or a nonspecific reaction with distinct organisms, although data suggests this is not the case [10]. The results obtained by this method must be considered presumptive and confirmed by culture, as it does not make it possible to distinguish between living and dead cells [14].

Diverse molecular targets have been used for the diagnosis of *Legionella pneumophila*, such as the 5S ribosomal DNA, 16S ribosomal DNA, the 23S-5S spacer, the macrophage infectivity potentiator (*mip*), gyrase subunit β (*gyrB*), defective organelle trafficking (*dotA*) [120] and ribonucleic acid (RNA) polymerase β-subunit (*rpoB*) [121] genes. Nevertheless, some studies have been reporting that the 5S and 16S ribosomal ribonucleic acid (rRNA) genes are very conserved regions, which turns arduous to differentiate between *L. pneumophila* and other *Legionella* species without resorting of other tests in the real-time basis detection. Moreover, there is a risk of DNA amplification of other organisms that do not correspond to *Legionella* species [120]. The *mip* gene translates a 24-kDa protein virulence factor**,** which enables the entry of *Legionella* into macrophages and amoebae [122] and it is given as an example in ISO/Technical Specifications (TS) 12869 [113]. The *gyrB* gene encodes the β subunit of the bacterial gyrase, a type II DNA topoisomerase, dispersed in bacteria [123]. This gene has a horizontal transmission that occurs in low frequency [123], and its rate of evolution has been faster than the ribosomal and further housekeeping genes [123-127]. For the identification of bacteria at the species level, the *gyrB* gene has been showing as an appropriate phylogenetic marker [128-131]. The *dotA* gene translates an integral cytoplasmic membrane protein, named DotA, of *Legionella pneumophila* [132]. This gene is associated with the virulence of *L. pneumophila* [133, 134], inhibiting phagosome-lysosome fusion in macrophages [135, 136]. Regarding the comparison between the *mip* gene and the *dotA* gene, it was concluded that the *dotA* gene is very variable between *L. pneumophila* strains, with a nucleotide diversity four times superior than that of *mip* [120]. However, the macrophage infectivity potentiator gene may be used to perform specific PCR detection of *L. pneumophila* [122]. The *rpoB* gene encodes the β subunit of DNA-dependent RNA polymerase [137] and mutations in a specific region of this gene are related to rifampin resistance [138]. It was proposed that the *rpoB* could avoid the restrictions imposed when the 16S rRNA gene is used [139].

Genotyping

The principal reason for genotyping *Legionella pneumophila* is to help identifying environmental sources that can potentially give origin to LD cases, facilitating control measures to be implemented and preventing new cases. Since this species is common in the environment, a variety of methods have been established with the attempt to differentiate the strains. The context within which the various methods are to be applied is what, most times, defines their suitability [140]. In the list of possible procedures are multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP) analysis, restriction fragment length polymorphism (RFLP) analysis, sequence-based typing (SBT), among others. Nevertheless, none of these methodologies is entirely satisfactory and failures in identifying the source of disease can occur [141].

ISO - International Organization for Standardization

ISO 19458:2006, Water quality — Sampling for microbiological analysis.

This International Standard focuses on the sampling phase of the microbiological investigations, providing guidance on planning water sampling regimes, on sampling procedures for microbiological analysis and on transport, handling and storage of samples until analysis begins. General information about the different water bodies sampling is found in the respective parts of ISO 5667 [142].

ISO 11731:2017, Water quality — Enumeration of *Legionella*.

This document described culture methods for the isolation of *Legionella* spp. and also the estimation of their number in water samples. However, the methodologies are inefficient for some *Legionella* species, once that not all of them are culturable. The information can be applied not only to all kinds of water samples, including potable, natural, waste and industrial waters, but can be also used for water-related matrices, such as sediments, biofilms, among others [143].

ISO/TS 12869:2019, Water quality – Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR).

The present method is intended for the detection and quantification of *Legionella* spp. and *L. pneumophila* through qPCR. General performance evaluation requirements,

quality control requirements and methodological requirements are here specified, but the technical details are only informative, what makes possible other technical solutions. Due to the qPCR methods nature, it is not given any information about the physiological state of the bacteria. Similar to the previous ISO here described, this document is also intended to be applied in all types of water, such as cooling tower water, hot or cold water, among others, except if the content of suspended matter, nature or accompanying flora interfere with the analysis. The detection and quantification limit can be adversely affected by this interference. Furthermore, some additives, like chemicals for water treatment, have the potential to affect and interfere with the sensitivity of the method [113].

Treatment Methods for Different Water Systems

The microorganisms' growth prevention or destruction is crucial for the control and inhibition of infectious disease transmission. Typically to achieve this goal, the most common treatment methods are based on heat, chemicals, radiation or filtration, and each one of them has their own way of actuation. The heat inactivates or kills by denaturation of nucleic acids and essential proteins (enzymes, viral capsids). The chemicals may interfere with not only the replication of nucleic acids but also with enzymatic action also destroying membranes and cell walls. The radiation, gamma radiation or UV light, act directly on nucleic acids, and finally, the filtration physically removes the organisms by size selection and does not destroy the organism [144].

The efficacy of each method depends on its nature, temperature, water chemistry, cleanliness, contact time, pH, presence of corrosion, scale and biofilms. Attention should be given to the duration of contact time and dosage, since the treatment may result in degradation of system components and pipework. The building water systems materials should be chosen, thinking in the possible sequels of the treatment method. Nonetheless, it is still imperative to use good quality water source and preserve the right conditions of the systems, assuring that they are well maintained, operated and designed for a good flow. A critical factor is the treatment ability to reach and be maintained at the adequate target levels in all parts of the system, and that is why it is crucial to optimize the flow and escape create areas where exist no or reduced flow around the system [37]. Advantages and disadvantages of methods for controlling *Legionella* in reticulated water systems and cooling towers are described in Table 4.

The biocidal products used for water systems treatment should fulfil the requirements set out in Regulation (EU) Nº 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the use of biocidal products and availability on the market. The legal act lays down the national procedures for authorizing the placement of these products on the market. Companies responsible for this placement must submit a dossier for each biocidal product that complies with the regulatory provisions to the DGS through its website [145].

Law (*Decreto-Lei*) N. º 306/2007, of 27 August

This decree-law establishes the quality regime for water intended for human consumption. It is possible to find in the article 9 of this document that: the managing entities must ensure an adequate treatment of water intended for human consumption; the distributed water must be subjected to a disinfection process; the health authority may exempt management entities from complying with the provisions if, through the analytical history, they demonstrate that have not breached microbiological parameters without resorting to disinfection; the management entities are responsible for ensuring the effectiveness of disinfection and ensuring, without compromising disinfection, that contamination by water sub-products is kept as low as possible and does not jeopardize its quality for human consumption [146].

Table 4 - Advantages and disadvantages of methods for controlling *Legionella* in reticulated water systems and cooling towers. Adapted from WHO [10].

Method	Advantages	Disadvantages
Keeping temperature <20ºC	- Straightforward, effective and easily monitored - Little significant growth of *Legionella*	- Only really applicable to drinking water systems
Keeping temperature >50ºC	- Straightforward, effective and easily monitored	- Does not eliminate legionellae - Requires circulation temperature to be near 60ºC - Difficult to maintain temperatures in old systems - Requires protection against scalding
Periodic flushing with hot water at 50-60ºC (usually an essential part of control by high temperature, above)	- Simple, effective and easy to monitor	- Not applicable in cold-water systems - Requires protection against scalding - Must be maintained and inspected to achieve consistent control - Recolonization occurs within days
Dosing with sodium hypochlorite	- Proven, effective disinfection technique - Simple to use - Relatively cheap	- Formation of trihalomethanes - Needs protection (e.g. carbon filter) for dialysis patients - Toxic to fish - Affects taste and odour - Not stable, particularly in hot water - Increases corrosion of copper
Dosing with monochloramine	- More persistent than chlorine - Simple to use in mains distributions - Penetrates biofilms	- Needs protection (e.g. carbon filter) for dialysis patients - Toxic to fish - Affects rubber components
Dosing with chlorine dioxide	- Proven disinfection technique - Simple to use	- Formation of chlorite - Needs protection (e.g. carbon filter) for dialysis patients - Safety considerations (depending on the method of generation)
Dosing with hydrogen peroxide	- Simple to use	- Weak disinfectant - Suspected of mutagenicity
Copper and silver ionization	- Effective when prescribed concentrations are maintained	- Frequent monitoring of copper and silver needed

Table 4 (continuation) **-** Advantages and disadvantages of methods for controlling *Legionella* in reticulated water systems and cooling towers. Adapted from WHO [10].

		- Pretreatment needed (pH, hardness) - Increased concentrations of copper and silver in water
Anodic oxidation	- Disinfection demonstrated	- Pretreatment needed (depending on the effect of pH and hardness) - Effect on *Legionella* in biofilms not known
UV disinfection	- Proven disinfection technique - Simple to use	- Effective only at the point of application; no control downstream (no residual) - Not suitable for turbid water - No effect on biofilm formation
Ultrafiltration at point of entry to the building or system	- Physical disinfection barrier - Effective removal of biomass and particles	- No inactivation of *Legionella* downstream of the filter within system - Effect on the formation of biofilms and sediment not known
Point-of-use filters	- Physical barrier - Easy to install (may require some modification of the outlet) - Suitable for hot and cold-water systems - Good for use in systems exposing high-risk patients	- Only suitable at point of use - Must be replaced regularly - Particle in water may reduce flow and operational life - Expensive
Pasteurization heat with flushing	- Disinfection barrier - Useful as a short-term remedial measure - Simple to apply in hot-water installation	- Transient effect on *Legionella* - No limitation of biofilm formation - Scalding risk
Non-oxidizing biocides	- A proven technique for cooling systems	- Not suitable for potable water systems - Most not applicable to spa pools - Resistant populations may develop - Need to alternate two different biocides - Often concentrations cannot be readily monitored - Difficult to neutralize for sampling purposes

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Objectives

The present report results from the work that was done as part of an internship at the Environmental Health Department in *Instituto Nacional de Saúde Doutor Ricardo Jorge*. This state laboratory has the mission to contribute to public health gains, through research and technological development activities, reference laboratory activity, health observation and epidemiological surveillance, as well as coordinating the laboratory quality external evaluation, disseminating scientific culture, fostering training and education and also ensuring the provision of the differentiated services in the fields mentioned above. And it was in this context that this theme was built.

With this internship it was expected to understand how the situation regarding water systems, possible contaminated by *Legionella* spp., including *Legionella pneumophila*, is found in Portugal. An essential subject after what occurred in Vila Franca de Xira. Following this objective, the work was divided into two main tasks:

1. Organizing the information from two *Instituto Nacional de Saúde Doutor Ricardo Jorge* (INSA) databases regarding *Legionella*'s contamination analysis by industry/activity sector, concerning the time period between March 2019 to March 2020;
2. Understanding and promoting the improvement of the surveillance of this bacterium's dissemination.

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Materials and Methods

Sampling

This part of the work was done by the INSA laboratory technicians, or by the requesting entity itself between March 2019 and March 2020, using the laboratory internal method instructions for samples collection, conservation and transport (DSA ASMI-PE33_03 P). The harvest report's layout that is used by the INSA technicians is shown in Supplemental Figure 1.

If the samples were only intended for analysis by the cultural or qPCR methods, it was necessary to collect 1L of water, whenever possible. However, if the samples were going to be analysed by the two methodologies, then it was necessary to collect 2L of water. Hot tap water was always chosen, whenever possible. The sterile container should have added a biocide neutralizer [14], for example, sterile sodium thiosulphate, in order to not influence the analysis. The water samples analysis should start as soon as possible after collection. If samples are delivered to the laboratory within 24 hours, transport must be carried out under refrigeration or at room temperature and protected from sunlight. If such time period goes instead from 24h to 48h, transportation must be carried out at 5ºC ± 3ºC. It is also important to note that in cases where the water system has been subjected to disinfection, it is advisable to wait at least 72 hours before harvesting, or the necessary time until the treatment product return to its normal values.

Collection of water samples from taps

After opening the tap, the water first jet was collected directly into a sterile 1L flask (a space of approximately 2 fingers was left in the container neck, to allow the sample homogenization with the thiosulfate). In cases where the sample was also needed for qPCR analysis, the previous procedure was repeated for the second 1L bottle.

Collection of water samples from showers

The shower head was inserted into a sterile plastic bag, from which, one of the corners was previously cut. The sterile 1L flask was removed in the shower vicinity. The

bottle was filled with the initial water flow, keeping it tilted (2 fingers space was left in the container neck, to allow the sample homogenization with the thiosulfate). In cases where qPCR analysis was also required, the previous procedure was repeated for the second 1L bottle.

Collection of water samples from pools

For this harvest, the inclined, closed bottle was submerged until the desired depth (from 10 to 30 cm). The bottle was then opened, tilted horizontally and slid smoothly forward in the water until the required sample volume was collected. After closing it and removing it to the outside, the flask was shaken.

Collection of water samples from deep tanks without tap

For these cases, a diving flask was used. Ropes were attached to the bottle's frame devices, keeping it inside the protective box. A sterile 1L bottle or two 0.5L bottles were used. The bottle was submerged as low as possible, up to the limit of the strings but without touching the bottom of the tank. When filled, the bottle was closed still immersed, and smoothly homogenized. In cases where qPCR analysis was also intended, the previous procedure was repeated for a second 1L bottle or two 0.5L bottles.

Collection of water samples from shallow tanks (for example, a cooling tower tank)

The bottle was dipped and opened, tilted upwards to fill. The bottle was filled with the initial water flow, keeping it tilted (2 fingers space was left in the container neck, too allow the sample homogenization with the thiosulfate). The flask was closed and, already on the surface, homogenized with the thiosulfate, shaking the sample gently. Whenever the qPCR analysis was also required, the previous procedure was repeated for a second 1L bottle.

Collection from condensate trays with a swab

Water was collected from the condensate trays directly into a sterile collection bottle. If necessary, a sterile syringe was used. Then, the biofilm was collected with a

swab and placed inside the used bottle, always taking care to cut the part of the stem that was touched.

For each one of the previews described collection methods, it is always advised to measure the water-free residual chlorine, bromine, temperature and pH, to guarantee that the results are trustworthy. When the technicians collect the sample, measurement of the pH in the laboratory is only performed when requested by the entities. In Table 5 it is described the acceptable range of values for bromine and free residual chlorine in cooling systems, hot and cold-water networks, air-conditioned water systems for recreational use and pools. Values of free residual chlorine higher than 1 mg/L contribute to the corrosion phenomenon, being recommended the addition of chemicals that prevent this effect [14].

Table 5 - Acceptable range of values for bromine and free residual chlorine in cooling systems, hot and cold water networks, air-conditioned water systems for recreational use and pools. Adapted from IPQ and EPAL [14] and Portuguese Standard 4542:2017 [111].

	Cooling Systems	**Hot and cold-water networks**		**Air-conditioned water systems for recreational use**	**Pools**
		Hot water building network	**Cold water building network**		
Free residual chlorine	0.5 – 1.0 mg/L (pH between 7 and 8)	0.2 – 0.6 mg/L*	0.2 – 0.6 mg/L*	0.8 – 2.0 mg/L	Covered tanks: 0.5 - 1.2 mg/L (pH between 6.5 and 7.4); 1.0 - 2.0 mg/L (pH between 7.5 and 8.0) Outdoor tanks: 0.8 - 1.5 mg/L (pH between 6.5 and 7.4); 1.5 - 3.0 mg/L (pH between 7.4 and 8.0)*[2]
Bromine				2 – 4 mg/L (recommended in tepid water), keeping the pH values between 7.2 and 7.8	2 - 4 mg/L*[2]

* Decree-Law n°152/2017, of December 7

*[2] Portuguese Standard 4542:2017

Microbiological Analysis

This part of the work was done by INSA laboratory technicians, accredited to ISO 17025:2017, between March 2019 and March 2020. All samples analysed in this laboratory are at the customer's request and motivated mainly by good internal practices.

Culture method

For "*Legionella pneumophila* research and quantification" and "*Legionella* spp. non-*pneumophila* research and quantification" two methods are used. The DSA ASMI PE09_05 P method is used in cleaner waters (e.g. swimming pool water, treated consumption water, thermal water), in which the samples are filtered and centrifuged (Figure 12), with a detection limit (DL) of 10 colony forming unit (CFU)/L. When exists filtering impossibility (e.g. cooling towers water), many particles in suspension, oil drops (Figure 13), or in swab's utilization cases (Figure 14) is used the DSA ASMI PE20_05 P method, in which the samples are only centrifuged. In this method, the water samples have a DL of 100 CFU/L and the swabs of 10 CFU/swab. In samples from epidemiological surveys, cooling towers and other non-potable water, when is expected a higher concentration of *Legionella*, direct culture of the sample (without concentration) can also be carried out in glycine vancomycin polymyxin cycloheximide (GVPC), proceeding in the same way, without treatment, heat treatment (optional) and acid treatment (optional) (right arrow from step 2 in Figures 12,13 and 14). The agglutination step, used in both methods, is usually performed with one of two kits, Microgen Bioproducts® Legionella or Oxoid Legionella Latex Test. The total analysis has a minimum time required of 10 days.

Examples of *Legionella* spp. non-*pneumophila* and *Legionella pneumophila* cultures can be found in Figure 15. The layout of the annotation sheet for *Legionella*'s analysis results that is used by the INSA technicians is presented in Supplemental Figure 2.

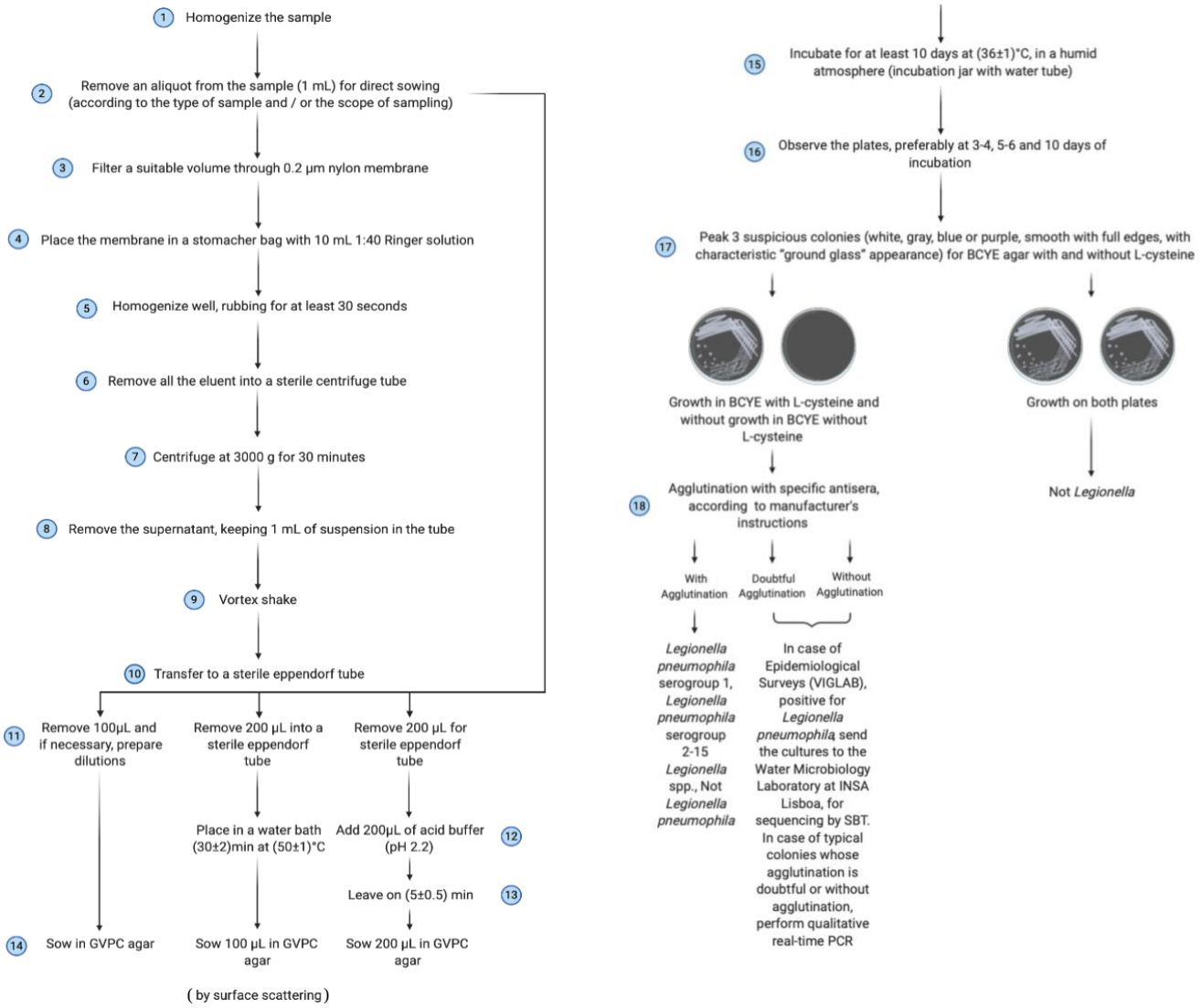

Figure 12 - Flowchart of the research and quantification method DSA ASMI PE09_05 P for *Legionella* spp. non-*pneumophila* and *Legionella pneumophila*.

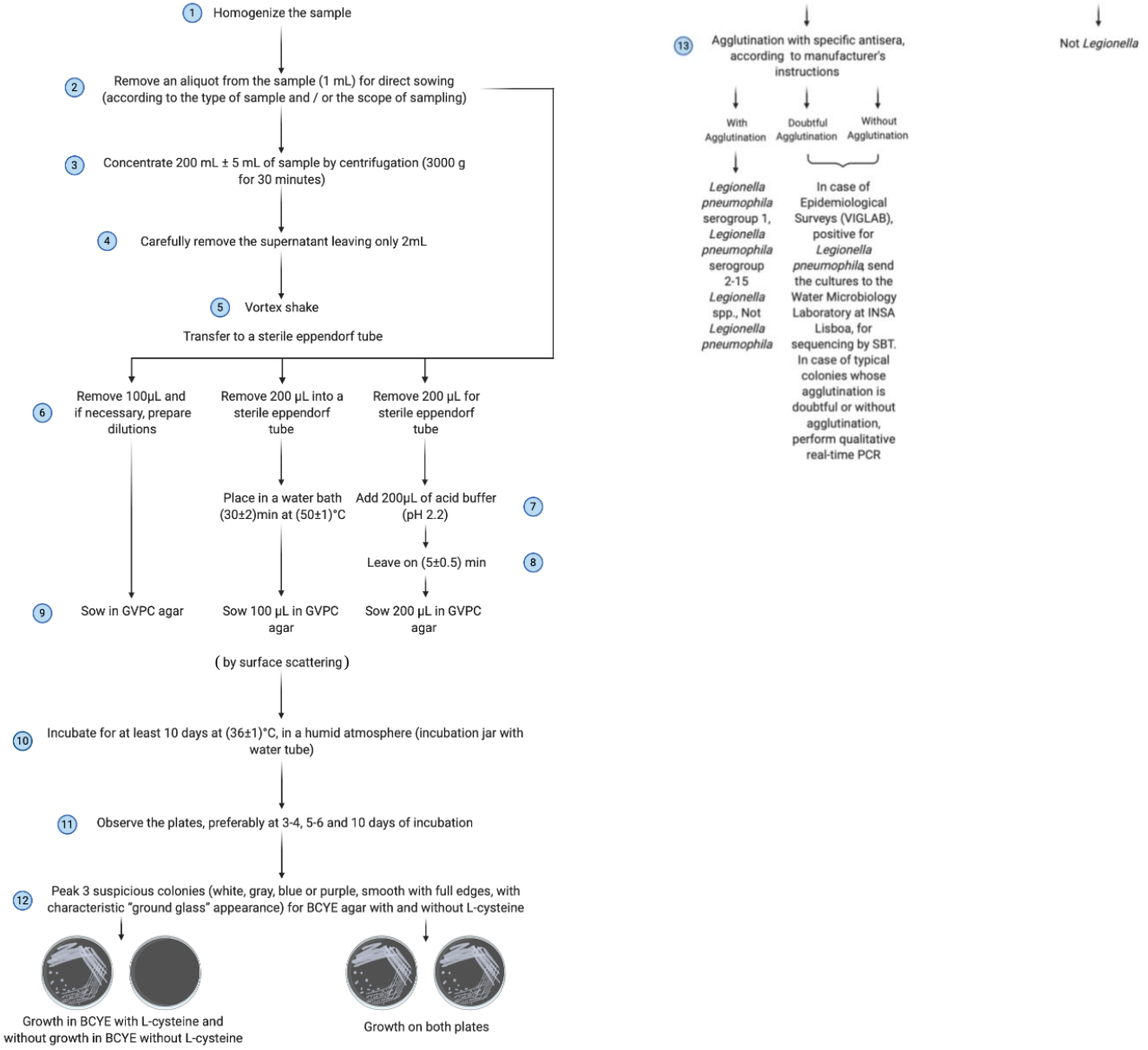

Figure 13 - Flowchart of the research and quantification method DSA ASMI PE20_05 P for *Legionella* spp. non-*pneumophila* and *Legionella pneumophila*.

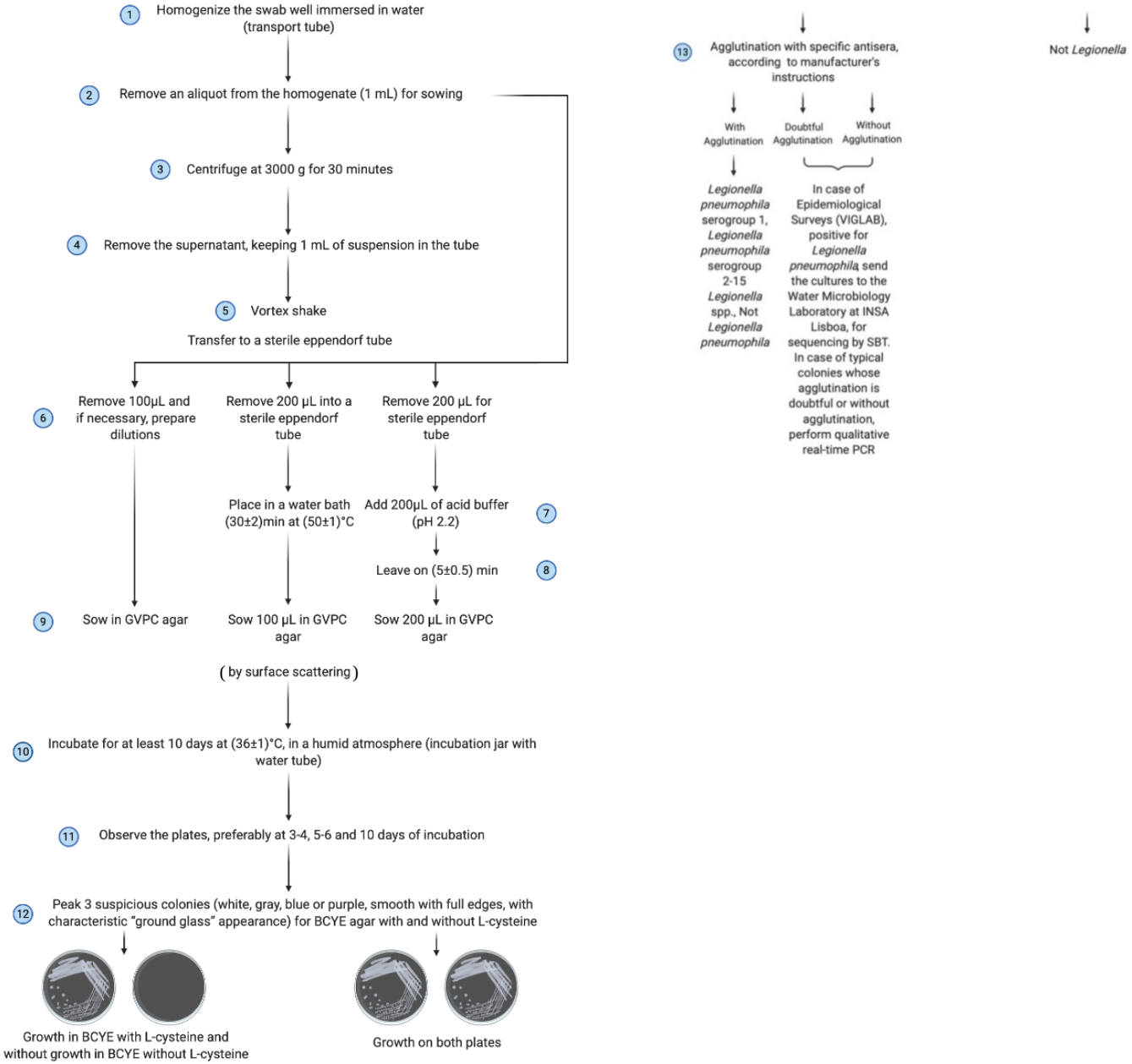

Figure 14 - Flowchart of the research and quantification method DSA ASMI PE20_05 P when using the swab for *Legionella* spp. non-*pneumophila* and *Legionella pneumophila*.

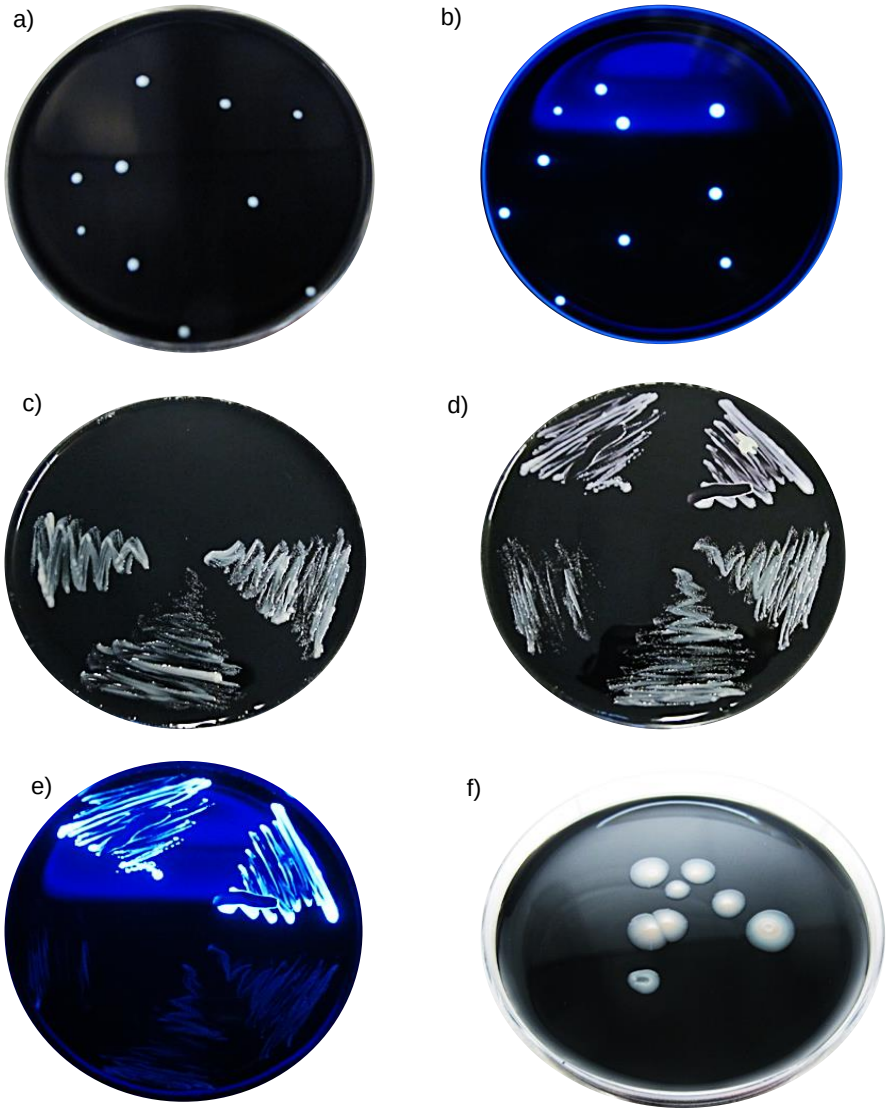

Figure 15 - Examples of *Legionella* spp. non-*pneumophila* and *Legionella pneumophila* cultures: a) GVPC culture medium plate with direct sowing of the sample after heat treatment, in this sample *Legionella pneumophila* serogroup 1 and *Legionella* spp. non-*pneumophila* were detected; b) GVPC culture medium plate with direct sample sowing after heat treatment under ultraviolet light, in this sample *Legionella pneumophila* serogroup 1 and *Legionella* spp. non-*pneumophila* were detected; c) BCYE culture medium plate without L-cysteine (where Legionella is incapable of growth), in this sample was detected *Legionella* spp. non-*pneumophila*; d) the previous sample in BCYE culture medium plate with L-cysteine; e) the previous BCYE culture medium plate with L-cysteine under ultraviolet light; f) GVPC culture medium plate with direct sowing of the sample after heat treatment, in this sample *Legionella pneumophila* serogroup 2-15 was detected.

Real-time PCR method

The *Legionella* detection and quantification by qPCR takes place in three phases: the concentration of 50 to 1000 ml of water samples by filtration with 0.45 µm polycarbonate filters; DNA extraction using the Aquadien™ DNA Extraction and Purification Kit; Amplification, detection and quantification of specific DNA sequences belonging to the *Legionella* genus and/or *Legionella pneumophila* species by qPCR in the sample. The PCR detection limit is 5 genome unit (GU)/5µL.

***Legionella* spp. and/or *Legionella pneumophila* detection**

For "*Legionella* spp. detection" and "*Legionella pneumophila* detection" it is used the ISO/TS 12869 method (Real-time PCR). The iQ-Check® Screen *Legionella* spp. Real-Time PCR Detection Kit and the iQ-Check® Screen *L. pneumophila* Real-Time PCR Detection Kit were used to accomplish these goals. Information about the fragments amplified and screened with these kits was not found. The DL for the qualitative methods is 160 GU/L, and the expression of results is: in case of a negative result "Not detected (< DL)"; and in case of a positive result "Detected". For executing qPCR and analysing results it is used the Bio-Rad Manager CFX - Industrial Diagnostic Edition (IDE) software.

***Legionella* spp. and/or *Legionella pneumophila* quantification**

For "*Legionella* spp. detection and quantification" and for "*Legionella pneumophila* detection and quantification" is applied ISO/TS 12869 method (Real-Time PCR). The iQ-Check® Quanti *Legionella* spp. Real-Time PCR Quantification Kit and the iQ-Check® Quanti *Legionella pneumophila* Real-Time PCR Quantification Kit were applied. The iQ-Check® Quanti *Legionella* spp. includes reagents to amplify and quantify nearly 100 base pairs (bp) fragment from the 5S rRNA gene of *Legionella* spp. It is considered that a genomic unit contains all gene copies, the 3 copies of the 5S rRNA gene present in a cell [98]. The amplification mix has the internal control (IC), a linear plasmid that adds to respectively PCR reaction and that should be amplified in all conditions. In the reaction mix, the control alerts to any inhibitory effects that may occur. Both the *Legionella* target and the IC are amplified in the same PCR well [147]. Regarding the iQ-Check® Quanti *Legionella pneumophila*, it was not found any information about the amplify and quantify fragment. The DL for the quantitative methods is 80 GU/L and the Quantification Limit (QL) is 608 GU/L, and the expression of results is: in case of a negative result "Not detected (< DL)"; in case of a positive result: when the value is > QL,

the result is expressed in GU/L, otherwise is expressed as "Detected (< QL)". The execution of qPCR and the result analysis is done with resource to the Bio-Rad Manager CFX - Industrial Diagnostic Edition (IDE) software.

Report Emission to the Requesting Entities

The results are communicated to the requesting entities by sending the respective test reports by email, in the vast majority of cases. When the results of the samples analysis are positive, the customer is informed in three different ways, according to his preference, by phone, email or by sending a provisional test report. In these cases, the laboratory usually sends to the client the *Instituto Português da Qualidade* (IPQ) 2018 manual "*Legionella Prevention and Control Plan*", with the measures that should be taken. Furthermore, it also suggests contacting the local health authority to be informed about the existence of a possible public health risk, and to make the recommendations and requirements it deems convenient. The final report's layout, that is sent to the requesting entities, is presented in Supplemental Figure 3.

New Database Structure

Originally, there existed two INSA databases, one from March 2019 to August 2019 (sample record system: LabWay-LIMS®) and other from August 2019 to March 2020 (sample record system: INSALAB). They were not uniform and presented requesters and harvest points that were not consistent within the period from March 2019 to March 2020, determined for the analysis. For that reason, the information from these two parameters was selected, prioritizing their existence in both databases, which facilitates their one-year tracking. After this filtration, the institutions were organized by their industry/activity sector for a better understanding of the requester type.

The industry/activity sector, requesting entity, harvest point, water nature, parameter, collection date, sample number, result, output and method were all represented in the new database. In some cases, when the requesting entity provided the treatments used in their system, these data were also added between the treatment's previous sampling and the posterior one. For this, an informative sheet was developed to be filled by the various requesting entities, as can be seen in Supplemental Figure 4.

Results and Discussion

Data Analysis

Looking at the data gathered from these technical reports on *Legionella* spp. surveillance, few conclusions can be drawn so far, for various reasons. Not all samples have an explicit collection point, and several sampling points have been identified with a code for which the meaning is unknown, making it difficult to recognize the type of the sampled system, important to understand and interpret the results in a complete manner. Furthermore, several requesting entities send samples from their customers with a low degree of identification information, and for this reason, it is very challenging to know who they are, to what industry they belong and where they are located, elements that are absolutely needed if outbreaks occur or significant bacterial concentrations are verified. In the vast majority of samples collected by clients, parameters such as pH, temperature, bromine and free residual chlorine measured at the collection point are not known, and, as previously mentioned, in the case of samples collected by technicians, the pH is only measured when demanded by the requesting entity. Since the request of measuring the pH is rarely made, this limitation may compromise the interpretation of bromine and free residual chlorine values measurements, as well as bacterial concentration levels. A significant gap is also pointed out in the knowledge of the description and registration of water system's equipment where the collection is carried out, an obligation established by Law Nº 52/2018 of 20 August. Such equipment may escape audits and be neglected from a specific type of adequate treatment. Finally, most of the requesting entities did not provide information about the treatments carried out in the systems, which does not allow the study of its effectiveness, essential to implement corrective measures.

Nonetheless, some observations can be discussed. For example, the requesting entity Textile A displays the harvest point "*Torre 2 (torneira*)" with a time-spaced contamination by *Legionella pneumophila*. However, there is no information on treatment intervention, what leads to the lack of evidence about the treatment's efficacy and to the incapacity of INSA correctly instructing about the treatment that would be suitable. In the Chemical A's harvest point "*Torre de Arrefecimento 1*", the responsible did not explore the positive case of *Legionella* spp. non-*pneumophila*, or at least did not report any kind of treatment. From this same requesting entity, the harvest point "*Torre de Arrefecimento 2*" had a time-spaced contamination by *Legionella pneumophila* even after treatment,

unveiling its inefficacy and urgence to be modified. It can also be observed the case of Laboratory A's harvest point "*Torre de Arrefecimento 3*" that from March 14th of 2019 to March 10th of 2020 had continuous *Legionella pneumophila* contamination and no intervention is known to reverse this effect, making it, at least, a worry situation. This same requester also has the "*xxx2*" harvest point with constant *Legionella* spp. contamination over time and no sanitation intervention was reported. The Local Health Unit A's harvest point "*Chuveiro*" from March 26th to November 13th had exponential growth of *Legionella* spp. non-*pneumophila*, and also there is no known intervention to correct this situation. At last but not least, harvest point "*Chuveiro xxx2*" from Hospital A is an example of local contamination.

Framework for *Legionella* Research Harvest Database

To overcome the previously described problem, a database framework for *Legionella* surveillance was designed and proposed, aiming for a better and accurate organization of the data and to meet the legal requirements (Figure 16). In this annotation and history file, it is possible to find different categories, each one with their parameters. It is divided into Requester, Equipment, Treatment, Sampling and Analysis, and the goal is to characterize each one of them in a deep manner (Figure 16). Some documents, such as the Law N. º 52/2018 of 20 August, internal method DSA ASMI-P33_03 P and the databases from the sample record systems LabWay-LIMS® and INSALAB were used for the development of this framework. This framework differs from the new database, requiring a more detailed information about the requesting entity, equipment, treatment and sampling, what favours full and detailed knowledge, as can be seen represented in bold in Figure 16.

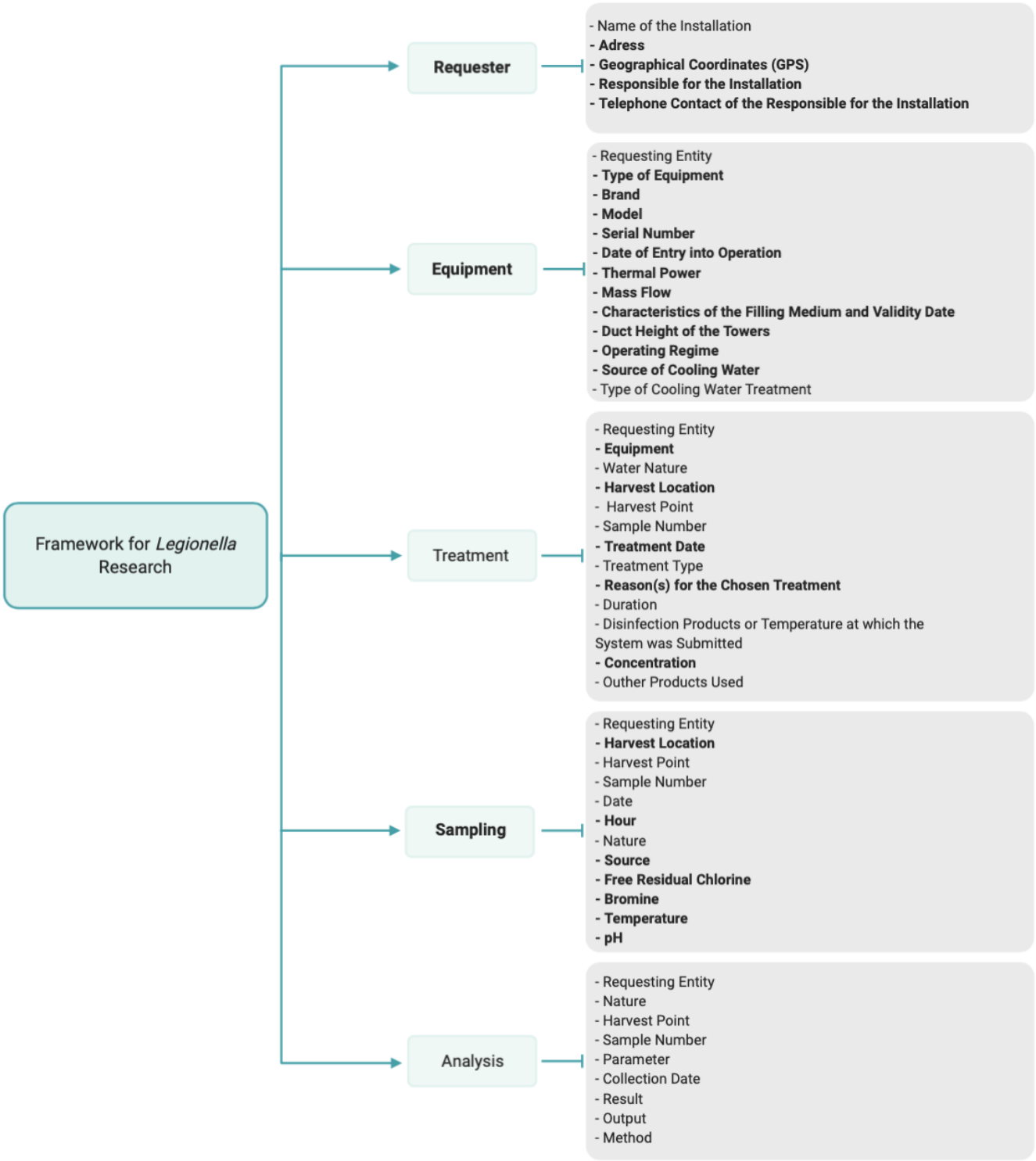

Figure 16 - Schematic representation of the framework for *Legionella* research, with the information missing from the database in bold.

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Conclusion

During this process, several weaknesses and inconsistencies in the data provided became clear. Alterations and uniformizations on the current treatment of *Legionella* analysis information are necessary, mainly because of their value in epidemiological analysis. To achieve these goals, it is important to establish legal obligations to record temperature, pH, bromine and free residual chlorine levels. Besides, water systems' equipment should be registered and characterized on the laboratories databases, as well as the treatments applied to these same systems. Similarly to the clinical analysis' laboratories response system, notification systems from the water analysis' laboratories to the health entities when environmental *Legionella* exceeds the eligible legal values should exist, and, furthermore, mandatory notification of new cooling towers' construction and consequent authorization would be a good idea for monitoring potential origins of outbreaks.

These actions would benefit both the client and INSA, since the latter would be capable of informing some irregularities to the requester, not only about the data supplied, but also concerning the measurements and developed analysis in the field and laboratory with a higher degree of certainty. On the other hand, the client side could also improve their water systems control and prevent any prominent issue.

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Supplemental Data

Supplemental Table 1 - Legionnaires' disease and Pontiac fever main characteristics. Adapted from WHO [10].

Characteristic	Legionnaires' disease	Pontiac fever
Incubation period	2 - 10 days, rarely up to 20 days	5h - 3 days (most commonly 24-48h)
Duration	Weeks	2 - 5 days
Case-fatality rate	Variable depending on susceptibility; in-hospital patients can reach 40 - 80%	No deaths
Attack rate	0.1 - 5% of the general population 0.4 - 14% in hospitals	Up to 100%
Symptoms	• Often non-specific • Strength loss (asthenia) • High fever • Headache • Nonproductive, dry cough • Sometimes expectoration blood-streaked • Chills • Muscle pain • Difficulty in breathing, chest pain • Diarrhoea (25 - 50% of cases) • Vomiting, nausea (10 - 30% of cases) • Central nervous system manifestations, such as confusion and delirium (50% of cases) • Renal failure • Hyponatremia (serum sodium <131 mmol/L) • Lactate dehydrogenase levels > 700 units/mL • Failure to respond to beta-lactam antibiotics or aminoglycosides • Gram stain of respiratory specimens with numerous neutrophils and no visible organisms	• Influenza-like illness (moderate to severe influenza) • Strength Loss (asthenia), tiredness • High fever and chills • Muscle pain (myalgia) • Headache • Joint pain (arthralgia) • Diarrhoea • Nausea, vomiting (in a small proportion of people) • Difficult breathing (dyspnea) and dry cough

Supplemental Table 2 - Summary of regulations and guidelines in European countries. The object of the regulations is marked with green when applied and with red when not applied. Adapted from Van Kenhove *et al*. [102].

Country	Object of regulations/guidelines						Critical levels If deviant from EWGLI*	Context of regulation	Document
	Drinking water systems	Spa pools	Swimming pools	Cooling towers	Air conditioning systems	Process water			
Austria								- Health - Bathing hygiene	- Aspects of drinking water (decree of Ministry of Health) - Special decree for prevention in spa pools and water systems of swimming pools - Some provinces: regulations by public health authorities
Belgium (Flanders)							1,000 – 100,000 CFU/L	- Environment - Labour safety - Public health - Biosafety	- Guidelines for hospitals and policy rule on working conditions (KB biological agents) - *Legionella* decree and code of practice for prevention of LD (BAT) (Flanders) - Regulation for pools and cooling towers (Brussels and Wallonia) - Different risk levels covered
Bulgaria								- Public health	
Croatia								- Public health	- Guidelines - law on communicable diseases
England and Wales								- Health and (management of) safety at work - Health	- Primary legislation: Approved Code of Practice and Guidance. Other legislation: reporting of diseases, water supply (water fittings), notification of cooling towers, TM13, HPA, HPSC, BS
Finland								- Health protection - Housing health - Building code - Communicable diseases	
France	partially						250 - 10,000 CFU/L	- Public health - Drinking water - Environment	
Germany							10,000 CFU/100mL	- Public health - Drinking water	- Code of practice W551 (April 2004), W556 (2015), VDI/DVGW Guideline 6023 (2012)
Hungary									- There are plans to develop regulations on general prevention of legionellosis
Ireland								- Labour safety	- Guidelines - Special attention is given to potential risks of dentist systems and high risk in hospitals
Italy								- Public health	- Guidelines for the prevention and control of legionellosis
Latvia								- Labour safety	

								- Public health	
Lithuania	hot water only							- Public health - Drinking water	- Recommendations mainly aimed at clinical manifestation, diagnostics, and treatment of legionellosis - Lithuanian hygiene standard - Draft of regulations for legionellosis aimed at prevention in institutions and accommodation where water is stored or used for work
Malta								- Public health	- Code of practice for prevention of LD in hotels and other establishments
The Netherlands							100 CFU/L	- Drinking water - Bathing hygiene - Safe labour - Infectious diseases - Public health	- Drinking water decree and guidance document (ISSO- publicatie 55) - Decree on bathing locations and guidance document - Policy rule on working conditions - Public Health Act - Act on infectious diseases
Poland									- Regulations on *Legionella* prevention in drinking water under discussion - Regulation of new building construction under discussion - Act on infectious diseases and infections
Portugal									- Elaboration of legislation concerning installation and use of air-conditioning and cooling tower equipment - Prevention guidelines
Slovenia								- Environment - Water - Building construction	
Sweden								- Public health - Building construction	- Mandatory regulations and general recommendations
Turkey									

BAT, best available techniques; Bruss. and Wall., Brussels and Wallonia; BS, British Standards; EWGLI, European Working Group for *Legionella* Infections; Fland., Flanders; HPA, Health Protection Agency; HPSC, Health Protection Surveillance Center; ISSO, Instituut voor Studie en Stimulering van Onderzoek: KB, Koninklijk Besluit; TM13, Technical Memorandum 13; VDI/DVGW, Association of German Engineers/German Technical and Scientific.

*It is not clear whether these values are referent to *Legionella pneumophila* or *Legionella* spp.

Instituto_Nacional de Saúde Doutor Ricardo Jorge

Departamento de Saúde Ambiental
Unidade de Água e Solo

Relatório de Colheita

Código – Parâmetro/ Lista de parâmetros	**Requisição / Nº amostra**
_____	_____
_____	_____

Controlo de temperatura do transporte das amostras		Mala Térmica:
No local da colheita:	Na chegada ao INSA:	Amostras correspondentes:
Conforme: Sim ❑ Não ❑	Hora de entrega das amostras no INSA:	

Dados do cliente:

Cliente: Telefone/ telemóvel:

Morada: e-mail: NIF:

Dados da colheita:

Local de colheita: Técnico Certificado N.º:_____

Ponto de colheita: _____ Data: ___/___/____ às _____horas Técnico de Colheita INSA:_____

Proveniência	Poço ❑	Mina ❑	Nascente ❑	Furo ❑	Rede Pública ❑	Rio ❑	Outra: _____
Matriz / Natureza	Água de Piscina ❑	Água MineralNatural ❑	Água de Nascente ❑	Água de Rega ❑	Água Termal ❑	Água de consumo tratada ❑	Água de consumo não tratada ❑
	Biofilome ❑	Água de processo ❑	Água Balnear ❑	Água Superficial ❑	Torre arrefecimento ❑	Areia ❑	Outra: _____

Dados de campo

Cloro residual livre:_____ mg/L (Fotómetro Nº. Inv.:_____) Temperatura: _____ °C (Termómetro Nº. Inv.:_____) Lote dos frascos de colheita:_____

Bromo:_____ mg/L (Fotómetro Nº. Inv.:_____) pH:_____ (Fotómetro Nº. Inv.:_____) Lote das Zaragatoas: _____

Assinatura do cliente ou da pessoa que acompanhou a colheita: **Assinatura do técnico de colheita:**_____ **Data:** ___/___/____

_____

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Supplemental Figure 1 - The harvest report's layout that is used by the INSA technicians.

Instituto_Nacional de Saúde Doutor Ricardo Jorge

Departamento de Saúde Ambiental
Laboratório de Microbiologia

FICHA DE NOTAÇÃO DE RESULTADOS-LEGIONELA

Data de entrada: / / Nº amostra:

SEMENTEIRA - DIRECTO Data: / /						**SEMENTEIRA - CONCENTRADO Data: / /**						OBS
N.º Colónias **1**	Data / Rubrica	Confirmações				N.º Colónias **1**	Data / Rubrica	Confirmações				
		BCYE	BCYE S/ Cisteína	Aglutinação	Data/ Rubrica			BCYE	BCYE S/ Cisteína	Aglutinação	Data/ Rubrica	
N.º Colónias **2**	Data / Rubrica	Confirmações				N.º Colónias **2**	Data / Rubrica	Confirmações				OBS
		BCYE	BCYE S/ Cisteína	Aglutinação	Data/ Rubrica			BCYE	BCYE S/ Cisteína	Aglutinação	Data/ Rubrica	
N.º Colónias **3**	Data / Rubrica	Confirmações				N.º Colónias **3**	Data / Rubrica	Confirmações				OBS
		BCYE	BCYE S/ Cisteína	Aglutinação	Data/ Rubrica			BCYE	BCYE S/ Cisteína	Aglutinação	Data/ Rubrica	

1 - Sem tratamento; 2 - Calor; 3 - Ácido

Resultado do PCR: _____ Data: / / Rubrica:

Resultado do Método Cultural: *L. pneumophila* ____ _____ UFC/L Data: / / Rubrica:

L.spp não *pneumophila* _____ UFC/L

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Supplemental Figure 2 - The layout of the annotation sheet for *Legionella*'s analysis results that is used by the INSA technicians.

a)

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Relatório de Ensaio N.º 27935 V1 — Data de Emissão: 25-03-2020

Identificação da amostra
Local de colheita: -
Natureza: Água de consumo tratada
Ponto de colheita: -
Responsável pela colheita: Técnico de colheita do INSA
Método de colheita: *ISO 19458:2006; DSA ASMI PE33_04 P (Microbiologia)
Data/Hora da colheita: 12-03-2020 11:20
Origem: Rede pública de abastecimento
Localidade: -

Entidade: -
Nº da requisição:
Nº da amostra:
Data de entrada: 12-03-2020
Data de início dos ensaios: 12-03-2020
Data de fim dos ensaios: 25-03-2020

Laboratório de Microbiologia (Porto)
Departamento de Saúde Ambiental
Unidade de Água e Solo

Ensaio	Resultado / Unidades	Incerteza	VR
Pesquisa e Quantificação de *Legionella pneumophila* Método: DSA ASMI-PE09_05 P	Não Detetada/L (LD=10 ufc/L)		
Pesquisa e Quantificação de *Legionella* spp. não *pneumophila* Método: DSA ASMI-PE09_05 P	Não Detetada/L (LD=10 ufc/L)		
***Cloro residual livre determinado no local (Cl$_2$)** Método: Colorimetria / DSA ASQT-PE36_02 P	0,09 mg/L	± 27%	0,2 - 0,6
***Temperatura no local de colheita** Método: DSA ASQT-PE36_02 P	21,5 °C	± 0,59 (valor abs)	

Cristina Pizarro
Responsável Técnico

VR = Valor Recomendado, VP = Valor Paramétrico do Decreto Lei 306/2007 de 27 de Agosto alterado pelo Decreto Lei 152/2017 de 7 de Dezembro; a) Sem alteração anormal - ver histórico da água.

Observações Gerais
Chama-se a atenção para o valor de cloro residual livre.

Legenda de siglas e abreviaturas: ASTM - American Society for Testing and Materials; CI - Cromatografia Iónica; C.INF - Combustão e infravermelho; COT – Carbono Orgânico Total; Dic.Pot. - Dicromato de Potássio; DIL - Diluição; DLLME - Micro Extração Líquido-Líquido Dispersiva; DSA ASQT-PExx L/P, DSA UAS-PExx L e DSA ASMI-PExx L/P – métodos internos dos laboratórios/unidades; EAA - Espetrometria de Absorção Atómica; EAM – Espetrometria de Absorção Molecular; Eb.M.Ácido - Ebulição em meio ácido; ECD – Detetor de Captura Eletrónica; EE - Espetrometria de Emissão; UE– Unidades de Endotoxinas; Fat.Dil - Fator de Diluição; FC – Fotometria de Chama; FCS – Fluxo Contínuo Segmentado; GC – Cromatografia Gasosa; GH – Geração de hidretos; HPLC-FLD – Cromatografia Líquida de Alta Resolução com detetor de Fluorescência; HPLC-UV – Cromatografia Líquida de Alta Resolução com detetor de ultra-violeta; HS – Extração em espaço de cabeça; ISO-International Organization for Standardization; LAE – L' Analise de l' eau, Rodier; LD-Limite de Deteção; LQ - Limite de Quantificação; MI – Método interno; MS – Espetrometria de Massa; MW - Método Winkler; MR - Método Respirométrico; NMP – Número Mais Provável; NP – Norma Portuguesa; NP EN - Norma Portuguesa / European Norme; OXI - Oxidação; SMEWW - Standard Methods for the Examination of Water and Wastewater; SPE – Extração em Fase Sólida; SPME – Micro Extração em Fase Sólida; ufc- unidades formadoras de colónias; UG- Unidades Genómicas; UNT - Unidades Nefelométricas de Turvação; UV – Ultra violeta; VF – Vapor Frio
O laboratório não contabiliza a incerteza do método de ensaio na declaração de conformidade.
Incerteza expandida (k=2) para um intervalo de confiança de 95% para a componente analítica. Os ensaios/métodos assinalados com (*) não estão incluídos no âmbito da acreditação. Os ensaios assinalados com (#) são contratados e acreditados. Os ensaios assinalados com (##) são contratados e não acreditados. Os comentários/interpretações expressos neste relatório não estão incluídos no âmbito da acreditação. Os resultados constantes do presente relatório referem-se apenas aos itens analisados e nas condições em que a amostra foi rececionada. Assumiram-se como verdadeiras as declarações do cliente/entidade requisitante relativas à sua identidade e à identificação dos itens analisados, sendo estes dados da sua inteira responsabilidade. A reprodução parcial do relatório é proibida.

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b)

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Relatório de Ensaio Nº 68823 — Data de Emissão: 29-06-2020

Identificação da amostra
Natureza: Água de torres de arrefecimento
Ponto de colheita: -
Responsável pela colheita: Entidade requisitante - Cliente
Data/Hora da colheita: 15-06-2020 10:00
Dióxido de cloro (mg/L): 0.10
Temperatura (ºC): 25.5

Entidade: -
Nº da requisição:
Nº da amostra:
Data de entrada: 16-06-2020
Data de início dos ensaios: 16-06-2020
Data de fim dos ensaios: 29-06-2020

Laboratório de Microbiologia (Porto)
Unidade de Água e Solo
Departamento de Saúde Ambiental

Ensaio	Resultado / Unidades
Pesquisa e Quantificação de *Legionella pneumophila* Método: DSA ASMI-PE20_05 P Identificou-se serogrupo 1 Identificou-se serogrupo 2-15	$7{,}0x10^2$ ufc/L
Pesquisa e Quantificação de *Legionella* spp. não *pneumophila* Método: DSA ASMI-PE20_05 P	Não Detetada/L (LD=100 ufc/L)

Cristina Pizarro
Responsável Técnico

Legenda de siglas e abreviaturas:
ASTM - American Society for Testing and Materials; CI - Cromatografia Iónica; C.INF - Combustão e infravermelho; COT – Carbono Orgânico Total; Dic.Pot. - Dicromato de Potássio; DIL - Diluição; DLLME - Micro Extração Líquido-Líquido Dispersiva; DSA ASQT-PExx L/P, DSA UAS-PExx L e DSA ASMI-PExx L/P – métodos internos dos laboratórios/unidades; EAA - Espetrometria de Absorção Atómica; EAM – Espetrometria de Absorção Molecular; Eb.M.Ácido - Ebulição em meio ácido; ECD – Detetor de Captura Eletrónica; EE - Espetrometria de Emissão; UE– Unidades de Endotoxinas; Fat.Dil - Fator de Diluição; FC – Fotometria de Chama; FCS – Fluxo Contínuo Segmentado; GC – Cromatografia Gasosa; GH – Geração de hidretos; HPLC-FLD – Cromatografia Líquida de Alta Resolução com detetor de Fluorescência; HPLC-UV – Cromatografia Líquida de Alta Resolução com detetor de ultra-violeta; HS – Extração em espaço de cabeça; ISO-International Organization for Standardization; LAE – L' Analise de l' eau, Rodier; LD-Limite de Deteção; LQ - Limite de Quantificação; MI – Método interno; MS - Espetrometria de Massa; MW - Método Winkler; MR - Método Respirométrico; NMP – Número Mais Provável; NP – Norma Portuguesa; NP EN - Norma Portuguesa / European Norme; OXI - Oxidação; SMEWW - Standard Methods for the Examination of Water and Wastewater; SPE – Extração em Fase Sólida; SPME – Micro Extração em Fase Sólida; ufc- unidades formadoras de colónias; UG- Unidades Genómicas; UNT - Unidades Nefelométricas de Turvação; UV – Ultra violeta; VF – Vapor Frio
O laboratório não contabiliza a incerteza do método de ensaio na declaração de conformidade.
Incerteza expandida (k=2) para um intervalo de confiança de 95% para a componente analítica. Os ensaios/métodos assinalados com (*) não estão incluídos no âmbito da acreditação. Os ensaios assinalados com (#) são contratados e acreditados. Os ensaios assinalados com (##) são contratados e não acreditados. Os comentários/interpretações expressos neste relatório não estão incluídos no âmbito da acreditação. Os resultados constantes do presente relatório referem-se apenas aos itens analisados e nas condições em que a amostra foi rececionada. Assumiram-se como verdadeiras as declarações do cliente/entidade requisitante relativas à sua identidade e à identificação dos itens analisados, sendo estes dados da sua inteira responsabilidade. A reprodução parcial do relatório é proibida.

Relatório de Ensaio Nº 68823
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Supplemental Figure 3 - The final report's layout that is sent to the requesting entities. Examples for treated consumption water (a) and cooling tower water (b).

Data de Colheita da Amostra	Tipo de Água (Ex.: rede pública, torre de arrefecimento, etc.)	Ponto de Colheita	Nº da Amostra	Resultado (Qualitativo e Quantitivo)	Tipo de Tratamento (Ex.: químico ou térmico)	Qual o Motivo do Tratamento Escolhido	Data do Tratamento	Duração do Tratamento (Horas ou Dias)	Produtos Utilizados na Desinfeção OU Temperatura a que o Sistema foi Submetido	Concentração Utilizada	Outros Produtos Utilizados	Resultado Obtido	Outras Intervenções

Supplemental Figure 4 - Information about the treatments applied to the water systems, to be filled by the requesting entities.