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Application of *Sequence Based Typing* (*SBT*) technique to typing strains of *Legionella* spp. : development of an environmental risk map

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FOREWORD

The main purpose of my PhD is the assessment of the environmental prevalence of *Legionella* spp. in artificial aquatic environments, considered a habitat at major risk of proliferation, in order to elaborate a risk map. The water distribution system for its characteristics: material of pipelines, aging of network, and absence of adequate maintenance procedures, become an habitat where the microorganism can survive protect by biofilm. During the course of 3 years I had the opportunity to study and evaluate the different habitats correlated to *Legionella* growth and dissemination; in particular, I have studied the ability of *Legionella* to colonize these aquatic environments in which, as reported by the WHO, there are suitable conditions for their survival and multiplication, creating a risk to public health. The focus of my research is the hot water network of a hospital, and at the same time I could assess the presence of the microorganism in other conditions as the water lines circuit of the dental unit and a cooling towers, recently correlated to single case and epidemic events.

The techniques used for detection and enumeration of *Legionella* are recognized and approved by the national and international scientific community: they are the traditional culture method (considered the *gold standard*) and molecular techniques (in particular PCR and sequencing *multilocus*), a main topic of my PhD activity. As suggested by Italian Guidelines for the Control and Prevention of Legionellosis published in May 2015, molecular techniques have been applied alongside the culture technique, according to the official or experimental protocols.

Plan of the thesis

This Thesis has a general introduction (Chapter 1) and 5 chapters where I represent the main arguments of my researches performed during PhD course.

Within of a research project followed by our group as supporting my PhD activity, I have elaborated data related to the colonization of *Legionella* spp. in a hospital located in the Emilia Romagna region and subjected to a strict environmental monitoring program for the *Legionella* risk. The Hospital adopted a new hot water disinfection system, based on a hydrogen peroxide and silver salts (WTP828) required by Regulation (EU) 528/2012 (regarding the use and trade of biocide products) and approved by the World Health Organization (WHO). The results collected during the two years of this project submitted for publication are included in Chapter 2 in the form of scientific paper – in the same form sent to selected journal.

The subsequent results of the above mentioned paper were developed as aim of my PhD project - providing interesting data, showed in Chapter 3, that will be submitted to the journal for publishing.

The work is based on application of the *Sequence Based Typing* (SBT) technique - developed by members of (European Legionnaires' Disease Surveillance Network (ELDSnet) for genotyping of *L. pneumophila* - also to strains of *Legionella species* (eg *L. anisa* and *L. rubrilucens*), in order to identify and study the pathogenicity of some strains, less known but strictly correlated to clinical cases. The second aim of this research, in addition to the SBT approach for *L. species* other than *L. pneumophila*, is the study of phylogenetic correlation to design a map of environmental risk in order to control *Legionella* contamination and support epidemiological investigations.

In Chapter 4, I show the work performed to test a new generation of disinfection system, based on monochloramine and its impact on *Legionella* spp. colonization in the plumbing system of a hospital in Emilia Romagna region, after the removal of the previous disinfection system, which was based on a ClO_2 bioreactor. The data collection was carried out for a year

and the results were published in 2015 in the journal Environmental Science and Technology (DOI: 10.1021/es506118e Environ. Sci. Technol. 2015, 49, 4551−4558).

In Chapter 5, I present another paper, waiting for publication, in a field of waste management. It is not related close to the main topic of my thesis, but I could take part to this project acquiring a new knowledges over these three years. The results concern the study of the Efficiency of a wetland pilot plant based on the use of earthworms and plants.

Chapter 6 is composed by Appendices.

CHAPTER 1

INTRODUCTION

1.1 History

In July 1976, when America was celebrating the bicentennial of its separation from Great Britain, in Philadelphia, about 4000 members of the Pennsylvania State American Legion, an organization of World War II military veterans were gathered to participate in the Legion's 58th annual convention at the Bellevue-Stratford Hotel. The day after the convention began, some of the Legionnaires started to become ill with pneumonia-like symptoms – high fever, cough, chest pains, and difficulty breathing. By the time the epidemic was over in mid-August, 221 individuals had contracted the still unknown disease and 34 of them had died.

Because epidemiological studies led by 20 medical detectives from the US Centers for Disease Control (CDC), as well as scores of Pennsylvania state health workers, were unable to quickly determine the cause of the outbreak, speculation ran wild. Some thought that the epidemic was caused by domestic terrorists through chemical or microbiological means; others, that it was a CIA experiment which had gone awry; still others suggested that the whole thing was a hoax, designed to further extend vaccinations to limit swine flu.

The final theory was that the cause of the outbreak was the air of the Bellevue-Stratford Hotel cooling units and air conditioning because the researchers identified victims among the people who had stayed at the hotel. Because of the time elapsed between the outbreak and investigation, this has never been fully proven. The investigation of the source took a tremendous amount of time and after six months, on December 1976 Dr. Joseph McDade, a CDC laboratory scientist, using the technique of guinea pig inoculation, was able to isolate the bacterium which caused the disease and identify it as *Legionella pneumophila* the *Legionella* bacteria was discovered. In April 1977, the term Legionnaires' Disease (LD) was

first published by the CDC as the official name of the epidemic disease that had caused 34 died.

The event would actually go down as one of the worst medical tragedies of the 20th century.

Many more outbreaks of Legionnaires' disease continued to occur in the U.S. and other outbreaks were confirmed in foreign countries after 1976: in all cases, the illness emanated from some kind of contaminated water system. [1, 2]

1.2 Taxonomy

Legionella is the only genus of the family *Legionella*ceae belonging to the order of taxonomic *Legionella*les. Currently 59 species belong to the genus *Legionella* divided in more than 70 serogroups and about half of these results to be pathogenic opportunist.

There are 16 serogroups of *L. pneumophila*, two each in *L. bozemanii, L. longbeachae, L. feeleii, L. hackeliae, L. sainthelensi, L. spritensis, L. erythra*, and *L. quinlivanii*, and a single serogroup in each of remaining species. The most implicated species in the pathology human it is *L. pneumophila* which is responsible for more than 90% of cases, and in particular sg 1 of more than 84%, followed by *L. longbeachae* (3.9%) and *L. bozemanii* (2.4%), whereas other species involved, although less frequently, are *L. micdadei*, *L. dumoffii*, *L. feeleii*, *L. wadsworthii* and *L. anisa* (2.2% in total). [3, 4]

Legionella species	Sero-groups	Association with clinical cases	Reference
L. adelaidensis		Unknown	Benson et al., 1996a; Benson & Fields, 1998
L. anisa		Yes	Bornstein et al., 1989a; Fenstersheib et al., 1990; Thacker et al., 1990
L. beliardensis		Unknown	Lo Presti et al., 2001
L. birminghamensis		Yes	Wilkinson et al., 1987;
L. bozemanii	2	Yes	Boldur et al., 1985; Bornstein et al., 1987; Bazovska & Spalekova, 1994
L. brunensis		Unknown	Wilkinson et al., 1988
L. busanensis		Unknown	Park et al., 2003
L. cherrii		Unknown	Brenner et al., 1985; Edelstein & Edelstein, 1989
L. cincinnatiensis		Yes	Thacker et al., 1988a; Jernigan et al., 1994; Spieker et al., 1998
L. drozanskii		Unknown	Adeleke et al., 2001
L. dumoffii		Yes	Edelstein & Pryor, 1985; Fang, Yu & Vickers, 1989
L. drancourtii		Unknown	La Scola et al., 2004
L. erythra	2	Yes	Brenner et al., 1985; Saunders, Doshi & Harrison, 1992; Fields, Benson & Besser, 2002
L. fairfieldensis		Unknown	Thacker et al., 1991
L. fallonii		Unknown	Adeleke et al., 2001
L. feeleii		Yes	Herwaldt et al., 1984
L. geestiana		Unknown	Dennis et al., 1993
L. genomospecies 1		Unknown	Benson et al., 1996b
L. gormanii		Yes	Lode et al., 1987; Griffith et al., 1988
L. gratiana		Unknown	Bornstein et al., 1989b
L. gresilensis		Unknown	Lo Presti et al., 2001
L. hackeliae	2	Yes	Wilkinson et al., 1985; Brenner et al., 1985
L. israelensis		Unknown	Bercovier et al., 1986; Sonesson et al., 1994
L. jamestowniensis		Unknown	Wilkinson et al., 1990; Brenner et al., 1985
L. jordanis		Yes	Cherry et al., 1982; Thacker et al., 1988b
L. lansingensis		Yes	Thacker et al., 1992
L. londiniensis	2	Unknown	Dennis et al., 1993
L. longbeachae	2	Yes	McKinney et al., 1981; Boldur et al., 1985; Chereshsky & Bettelheim, 1986; Eitrem, Forsgren & Nilsson, 1987; Lode et al.,1987
L. lytica (comb. nov.)		Unknown	Birtles et al., 1996
L. maceachernii		Yes	Brenner et al., 1985; Merrell et al., 1991
L. micdadei		Yes	Hebert et al., 1980
L. moravica		Unknown	Wilkinson et al., 1988
L. nautarum		Unknown	Dennis et al., 1993
L. oakridgensis		Yes	Orrison et al., 1983; Tang, Toma & MacMillan, 1985
L. parisiensis		Yes	Lo Presti et al., 1997
L. pneumophila	16	Yes	Brenner et al., 1985; Yu, 2000
L. quateirensis		Unknown	Dennis et al., 1993
L. quinlivanii	2	Unknown	Benson et al., 1989; Birtles et al., 1991; Wilkinson et al., 1990
L.rowbothamii		Unknown	Adeleke et al., 2001
L. rubrilucens		Unknown	Brenner et al., 1985; Saunders, Doshi & Harrison, 1992
L. sainthelensi	2	Yes	Benson et al., 1990
L. santicrucis		Unknown	Brenner et al., 1985; Lee et al., 1993
L. shakespearei		Unknown	Verma et al., 1992
L. spiritensis	2	Unknown	Brenner et al., 1985; Harrison et al., 1988
L. steigerwaltii		Unknown	Brenner et al., 1985; Edelstein & Edelstein, 1989
L. taurinensis		Unknown	Lo Presti et al., 1999
L. tusconensis		Yes	Thacker et al., 1989
L. wadsworthii		Yes	Edelstein, 1982a
L. waltersii		Unknown	Benson et al.,1996b
L. worsleiensis		Unknown	Dennis et al.,1993

Figure 1. *Legionella* species and serogroups associated with disease (source WHO, 2011 [5])

1.3 General description

It is Gram-negative bacteria, aerobes, non -spore-forming, mobile for the presence of one or more flagella, rod shaped and vary in size from 0.3 to 0.9 micrometers (µm) in width, and 1.5 than 5 m of length (while in culture are frequent filamentous forms long up to 20 µm). The cell wall is characterized by presence of branched-chain fatty acids not present in gram-negative.

They are to be considered facultative intracellular parasites as they are able to multiply within free amoebas, ciliated protozoa in the water and phagosomes of monocytes and macrophages human alveolar. [6]

From a biochemical point of view, the *Legionella* is relatively inert, do not present any fermentative activity of sugars and most shows a weak oxidase and catalase activities; in addiction, *Legionella* is urease and nitrates negative, but positive to gelatinase tests, the β-lactamase and hippurate hydrolysis. Furthermore, *Legionella* is difficult cultivable and require L-cysteine for growth and primary isolation. *Legionella* use different aminoacids as an energy source, including cysteine, arginine, isoleucine and methionine and its growth is stimulated by iron compounds.

Some species of *Legionella* are fluorescent when illuminated with light UV such as *L. bozemanii*, *L.anisa* or L. *gormanii* while *L. pneumophila* and *L. micdadei* are not fluorescent.

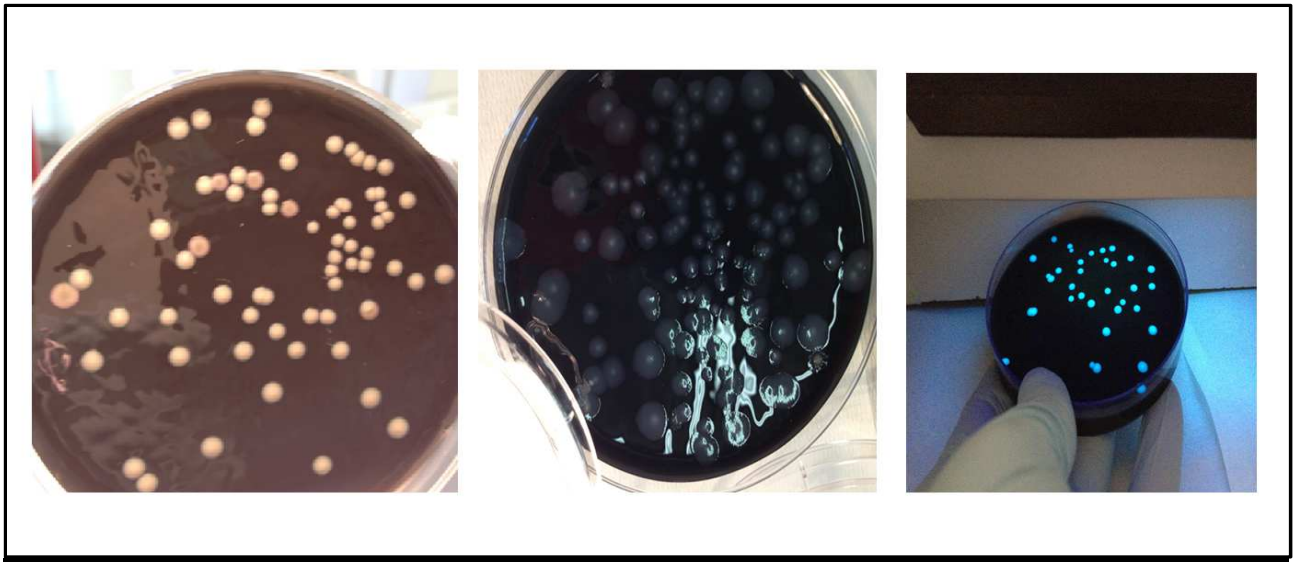


Figure 2. Colonies of *L. pneumophila* and *L. species* grown on selective medium

1.4 Source and occurrence

Legionella spp. are omnipresent in both natural and anthropogenic aquatic environments. Natural environments (like surface waters of lakes, rivers, hot springs and even in moist soil near water sources) are rarely related to legionellosis because habitat conditions do not support extensive *Legionella* spp. growth but anthropogenic systems can promote its proliferation to high concentrations. [7, 8]

The bacterium prefers hot aquatic habitats, where reproduces at a temperature between 25 ° C and 45 ° C, but it is also able to survive in a much larger range of temperature, between 5 ° C and 60 ° C; also it has a good survival in acidic environments and alkaline, enduring pH values between 5.5 and 8.1.

More ideal conditions for multiplication, in environments artificial water are represented by presence of dead branches in the pipes causing phenomena like stagnation or obstruction as well as by the presence of incrustations and sediments and biofilm. Biofilm is defined as complex microbial communities characterized by cells that are attached to a substratum or

phase boundary and to each other by means of a matrix of self-produced extracellular polymeric substances. [9]

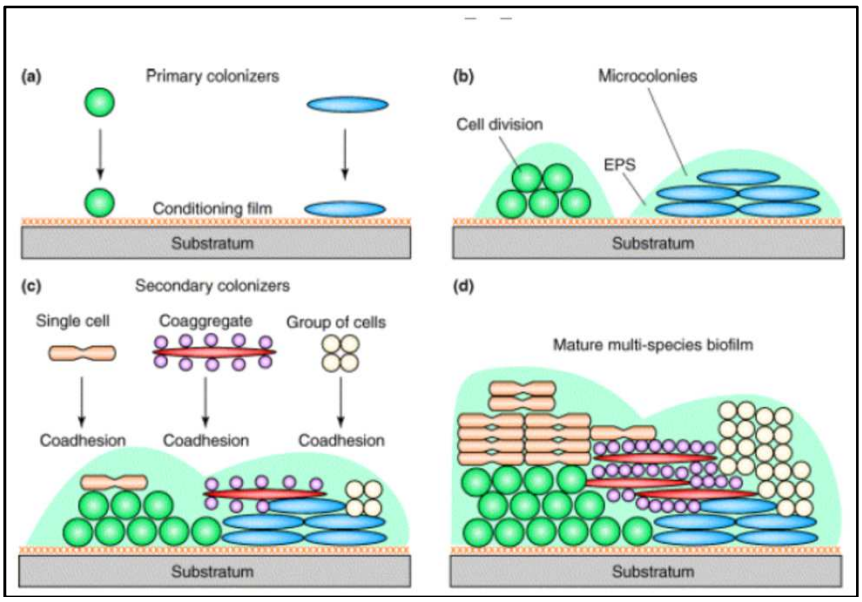


Figure 3. The formation mechanism of biofilm (source TRENDS in Microbiology, 1997)

Biofilms are highly efficient and stable ecosystems where resident microorganisms are well adapted to survive prolonged periods of environmental. [10] Because of their dynamic character, biofilm communities can continuously change in time and space, allowing transport of nutrients, oxygen, genes and even antimicrobial agents.[11]

The presence of biofilm in water distribution systems increases the persistence and associated risks of pathogens [12] because it provides a favorable environment for capture, growth, propagation, and release of pathogens, such as *L. pneumophila* by supplying nutrients and by protecting from disinfection. [13] Biofilm prevention is an important control measure against the proliferation of *Legionella* since, once established, it is difficult to eliminate.

When an infected source can disseminate sprays or droplets of water containing *Legionella*e, most or all of the water in the droplet evaporates quickly, leaving airborne particulate matter (particles of less than 5 μm in diameter) that can be deeply inhaled and be entered the respiratory airways causing legionellosis. [14] The acquisition of nosocomial Legionnaires

disease has been linked to inhalation of aerosols containing the bacteria. It is not possible to predict whether a source will cause infection based solely on the *Legionella* count. The likelihood that a source will cause an infection depends on the load of bacteria, the effectiveness of dissemination, the way in which it multiplies, and its ability to form aerosols.[5]

1.5 Epidemiology

Legionnaires Disease surveillance in the European Union (EU) Member States is carried out by the European Legionnaires' Disease Surveillance Network (ELDSNet), which continuously updates the reported LD status.

In Europe, in 2014, 30 countries reported 6943 cases, 6412 (92.4%) of which were classified as confirmed. The remaining 531 (7.6%) cases were reported as probable. The number of notifications per 100.000 inhabitants was 1.4 in 2014, which was the highest ever observed. Of 5505 cases with known outcome, 456 were reported to have died, giving a case fatality of 8%.

L. pneumophila serogroup 1 was the most commonly identified pathogen, accounting for 81% of culture-confirmed cases.

France, Germany, Italy, Portugal and Spain have been notified 74% of cases. Notification rates ranged from less than 0.1 per 100.000 inhabitants in Bulgaria, Poland and Romania to 5.6 per 100000 in Portugal. The high rate in Portugal was mainly driven by the large community outbreak that occurred in Vila Franca de Xira near Lisbon in October and November 2014. [15]

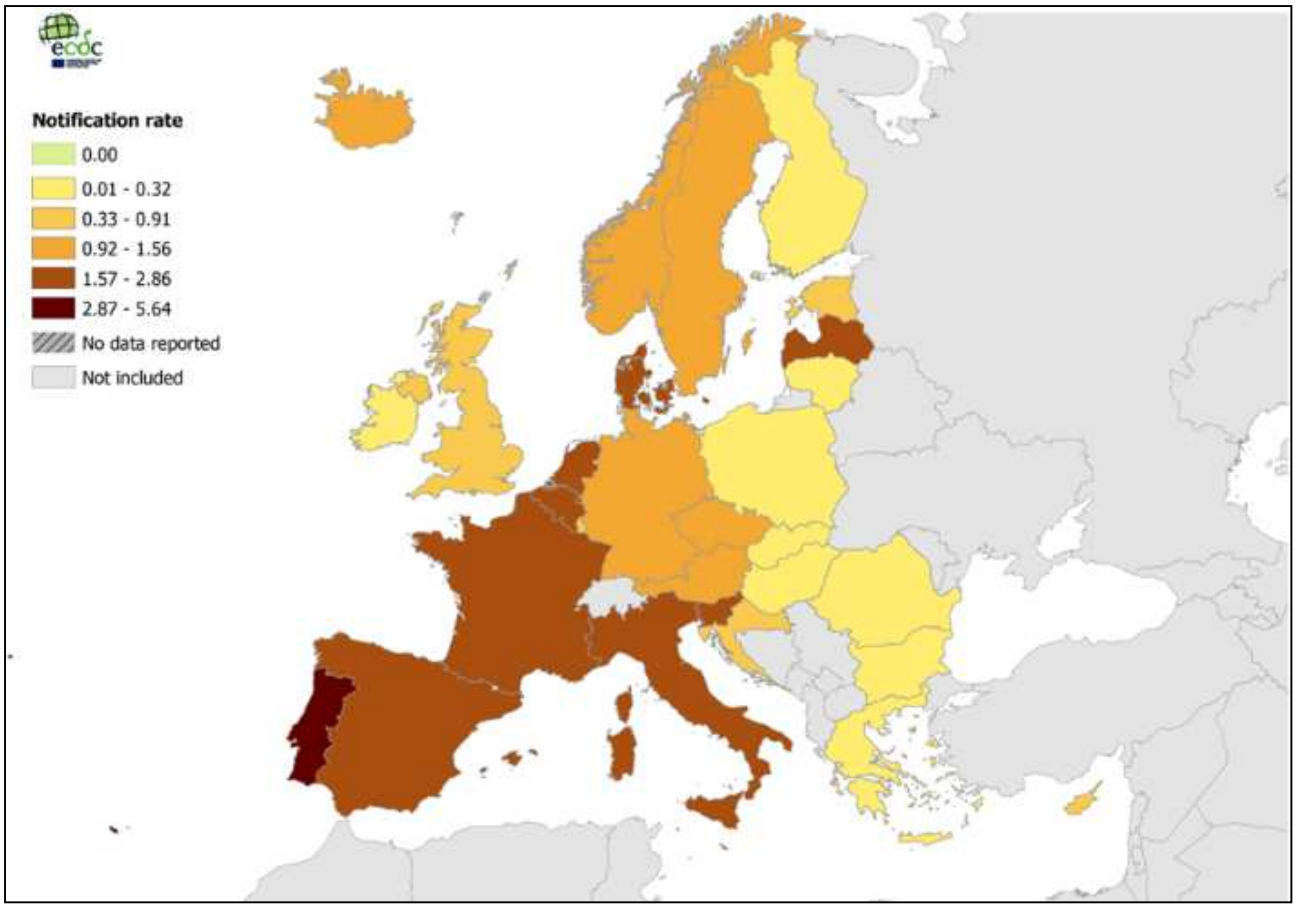


Figure 4. Rate of confirmed Legionnaires' disease cases per 100 000 populations by country, EU/EEA, 2014 (source ECDC. Annual Epidemiological Report 2015 – Legionnaires' Disease)

In Italy, LD is a class II statutorily notifiable disease, as defined by the Infectious Disease Regulations. [17] From 1983, it has also been subject to a reporting system that gathers detailed information on contamination cases in the appropriate national register, based at the Istituto Superiore di Sanità (ISS), Italy. However, according to the Italian National Centre for Epidemiology and the Department of Infectious, Parasitic, and Immune-Mediated Diseases of ISS which produces annual reports on the incidence of the disease in Italy, the number of LD cases is under-diagnosed and under-reported, leading to a significant underestimation of the real incidence of LD. In 2014, the ISS was notified of 1497 new LD cases, of which 1451 were confirmed and 41 were probable. The LD incidence rate in 2014 was equal to 25.1 cases per million. From the 1497 cases reported, 62 (4.1%) patients had been admitted to a hospital,

151 (10.1%) cases were travel-related, 38 (2.5%) patients were living in day care centers, and five (0.3%) cases were associated with other risk factors.

The causative agent in all cases was *Legionella pneumophila*. [18]

1.6 Virulence and pathogenicity

Legionella is a nonconventional waterborne pathogen, as it is not transmitted orally.

Transmission is through mechanical means by many systems that generate aerosols including cooling towers, hot tubs, industrial equipment, domestic plumbing systems, thermal spas, water outlets, respiratory devices and nebulisers, or nasogastric tubes in hospitals. [19] Once inhaled in aerosols, the bacteria are internalized in the lungs by alveolar macrophages and epithelial cells, replicate within the phagosomes and eventually lyse the host macrophages. This process is similar but not identical to the organism being parasitized by protozoa [20]: there are differences in the mechanisms used to enter and exit from the respective host cell types, but they using common genes and gene products. During phagocytosis, *Legionella* spp initiate a complex cascade of processes, including inhibition of the oxidative burst, reduced phagosome acidification, blocking of phagosome maturation, and modifications to organelle trafficking.

Not all of the species of *Legionella* that have been studied are able to infect macrophages. However, *L. pneumophila* that possess the relevant virulence factors can infect and replicate within various protozoa found in soil and in water [21], and by replicating in this way is the most virulent *Legionella* species and the most common cause of disease. [22]

The virulence mechanisms of *L. pneumophila* are complex and not fully understood: the pathogenesis of *L. pneumophila* has been made clearer by the identification of genes that allow the organism to bypass the endocytic pathways of host cells.

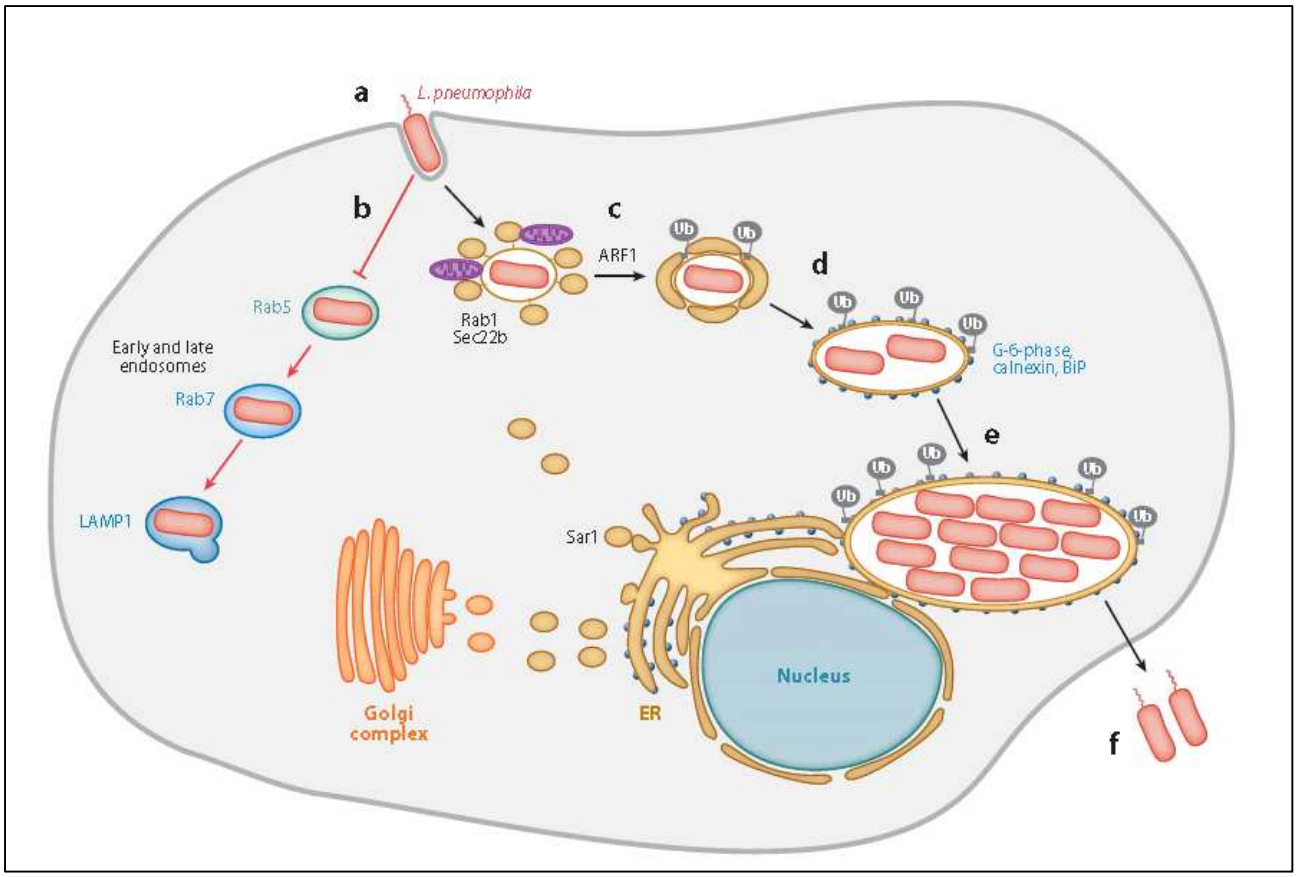


Figure 5. Mechanism of pathogenicity of *Legionella* in host cells (source TRENDS in Microbiology, 2007)

The type IV secretion system, a bacterial conjugation system used for transporting and injecting DNA or toxins into target cells, has a crucial role in the spread of pathogenicity. The type IV secretion systems (*dot/icm*) encoded by 26 *dot/icm* genes essential for infection of the host cell and, needed for *L pneumophila* to enter host cells, for intracellular multiplication of the pathogen, to modulate anti-apoptotic host cell signaling pathways, to disrupt and degrade the phagosome membrane, and to disrupt host cell membranes so that the bacteria egress into the extracellular environment. So far, more than 275 potential secreted effectors have been identified. [19]

The infection cycle starts with bacterial adhesion to host cells followed by cell entry as the most essential steps involving the flagellum, pili, and bacterial surface proteins. These proteins include: MOMP (the major outer membrane protein), Hsp60 (the heat shock protein) and macrophage infectivity potentiator (Mip) protein, coded for by the *mip* gene. [23]

MOMP binds the complement component C3, and mediates the uptake of *L. pneumophila* via macrophage receptors for the complement components CR1 and CR3. [24]

The *mip* gene was the first *L. pneumophila* virulence-associated gene detected. It is required for efficient host cell infection and conserved throughout the genus. [25, 26] The *mip* protein is thought to be conserved throughout the genus [27]:it is required for efficient infection of both mammalian phagocytic cells and protozoa, but its mechanism of action is unknown.

The 60-kDa chaperonins (also known as Hsp60 proteins) are a family of highly conserved proteins, present in all cellular forms of life, whose main function is to help other proteins fold properly. By exploring potential virulence functions of the surface- exposed (extracellular), it was determined that Hsp60 mediates the intracellular establishment of *L. penumophila* in HeLa cells. The ability of this protein to specific alter eukaryotic signaling pathways, cytoskeletal organization, and organellar traffic are indeed functional characteristics that fit well into its role as an *L. pneumophila* virulence effector. [28, 29]

The ability to infect host cells is also influenced by the expression of flagellin [30], although the flagellar protein itself is not a virulence factor [25]; moreover, genes such as *pilE* (coding for the pilin protein) and *pilD* (coding for prepilin peptidase) are important for unrestricted intracellular growth.

Additional virulence factors include several cytotoxins, phospholipases, lipopolysaccharides, compounds associated with iron uptake, metalloproteases, and β-lactamases.

1.7 Clinical manifestations and diagnosis

The generic term "legionellosis" is used to describe the *Legionella*'s infections, which can range in severity from a mild, febrile illness (Pontiac fever) to a rapid and potentially fatal pneumonia (Legionnaires' disease).

Legionnaires' disease is usually caused by *L. pneumophila* but in some cases one or more additional organisms may also be involved, resulting in a mixed (polymicrobial) infection.[31] Legionnaires' disease is an atypical pneumonia that might clinically resemble pneumococcal or other bacterial pneumonias for some similar clinical and radiographic findings. Symptoms range from mild disease to severe pneumonia requiring hospital admission. The incubation period is roughly 2–14 days. [32] General risk factors for the illness include gender (males are roughly three times more likely than females to contract LD), age (50 or older), chronic lung disease, cigarette smoking and excess consumption of alcohol.

The bacteria can also cause a less serious infection called Pontiac fever that has symptoms similar to a mild case of the flu. Pontiac fever is a febrile and generally benign, non-pneumonic disease associated with exposure to *Legionella* bacteria. Its pathogenesis remains obscure and there is no agreed-on definition, nor any specific clinical findings or laboratory tests for its diagnosis and antimicrobial treatment is usually not needed. [33]

The *Legionella* urinary antigen test and culture of lower respiratory secretions on selective media are the preferred diagnostic tests for Legionnaires disease. Isolation of *Legionella* from lower respiratory secretions or pleural fluid is confirmatory and an important method for diagnosis. In addition, if urinary antigen testing is negative but Legionnaires disease is still suspected, a respiratory culture is required for detection of other species and serogroups that the urinary antigen test does not cover. The urinary antigen test is designed to detect the most common cause of legionellosis, *L. pneumophila* serogroup 1. However, all species and

serogroups of *Legionella*e are potentially pathogenic so a patient with a negative urinary antigen result may have legionellosis caused by some other member of genus *Legionella*.

Legionnaires disease outbreak investigations rely on both clinical and environmental isolates. Because *Legionella* is commonly found in the environment, clinical isolates are necessary to interpret the findings of an environmental investigation. Clinical and environmental isolates can be compared using serological and molecular techniques. [16]

There is no evidence of person-to-person transmission of either Legionnaires' disease or Pontiac fever. [5] In general, people do not spread Legionnaires disease and Pontiac fever to other people. However, this may be possible in rare cases. [34]

1.8 Prevention and risk assessment plan

There is no established dose–response relationship for *Legionella* infections, and the concentration of *Legionella*e necessary to cause an outbreak is unknown. Transmission may occur through inhalation or aspiration or directly from contaminated water from a wide variety of sources.

There are no vaccines that can prevent legionellosis. Instead, the key to prevent legionellosis is making sure that the water systems in buildings are maintained in order to reduce the risk of growing and spreading *Legionella*. Examples of water systems that might spread *Legionella* include hot tubs, hot water tanks and heaters, large plumbing systems, cooling towers (air-conditioning systems for large buildings), medical device (e.g. dental unit waterline), decorative fountain.

Minimizing *Legionella* growth in water systems and devices is key to prevent infection. Timely identification and reporting of legionellosis cases is also important because this allows public health officials to quickly identify and stop potential clusters and outbreaks by linking new cases to previously reported ones.

The first step in the management of environmental prevention is timely detection of target organisms in the potential sources of infection. [35]

Following the publication of the new Italian Guidelines for the Control and Prevention of Legionellosis in May 2015, the importance of a surveillance program encompassing all facilities at risk of LD (hospitals, healthcare facilities, dental units, hotels, tourist facilities, and spas) has been acknowledged and the program has been implemented. [36] The guidelines support the development of a risk assessment plan based on the evaluation of "risk," i.e., risk management and risk communication. They recommend that the factors that influence the growth and environmental diffusion of *Legionella* spp. should be taken into account during the design and maintenance of water systems in every potential sources. The document also emphasizes the need for an adequate environmental surveillance plan that would include an appropriate number of sitesand the investigation from a variety of locations. These locations should roughly represent the entire distribution network of the cold/hot water system in hospital, healthcare facility, thermal spa, guest accommodation, and dental unit waterlines. [37] In particular, the number of samples must be proportionate to the planimetric area size and for each hot water plant, the points of hot tap water output, hot water return line, and storage tank must be considered, with at least three representative sites (i.e., distal sites located in different areas of a hospital; and one additional site for every 100 beds). Regarding the frequency of sampling aimed at primary prevention, in the absence of LD cases (presumptive or confirmed), the water system should be monitored every 6 months with an annual revision of the risk assessment plan. [36] The risk assessment plan should be prepared in conjunction with, and made available to, all concerned parties (e.g. health authorities, water suppliers, building managers and water treatment providers). The plan should be reviewed on a regular basis to reflect changes and ongoing improvements in the system, the available evidence base and the surrounding environment. [5]

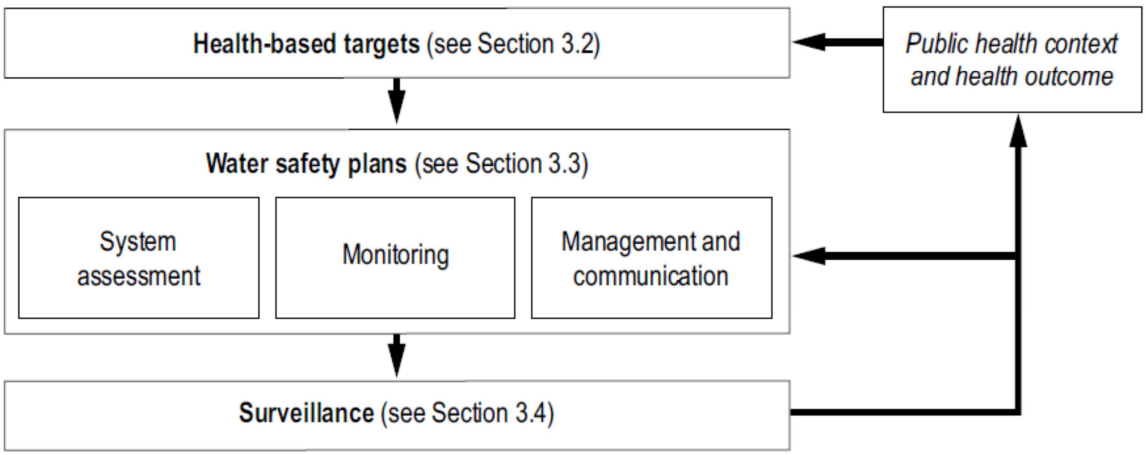


Figure 6. A framework for safe drinking-water **(**source: WHO Guidelines for Drinking-water Quality)

The best approaches to preventing *Legionella* spp. proliferation include, also, the following: increasing the temperature of hot water and maintaining biocide levels outside the optimal bacterial growth range; implementing controls to reduce stasis and aerosolization; and flushing, cleaning, and maintenance of potential sources. [38]

The supply of safe drinking water and adequate sanitation are the basis of preventing diseases which are transmitted through contaminated water; the physical, chemical, and biological indicators of water quality are all interconnected. [39, 40] To maintain water characteristics and its potability, it is essential that all parts of the drinking water distribution systems are properly designed and monitored. This particularly applies to the materials used for their construction and to the maintenance of the water system's working conditions, especially when disinfection treatment is employed.

1.9 Disinfection treatment

In order to control and remove pathogenic microorganisms from engineered water systems (e.g. tap water installations, distribution systems and cooling towers), a wide variety of sanitation practices such as chemical or physical disinfection has been employed worldwide.

Regarding the use of chemical disinfectants, in addition to the disinfection of drinking water, chlorination is the most frequently used approach for the disinfection of swimming pool water .[41] In fact, the main problem associated with water disinfection is the formation of secondary products, disinfection byproducts (DBPs), which pose a chronic and long-term risk to public health. [42, 43] Therefore, an optimal disinfection treatment should maximize the protection against microbes while minimizing DBP levels [44, 45] and should maintain the microbiological and chemical characteristics of the water source. Efficacy of disinfection depends on water quality parameters such as pH and turbidity which may compromise the disinfection process.

One of the most popular chemical measures used to prevent colonization of water distribution systems focuses supplemental chlorination of drinking water. [46] Chlorine is the primary disinfectant of choice for the majority of water treatment plants and its ability to oxidize or rupture the bacterial cell wall and to diffuse into the cell is the primary factor that determines disinfection efficiency. [47]

Disinfection methods other than chlorination such as ozone treatment, copper and silver (Ag^+) ionization, chlorine dioxide (ClO_2) have been tested over the last 30 years and are effective in controlling the growth of *Legionella* spp. in drinking water systems [48] and in hot water system networks in hospitals. Recent studies conducted by Duda et al. [49]and Mancini et al. [45] focused on the effectiveness of monochloramine as an alternative to other disinfectants for the control of *Legionella* spp. contamination.

Different studies have focused on the role of oxidizing agents, notably, hydrogen peroxide (H_2O_2), in disinfection treatments. The use of H_2O_2 as a biocide is now widespread, and it is increasingly used in medical applications, as an antiseptic (particularly in wounds), and in food and industrial applications, as a general surface disinfectant, as well as in environmental applications, such as water treatment, and in commercial dental disinfectant formulations. [50, 51]

2.0 Environmental artificial sources

- *Hospital and health care facilities*

Infections acquired in a healthcare setting are referred to as “nosocomial”.

Within hospitals such as healthcare facilities and nursing homes the potable water supply is the most common source of exposure. [48] These institutions are settings in which people with predisposing risk factors for *Legionella* infections are more likely to be present, and in which medical devices that can disseminate *Legionella* into the lower respiratory tract are used (such as medical humidifiers, inhalation devices and respiratory therapy equipment).In these environments the maintenance of temperatures outside the 20–50 °C range in the network is the best way to prevent colonization of *Legionella* in distribution systems. Proliferation of *Legionella*e, in these networks, is promoted by stagnation, which occurs, for example, in the dead-ends of distribution system pipework, and in storage tanks. Growth has also facilitated their large size, their complexity and the different water use within the various units. The materials used to construct piped water distribution systems should be compatible with the chemical quality of water (after a corrective treatment) and should minimize bacterial growth. In the past, water supply systems were generally constructed of metallic materials such as cast iron, galvanized iron, brass or copper: recently these materials are replaced with synthetic materials such as polyvinyl chloride (PVC) and polybutylene, that support microbial growth and biofilms.

In these environments the risk assessment plan should assess the type of health care provided and the immune and health status of the individuals using the facilities. [5]

- *Cooling towers*

Cooling towers and evaporative condensers have historically been implicated in numerous outbreaks of Legionnaires disease. Cooling towers and evaporative condensers are heat-transfer devices in which warm water is cooled by evaporation in atmospheric air.

Air movement through the tower or condenser is produced by fans or, occasionally, by natural convection. Aerosols generated by the operation of cooling towers and evaporative condensers can transmit *Legionella*e to susceptible hosts. [52, 53] Water is heated and droplets which have been generated within the device is carried in the airflow. The water droplets will contain any dissolved salts or suspended particles, including organisms that were in the original water. It is these droplets that can create an infectious aerosol when the water evaporates in the open air outside the tower.

A significant proportion of outbreaks of Legionnaires' disease in these systems have been attributable to the presence of stagnant systems without adequate chemical treatment. The major risk factor for *Legionella* proliferation in this environment is, in the most cases, insufficient maintenance.

- *Dental units waterline*

Dental unit waterlines (DUWLs) consist of complex networks of thin tubes that facilitate the formation of microbial biofilms. [54] The microbial contamination of water circulating in DUWLs is the result of colonization by both environmental microorganisms from tap water and microorganisms from the oral cavity of patients following fluid suck-back through the hand-pieces. The quality of water is of considerable importance because both patients and dental team are regularly exposed to water and aerosols generated by dental equipment. Studies have demonstrated that DUWLs provide a favorable environment for microbial proliferation and biofilm formation, and that water is consequently often contaminated with high densities of various microorganisms (bacteria, fungi, protozoa, viruses). The presence of high levels of microbial contamination may be a health problem for dentists and patients, especially those who are immune-compromised. [55] *Legionella*, together with *Pseudomonas* and non-tuberculous mycobacteria are the most frequently found genera of bacteria in DUWLs. [56, 57]

2.1 Analytical methods

In 1998, an international standard (International Organization for Standardization ISO 11731) was developed to incorporate the different strategies used by a number of institutions for efficient recovery and detection of *Legionella*e. [58]

Official methods for *Legionella* detection are based on the growth of the microorganism in selective media. This method represents the gold-standard technique for the detection and quantification of *Legionella* in environmental samples but it is labour intensive, slow (up to 14 days) and can have poor reproducibility and poor sensitivity for *Legionella* non-*pneumophila* strains. [59] The sample must be concentrated in order to enhance the quantitation limit. This is usually done by filtering 2 liters through a sterile membrane filter (0,22 µm pore size). The filter is vortexed in 10 mL of the same water and heated. Aliquots are then taken of this distilled water for plating onto 6 different formulations of GVPC or BCYE agar.

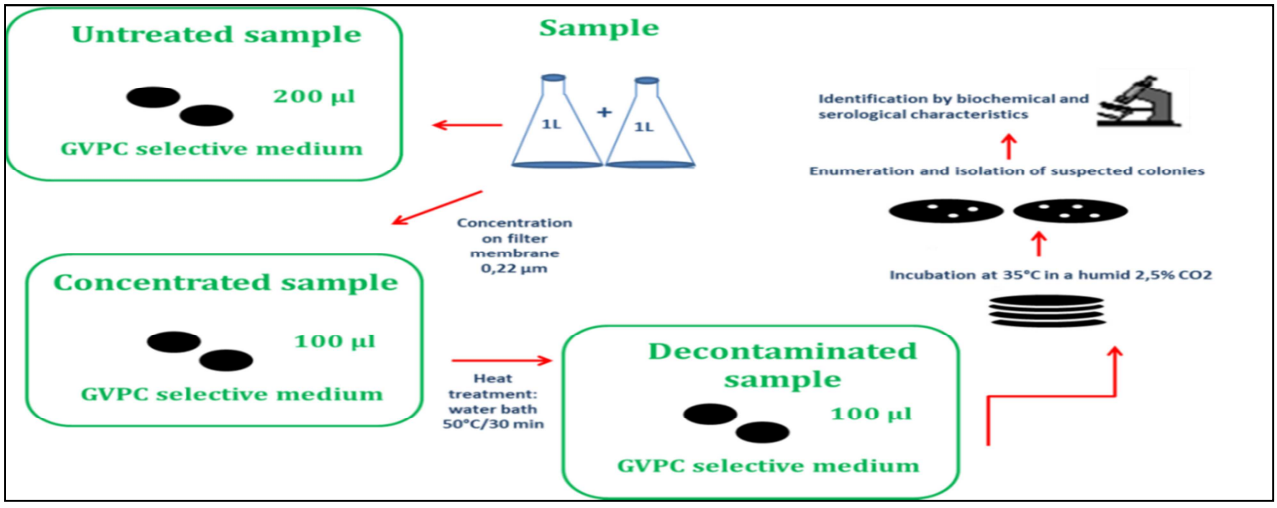


Figure 7. A schematic representation of the culture technique for detection of *Legionella* spp., in accordance with international standard method (ISO 11731)

Legionella spp. are Gram-negative bacteria with strict growth requirements. They grow on various solid selective and non-selective media. *Legionella* colonies are usually detectable

after days 3–5 of incubation. Young colonies are 0,5–1 mm in diameter, self-contained, flat, smooth, with a typical ground-glass appearance and an iridescent hue. [19]

When a colony is suspected to be *Legionella*, it should be Gram stained to check for small to filamentous Gram-negative rods and plated onto two different media in the presence and absence of L-cysteine to confirm its dependence on this amino acid. Commercially available latex agglutination kits may be used for identification and confirmation of *Legionella* species. Suspect colonies are simply emulsified as directed, and mixed with each latex reagent separately on a disposable reaction card. Each reagent is sensitized with antibodies specific to *Legionella*. In the presence of homologous antigens, the latex particles agglutinate to give a clearly visible positive reaction for some minutes. [60] The limit of culture method is the inability to detect viable but non-culturable cells (VBNC). [61]

In recent years to overcome the limitation of the standard technique have been developed alternative methods to identify and differentiate *Legionella* species [62, 63]: phenotypic characteristics, growth requirements, biochemical characteristics, fatty acid and carbohydrate analysis, serology, monoclonal antibodies reactions, various molecular techniques (including, recently, the use of sequencing techniques). Widely accepted alternative to rapidly detect *Legionella* in environmental samples is polymerase chain reaction (PCR). Several authors have described the use of PCR methods for the detection and enumeration of *Legionella* in water samples. It is possible to render the PCR technique quantitative by incorporation of standards with known amount of bacteria (genomes) and quantitative real-time variants of PCR (q(PCR) are often used today. Several commercial kits are available and several laboratories offer this analysis. Although qPCR can be useful in investigating potential sources of infection and in monitoring remedial actions, there is still no consensus on how and when qPCR should be used and how the results should be interpreted: the results of qPCR are expressed as genome units (GU)/mL or L and the correlation with the results of culture

(cfu/L) is very difficult. The discrepant results are especially pronounced for *Legionella* non-p*neumophila*. Studies are ongoing to establish the most appropriate interpretation of qPCR results. Starting from May 2015, the new Italian Guidelines introduce the possibility of using the technique for detecting genomic DNA in environmental samples, referring to the reference standard ISO/TS 12869:2012 "Water quality - Detection and quantification of *Legionella* spp. and/or *L. pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)", specifying that where samples were positive we must also analyze the culture method.

Nevertheless, unlike culture analysis where inter and intra-laboratory variability is high, PCR results are reproducible, accurate, precise, and very sensitive.

Recently sequence-based typing (SBT) has been described as a simple, rapid, discriminatory and portable method for typing *L. pneumophila* strains. [64] The SBT is a multilocus sequence developed by members of the European Legionnaires' Desease Surveillance Network (ELDSNet). It allows amplification of seven target genes: *flaA, pilE, asd, mip, mompS, proA, neuA* involved in different pathways of *Legionella* infection and evaluated for implementation in the investigation of outbreaks of legionellosis caused by *L. pneumophila*.

The choice of the seven target genes for typing *L. pneumophila* occurred as a result of three major studies. A first study showed that the combination of three genes expressed under selective pressure (*flaA, proA, mompS*) offered greater discrimination, sufficient to allow the typing epidemiology of *L. pneumophila* sg 1. [65] A second study led to the addition of three other genes (asd, mip, pilE) and the establishment of an online database for the 6 genes [66], a final study, published in 2007, proposed the addition of *neuA* gene. [67]

Portion of each gene is amplified by PCR and sequenced to identify all possible allelic polymorphisms present in it. At each allelic polymorphism. It is given a number, corresponding to polymorphisms already found and deposited in SBT online database. In the

case of anew polymorphism database assigns a new number. Based on the numbers assigned to the polymorphism of each gene, it is an allelic combination of the profile that corresponds to a Sequence Type (ST).

Genotyping was performed according to the 7-gene protocol from the EWGLI SBT scheme (http://www.hpabioinformatics. org.uk/*Legionella*/*Legionella*_sbt/ php/sbt_ homepage. php).

Gene	Primer name	Position	Primer sequence (5'-3')	Annealing temperature	Fragment size (bp)
flaA	flaA-587F	568-587	GCG TAT TGC TCA AAA TAC TG	55 °C	414
	flaA-960R	981-960	CCA TTA ATC GTT AAG TTG TAG G		
pilE	pilE-35F	12-35	CAC AAT CGG ATG GAA CAC AAA CTA	55 °C	460
	pilE-453R	471-453	GCT GGC GCA CTC GGT ATC T		
asd	asd-511F	487-511	CCC TAA TTG CTC TAC CAT TCA GAT G	55 °C	576
	asd-1039R	1062-1039	CGA ATG TTA TCT GCG ACT ATC CAC		
mip	mip-74F	58-74	GCT GCA ACC GAT GCC AC	55 °C	559
	mip-595R	616-595	CAT ATG CAA GAC CTG AGG GAA C		
mompS	mompS-450F	430-450	TTG ACC ATG AGT GGG ATT GG	55 °C	711
	mompS-1116R	1140-1116	TGG ATA AAT TAT CCA GCC GGA CTT C		
proA	proA-1107F	1090-1107	GAT CGC CAA TGC AAT TAG	55 °C	481
	proA-1553R	1570-1553	ACC ATA ACA TCA AAA GCC		
neuA	neuA-196F	176-196	CCG TTC AAT ATG GGG CTT CAG	55 °C	459
	neuA-634R	634-611	CGA TGT CGA TGG ATT CAC TAA TAC		

Figure 8. The figure shows the positions of the primers for each of the 7 genes with respect to the access number of the GenBank reference sequence

Sequences were analyzed with the use of the online available *Legionella* SBT Quality Assessment database (http://www.hpa-bioinformatics.org.uk/cgi-bin/*Legionella*/sbt/seq_assemble_*Legionella*1.cgi).

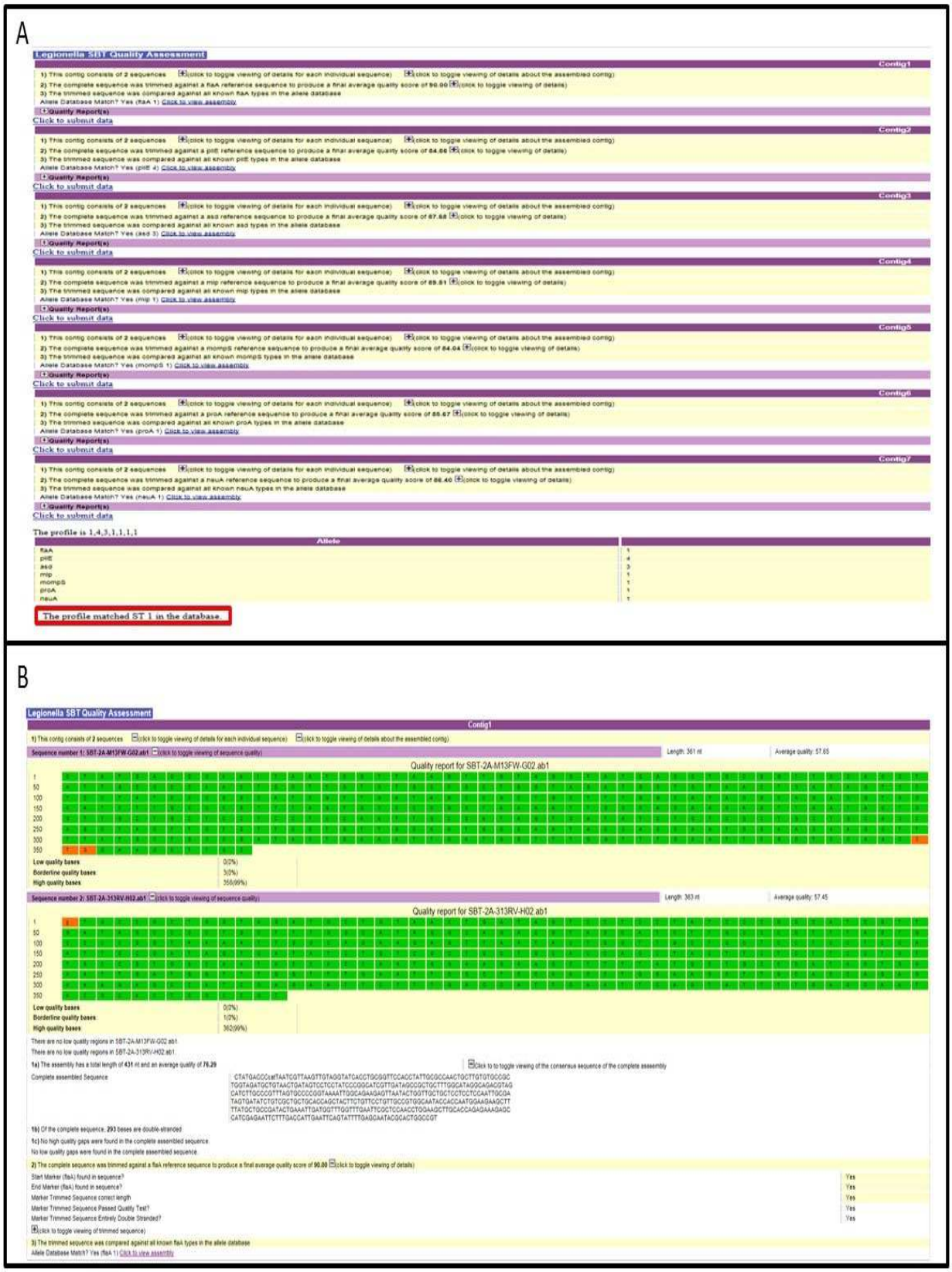
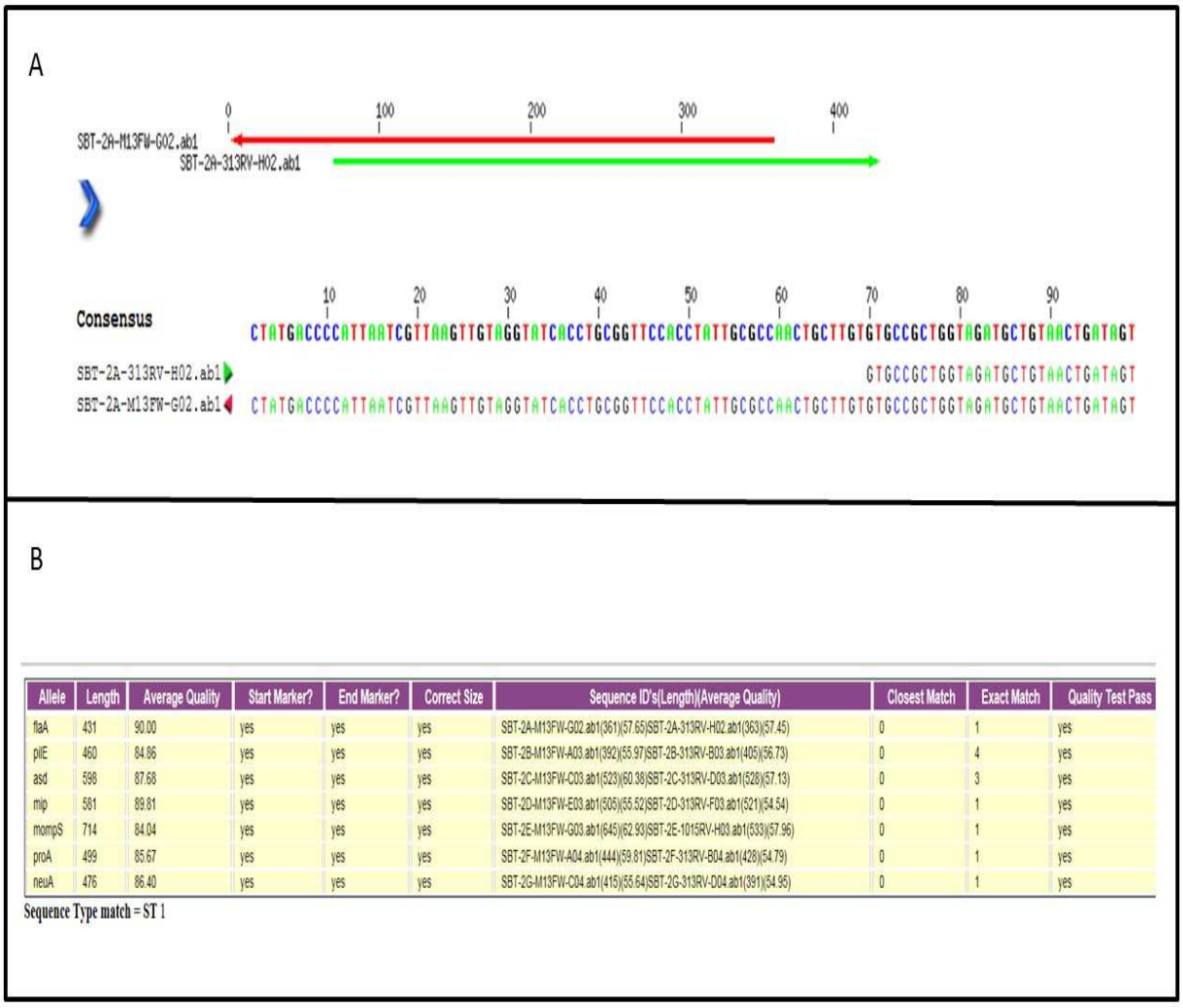


Figure 9. EWGLI Quality Assessment database: in section **A** submission data set sequences; in section **B** alignment of gene sequences – ST attribution

The assignment of the sequence type (ST) was carried out with the use of the SBT database checker

(http://www.hpa-bioinformatics.org.uk/ *Legionella*/*Legionella*_sbt/php/sbt_homepage.php).



Allele	Length	Average Quality	Start Marker?	End Marker?	Correct Size	Sequence ID's(Length)(Average Quality)	Closest Match	Exact Match	Quality Test Pass
flaA	431	90.00	yes	yes	yes	SBT-2A-M13FW-G02.ab1(361)(57.65)SBT-2A-313RV-H02.ab1(363)(57.45)	0	1	yes
pilE	460	84.86	yes	yes	yes	SBT-2B-M13FW-A03.ab1(392)(55.97)SBT-2B-313RV-B03.ab1(405)(56.73)	0	4	yes
asd	598	87.68	yes	yes	yes	SBT-2C-M13FW-C03.ab1(523)(60.38)SBT-2C-313RV-D03.ab1(528)(57.13)	0	3	yes
mip	581	89.81	yes	yes	yes	SBT-2D-M13FW-E03.ab1(505)(55.52)SBT-2D-313RV-F03.ab1(521)(54.54)	0	1	yes
mompS	714	84.04	yes	yes	yes	SBT-2E-M13FW-G03.ab1(645)(62.93)SBT-2E-1015RV-H03.ab1(533)(57.96)	0	1	yes
proA	499	85.67	yes	yes	yes	SBT-2F-M13FW-A04.ab1(444)(59.81)SBT-2F-313RV-B04.ab1(428)(54.79)	0	1	yes
neuA	476	86.40	yes	yes	yes	SBT-2G-M13FW-C04.ab1(415)(55.64)SBT-2G-313RV-D04.ab1(391)(54.95)	0	1	yes

Figure 10. Result of ST assignment provided by database :in section **A** alignment of gene sequences; in section **B** results of quality tool

For each isolate, the profile of 7 alleles at each of the loci was defined in the following order: *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*. ST was represented by a number.

With rare exceptions, all these methods have been used to compare clinical and environmental isolates of *L pneumophila*. [19, 68]

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CHAPTER 2

The Containment and Control of *Legionella* spp. in the Hospital Hot Water Network, by H_2O_2/Ag^+ (WTP 828).

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Abstract

Hydrogen peroxide and silver salts (H_2O_2/Ag^+) are widely advocated to control *Legionella* spp., but further investigations are needed into long-term efficiency, to evaluate its correct dosage and impact on water quality.

The performance of WTP 828, a new disinfectant based on H_2O_2/Ag^+, was evaluated in the hot water circuit of Maria Cecilia Hospital (MCH), Italy.

Hot water was analyzed for physical, chemical, and biological parameters. *Legionella* isolates were typed using agglutination test, Sequence-Based Typing (SBT), and *mip* gene sequencing.

Continuous treatment with WTP 828 (5–10 mg/L) and water demand-dictated dosage in individual buildings enabled the control of *Legionella* spp. colonization, with a reduction of positive sample numbers and bacterial mean concentration levels. Comparison with a previous, chlorine dioxide (ClO_2) mixture, disinfection revealed a significantly higher efficiency of WTP 828 in containing *Legionella* $p = 0.00001$. Typing results indicated the contamination by different species of *Legionella*, *L. pneumophila* serogroup 1 (ST1 and ST104), *L. anisa*, and *L. rubrilucens*.

WTP 828 can be used to control microbial contamination of hot water systems while preserving the water quality. The multidisciplinary approach used herein represents a promising strategy for identifying the real risks to public health and ensures the appropriate preventative measures.

INTRODUCTION

Hot and cold water systems (e.g., tap water installations, distribution systems, and cooling towers) are important sources of nosocomial and community acquired infections caused by pathogenic microorganisms. One of the diseases that are transmitted through water distribution systems is Legionnaires' disease (LD). *Legionella* spp. are water-based organisms that cause infection when inhaled in an aerosol form.[1]

Several national standards have been established to ascertain high water quality, including disinfection techniques to control and prevent *Legionella* colonization.[2]

A wide variety of sanitation practices, such as chemical disinfection, UV light, and temperature, have been employed worldwide [3,4] to reduce the risk of legionellosis.

In Italy, LD is a class II statutorily notifiable disease [5] and from 1983 it has also been subject to a reporting system that gathers detailed information on contamination cases in the appropriate national register, based at the Istituto Superiore di Sanità (ISS), Italy. However, according to ISS annual reports, the number of LD cases is under-diagnosed and under-reported, leading to a significant underestimation of the real incidence of LD, that in 2014 was equal to 25.1 cases per million. [6]

Following the publication of the new Italian Guidelines for the Control and Prevention of Legionellosis in May 2015, the importance of a surveillance program encompassing all facilities at risk of LD (hospitals, healthcare facilities, dental units, hotels, tourist facilities, and spas) has been acknowledged and the program has been implemented. The guidelines support the development of a risk assessment plan based on the evaluation of "risk," i.e., risk management and risk communication. The document also emphasizes the need for an adequate environmental surveillance plan that would include an appropriate number of sites that are potential sources of *Legionella* for patients/guests, and that should be investigated from a variety of locations. [7]

The best approaches to preventing *Legionella* spp. proliferation include the following: increasing the temperature of hot water and maintaining biocide levels outside the optimal bacterial growth range; implementing controls to reduce stasis and aerosolization; and flushing, cleaning, and maintenance of potential sources. [8,9]

Regarding the use of chemical disinfectants, in addition to the disinfection of drinking water, chlorination is the most frequently used approach for the disinfection of water. [10,11] However, increasing evidence suggests that the residual byproducts of water chlorination, such as trihalomethanes, can adversely affect health. [12,13]

Disinfection methods other than chlorination have been suggested for the control of *Legionella* spp. in water, such as ozone treatment, copper and silver (Ag^+) ionization, monochloramine, point-of-use filters or ultraviolet light. These measures have all been tested over the last 30 years and are effective in controlling the growth of *Legionella* spp. in drinking water systems [14] and in hot water system networks in hospitals.

Different studies have focused on the role of oxidizing agents, notably, hydrogen peroxide (H_2O_2), in disinfection treatments. The use of H_2O_2 as a biocide is now widespread, and it is increasingly used in medical , food, industrial applications, as a general surface disinfectant, as well as in environmental applications, such as water treatment. [15,16]

H_2O_2 is very stable in water because it is completely soluble; however, it must be stabilized in solution. A disadvantage of using H_2O_2 is that its potency is influenced by several factors: pH, temperature, or the presence of substances that hamper its reactivity. [17] Because H_2O_2 is a renowned disinfectant, the legislation [18] allows its use for the disinfection of water, as well as in food; also, this compound is generally considered to have low eco-toxicity, as well as no odor or color. [19,20]

To potentiate its activity, H_2O_2 is sometimes used in combination with other agents, such as ozone, Ag^+, or UV, to enhance and accelerate the disinfection processes. Silver, a biologically non-essential metal, has been investigated and used as a biocide for many years [21], and multiple strategies have been proposed for its treatment of drinking water. [22-24] The World Health Organization allows its use in generating drinking water. It is considered that up to 100 µg/L (ppb) of silver can be present in drinking water without posing health risks. [25]

The literature contains several accounts of the properties, germicidal effectiveness, and potential uses for stabilized H_2O_2 in healthcare facilities. [26-29] In 2015, Martin et al. demonstrated that HSP, a new generation peroxide stabilized with ionic silver and suitable for continuous disinfection of potable water, preferentially interacts with the bacterial cell surface

in a mechanism likely mediated by silver. [30] Furthermore, treatment of hospital hot water systems with various formulations of H_2O_2/Ag^+ compounds prevents contamination by *Legionella* and other microorganisms because of its bactericidal properties. [31,32] Different commercial formulations based on H_2O_2/Ag^+ are available to control *Legionella* spp. contamination, but many studies lack data about the hospital settings and long-term application.

This study evaluated the effectiveness of a new disinfectant, WTP 828 (Water Team S. r. l., Forlì, Italy), based on H_2O_2 and Ag^+ salts, in controlling *Legionella* spp. contamination in hot water distribution networks of Maria Cecilia Hospital (MCH), Cotignola (RA), Italy.

MATERIAL AND METHODS

MCH Facilities and the Hospital Water System

This study was conducted at MCH, an Italian hospital founded in 1973, called "Villa Maria," and located in Cotignola (RA, Emilia Romagna).

The structure of MCH is complex and it consists of three separate buildings (Buildings 1,2 and 3), covering an area of 27989.64 m^2 (Figure 1).

<Figure 1 – Site map of MCH, Cotignola (RA), Italy. >

The structural characteristics of each buildings are described in Figure 2.

<Figure 2 – MCH structure and water outlet characteristics >

The MCH plumbing system is very complex, partially antiquated, and predominantly made of galvanized iron and multi-layer or plastic materials, depending on age. A plan of the water distribution network is shown in Figure 3.

<Figure 3 – Plan of MCH water distribution network.>

All buildings are supplied by the same municipal water aqueduct bringing water from Ridracoli dam. The water is first collected outside the buildings in two 30 m^3 water reserves and, after filtration through a 150 µm pore size filter, fed into two pipelines: one to the cooling towers and refrigerant circuit (closed loop hydraulic system), and the other to the water treatment station (open loop hydraulic system).

A heat exchanger maintains the temperature of cold water below 18 °C in the treatment station; the cold water is treated with a general softener to reduce its hardness to 15 °f, in line with European Council directive [33,34]. Some of this water supplies the water to the sterilizers after a reverse osmosis treatment; another portion is used as cold water by the hospital. The cold water is sent to the substations at each building through a single tap water output. At each substation, three different heat exchangers produce the hot water. The cold and hot water circuits are independent of one another, and each building has its own hot water return line.

WTP 828

WTP 828 is a multi-component oxidizing biocide formulated using a stabilized combination of H_2O_2 (34%, wt/wt) and Ag^+ salts (0.003%, wt/wt) in demineralized water, for a highly effective disinfection solution. It is licensed by the European and Italian legislations [18,35] for

its application in drinking water. The synergistic action of H_2O_2 and Ag^+ salts renders the biocide more powerful than H_2O_2 alone. [36,37]

The WTP 828 is injected into mixed water (hot/cold) after hot water output downstream from the heat exchangers and dosed proportionally to the water supply.

The use of WTP 828 was introduced for the first time in October 2013 in MCH Building 2, following the removal of a previous disinfection system that was based on a ClO_2 mixture and was not effective in controlling *Legionella* colonization (see below). Three months later (January 2014), the use of WTP 828 was also implemented in Building 1 and also in Building 3. The WTP 828 concentrations during the study were modulated according to the microbiological results for each building. The initial injection dosage was ~30 mg/L, which resulted in a residual concentration of WTP 828 at distal outlets of 5–10 mg/L. During shock treatment, the injection dosage was increased to 50 mg/L, which resulted in a 25–30 mg/L concentration at distal outlets.

Study Design

This study was conducted in three phases: two experimental phases dedicated to sample and data collection, and one for data analysis, as follows.

- *Phase 1* (WTP 1): Disinfection treatment with WTP 828 in Building 2 (October 2013 to August 2014) and in some locations at Buildings 1 and 3 that were under construction or undergoing expansion at the time. The sampling was performed according to a risk assessment plan, approved by the MCH Health Director and the Local Authority, every 4 months and at 29 sampling points on a rotational basis. During this phase, 53 samples were analyzed.
- *Phase 2* (WTP 2): Once Buildings 1 and 3 were completed (September 2014 to October 2015), the risk assessment plan was revised. The adopted risk assessment

plan, starting from this phase (September 2014) and still in use for the control of *Legionella* contamination in all MCH buildings, comprised eight sampling points in the technical rooms [one in the aqueduct; two in the cold water reserve; one downstream from the general softener treatment; one tap water output; and three on the hot water return lines (1a for Building 1, 1b for Building 2, 1c for Building 3)], and another 55 sampling points in the offices, consulting and diagnostic rooms, wards, communal areas, and in-patient rooms, for a total of 63 points.

The sampling was performed monthly: each month, in rotation, only one building and sites at technical rooms were sampled. During this phase, according to the Italian Guidelines for the Control and Prevention of Legionellosis, [7] different sampling points were chosen at the following three locations: in the vicinity, mid-way to, and away from the technical room. This took account of the hospital setting: the size, numbers of in-patient rooms, health services provided, risk of patient and worker exposure, and epidemiological data. Despite the substantial number of in-patient rooms at MCH, the alternating sampling method enabled the sampling of almost all in-patient rooms in the three buildings over the 2 year period of the study. In total, 296 samples were analyzed during this phase.

- *Phase 3* (WTP 3): The data from the preceding phases were compared, using statistical analysis, with the data collected from September 2009 until September 2013 during the disinfection treatment based on ClO_2 mixture (i.e., WTP 1 phase vs. ClO_2 mixture, and WTP 2 phase vs. ClO_2 mixture). The data related to ClO_2 disinfection treatment were kindly provided by the MCH Health Director. The data collected during the two phases were also analyzed to evaluate the differences in efficacy of the WTP 828 treatment implemented in the MCH buildings (WTP 1 vs. WTP 2 phases).

Physical and Chemical Parameters of Water

Cold water (1 L from the aqueduct, water reserves, softener and tap water outputs) and hot water (1 L from the 3 hot water return lines and distal outlets) were collected every month from October 2013 to October 2015. The physical and chemical parameters of water, including temperature (°C), pH, hardness (°f), conductivity (µS/cm), turbidity (NTU), total iron content (mg/L), total phosphorus content (mg/L of P_2O_5), peroxide levels (mg/L), and Ag^+ content (µg/L), were also performed. The results are expressed as mean ± SD.

During sampling, the residual WTP 828 level was measured on-site using an MQuant™ Peroxide Test (Merck KGaA, Darmstadt, Germany) following manufacturing practices.

Sample Collection and Microbiological Analysis

Hot water (2 L) was collected in post-flushing modality (running water for 1 m) in sterile polytetrafluoroethylene (PTFE) bottles containing sodium thiosulphate solution (10%, v/v).

Microbiological analyses were performed in accordance with ISO11731-1998 method for the detection and enumeration of *Legionella*. [38] The plates were examined after 4, 8, and 14 days, and the presumptive colonies were enumerated and sub-cultured on BCYE agar, with and without cysteine. The isolates that grew on BCYE but failed to grow on the cysteine-free medium were verified serologically by an agglutination test (*Legionella* latex test kit; ThermoFisher Scientific, Oxoid Ltd.). The data are expressed as mean concentration ± SD, $\log_{10}$ colony forming units (cfu) per liter of water ($\log_{10}$ cfu/L).

Other microorganisms can affect the growth of cultivable *Legionella*, and the samples were simultaneously analyzed for the presence of *Pseudomonas aeruginosa*, a known competitor of *Legionella* that inhibits its growth on media. [39] The analysis was performed

according to UNI EN ISO 16266:2006, using a selective *Pseudomonas* agar (Biolife, Milan, Italy). [40]

As an indication of the actual bacterial contamination at the sampling point, heterotrophic plate count (HPC) analysis was also performed, at 36 °C, using a standard plate method on tryptic glucose yeast agar (Biolife), according to UNI EN ISO 6222:1999. [41] The results are expressed as mean concentration ± SD, $\log_{10}$ cfu/mL.

Legionella Typing

Colonies identified by the agglutination test as belonging to the genus *Legionella* were subsequently analyzed by DNA sequencing. In particular, all strains identified as *L. pneumophila* were analyzed by Sequence-Based Typing (SBT) to determine the Sequence Type (ST); strains identified as *Legionella species* were analyzed by *mip* sequencing. Genomic DNA was extracted from cultures using the InstaGene Purification Matrix (Bio-Rad, Hercules, CA). SBT was performed according to an ELDSNet protocol (http://bioinforatics.phe.org.uk/legionella/legionella_sbt/php/sbt_homepage.php). The protocol was based on sequencing of seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) and on the assignment of a ST allelic profile by ELDSNet database (http://www.hpabioinformatics.org.uk/cgibin/legionella/sbt/seq_assemble_legionella1.cgi).

The strains serotyped by agglutination as *L. species* were then genotyped by *mip* gene amplification by polymerase chain reaction (PCR) using degenerate primers, as described IN 1998 by Ratcliff et al., [42] and modified by M13 tailing to avoid noise in the DNA sequence. [43] Gene amplification was carried out in a 50 µL reaction containing DreamTaq Green PCR Master Mix 2× (ThermoFisher Scientific) and 40 pmol of each primer; 100 ng of DNA extracted from the presumptive colonies of *Legionella* spp. was added as template. The same amounts of DNA from *L. pneumophila* type strain EUL00137 provided by the European

Working Group for Legionella Infections (EWGLI) [44] and fetal bovine serum were used as positive and negative controls, respectively.

Following purification, DNA was sequenced using BigDye Chemistry and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Specifically, *mip* amplicons were sequenced using M13 forward and reverse primers (M13 FW, 5'-TGTAAAACGACGGCCAGT-3'; M13 RW, 3'-CAGGAAACAGCTATGACC-5') to obtain a complete coverage of the sequenced region of interest. Raw sequencing data were assembled using CLC Main Workbench 7.6.4 software (https://www.qiagenbioinformatics.com/). The sequences were compared with sequences deposited in the *Legionella mip* gene sequence database using a similarity analysis tool (http://bioinformatics.phe.org.uk/cgi-bin/legionella/mip/mip_id.cgi). The identification on species level was done on the basis of ≥98% similarity to a sequence in the database. [45]

Data Analyses

The bacteriological data were converted into $\log_{10}$ (x + 1) values to normalize non-normal distributions. Normality of continuous variables was assessed by the Shapiro-Wilk normality test. Variables following normal distribution are presented as means ± SD. Continuous variables were evaluated using one-way ANOVA and a post-hoc test (Bonferroni), and categorical variables were compared using the χ^2 and Mann Whitney test. One-way ANOVA and the post-hoc test (Bonferroni) were conducted to assess the differences between disinfectant treatments and between the buildings. Odds Ratio (OR) was calculated retrospectively for WTP 1 vs. ClO_2 mixture and WTP 2 vs. ClO_2 mixture, and Relative Risk (RR) was calculated for the prospective treatments WTP 2 vs. WTP 1. The statistical analyses were performed using STATA version 10.0 (Stata Corp., College Station, TX). A *p*-value below 0.05 was accepted as significant.

Hospital LD Surveillance

Throughout the entire period of monitoring, active legionellosis surveillance was conducted at MCH. In the presence of symptoms of an acute infection of the lower airways with clinical and/or radiological signs of focal pneumonia, the preliminary diagnosis was routinely confirmed by a urine antigen test (*Legionella* Urine Antigen EIA, Biotest, Milan, Italy) and a serological immunofluorescence test (*L. pneumophila* IFA, Meridian Diagnostic Europe, London, UK).

RESULTS

Physical and Chemical Parameters of Water

The physical and chemical parameters linked to the quality of water after disinfection with WTP 828 were measured throughout the tested period.

The measured parameters in the hot water return line and at randomly selected points in the three buildings were all in line with the recommendations of Italian legislation. [33] The hardness, turbidity, and conductivity of water, otherwise associated with the release of iron and total phosphorus, in the hot water system were not affected by the WTP 828 treatment. In particular, the mean Ag^+ concentrations were always under 3 µg/L, as prescribed by the World Health Organization. [25] The data for each building are reported in Table 1.

<Table 1 – Physical and chemical parameters of water in MCH representative samples in technical room.>

Legionella Contamination Results

The following results were obtained for the three phases of the study.

WTP 1 phase (October 2013 to August 2014)

The data were obtained from the analysis of 53 hot water samples collected in 1 year. We observed different *Legionella* contamination trends in the three buildings (Table 2): 16/25 positive samples (64.0%) in Building 1, 13/23 positive samples (56.5%) in Building 2, and 3/5 positive samples (60.0%) in Building 3.

<Table 2 – Legionella concentration in 3 buildings of MCH, numbers and percentage of positive samples for each study phases>

WTP 1 phase data were also compared with *Legionella* contamination data for the previous disinfection treatment involving ClO_2 mixture. We observed a change in the percentage of *Legionella*-positive samples in three MCH buildings, from 95.0% to 60.0%, when WTP 828 was introduced. Statistical analysis of *Legionella* contamination following different disinfection treatment in each building revealed a significant change only in the case of Building 2 ($p = 0.046$) (Table 3).

<Table 3 – Statistical analysis between WTP 828 (WTP 1 and WTP 2) and ClO_2 mixture during study phases in MCH buildings>

WTP 2 phase (September 2014 to October 2015)

In the second phase of the study, we observed a reduction in the number of *Legionella*-positive samples in all buildings (Table 2). We observed a drastic reduction in the number of *Legionella*-contaminated sites in Building 2 (from 56.5% to 7.0%) and in Building 3 (from 60.0% to 34.0%); by contrast, the percentage of positive samples was only slightly decreased in Building 1 (from 64.0% to 58.1%). The analysis of mean *Legionella* levels showed a decreasing trend in all of the buildings vs. WTP 1 phase (Table 2). Statistical analysis revealed significant differences in WTP 1 vs. WTP 2 comparisons, for Building 2 ($p = 0.001$)

and Building 3 (p = 0.037) (Table 3). No statistical differences were observed for these comparisons for Building 1.

We also compared the results from WTP 2 phase with *Legionella* contamination data after disinfection with ClO_2 mixture. The comparison revealed significant differences for Buildings 2 (p = 0.0001) and 3 (p = 0.045), and no significant correlation in Building 1 (Table 3).

WTP 3 phase (September 2009 to October 2015)

Finally, we compared the summative results from the different study phases with the outcomes of the ClO_2 mixture treatment (Table 4).

<Table 4 – Odds Ratio and Relative Risk during study phases>

The WTP 1 vs. ClO_2 mixture comparison indicated that WTP 828 treatment was not particularly effective in all MCH buildings (p = 0.15), while the comparison of WTP 2 vs. ClO_2 mixture revealed a significant improvement in *Legionella* control (p = 0.0001). In fact, the use of ClO_2 mixture significantly increased the risk of *Legionella* contamination vs. WTP 1 (OR, 3.5) and WTP 2 (OR, 13.25) (Table 4). A prospective study evaluation indicated that the WTP 1 regimen increased the risk of contamination vs. WTP 2 (RR, 1.46; p = 0.02).

We want to emphasize that, throughout the duration of the study, the reserve tanks, softener, and tap water output sites of all buildings were *Legionella*-free (below the detection limit of the techniques used, i.e., 0.5 $\log_{10}$ cfu/L).

Legionella **Typing Results**

The isolates from WTP 1 and WTP 2 were serotyped and genotyped using standard techniques. The agglutination test permitted us to identify two species of *Legionella*, *L.*

pneumophila serogroup 1 (SG1) and *L. species*, in 138/349 positive samples (39.0%) (Table 5).

<Table 5 – MCH Legionella spp. contamination: numbers of positive samples, serotyping and genotyping results.>

The SBT method assigned ST1 and ST104 to *L. pneumophila* SG1 isolates in 74/138 (53.6%) of the samples, while *mip* gene sequencing identified inner *L. species*, *L. anisa* and *L. rubrilucens*, in 35/138 (25.3%) of *Legionella*-positive samples; the remaining 29/138 (21.0%) samples contained a mixture of the previously described strains (Table 5).

The results revealed that every MCH building was colonized by a different mix of *Legionella* spp. Accordingly, Building 1 isolates were the most diverse, with *L. pneumophila* SG1 (ST1 and ST104) and *L. species* (*L. anisa* and *L. rubrilucens*). All Building 2 isolates belonged to *L. pneumophila* SG1 (ST1 and ST104), and Building 3 samples showed a presence of *L. pneumophila* SG1 (ST1), with some samples with a single *L. species* strain *(L. anisa* or *L. rubrilucens*).

The serotyping and genotyping data, and mean bacterial concentration ranges (log_{10} cfu/L), are given in Table 6.

<Table 6 – Serotyping and genotyping of Legionella spp. isolates in MCH buildings.>

During the study period, no significant association was found between *Legionella* colonization in the buildings and specific serogroups or strains.

P. aeruginosa and HPC Results

All samples were also analyzed for the presence of *P. aeruginosa* and HPC. *P. aeruginosa* was not detected (as defined in D. Lgs 31/2001) in the hot water system sampled at the distal outlets, water reserves, softener and tap water output, of MCH.

HPC bacterial count (at 36 °C) was analyzed at all sites. The mean concentration ± SD values (log_{10} cfu/mL) for each building were as follows: 0.82 ± 0.25 for Building 1; 0.77 ± 0.65 for Building 2; and 0.94 ± 0.35 for Building 3. At all sites, the contamination range was below the D. Lgs 31/2001 limit of 20 cfu/mL (1.3 log_{10} cfu/mL).

LD Surveillance at the MCH

During the study, 32 patients underwent urine antigen testing and other diagnostic tests because of suspect pulmonary signs of pneumonia. The Hospital Infections Committee considered the negative test results as evidence for the lack of nosocomial legionellosis.

DISCUSSION

This study focused on MCH for its peculiarity organization in three building. The water distribution system is characterized by a single tap water output, and each building is equipped with its own hot water return line, and water disinfection treatment. Before the introduction of WTP 828 (September 2009 to September 2013), MCH implemented a disinfection approach of an occasional hyper-chlorination shock with a continuous treatment with a ClO_2 mixture. This type of treatment led to corrosion of some parts of the plant and a visible decrement of the efficiency of *Legionella* colonization containment, as demonstrated by the high number of *Legionella*-positive samples in the three buildings (114/120, i.e., 95.0%). In October 2013, the MCH Health Director decided to introduce a new disinfectant, WTP 828, in Building 2, as well as at the available sampling points in Buildings 3 and 1.

In the WTP 1 phase of the study, the reduction of *Legionella*-positive samples to 60.0% following the introduction of WTP 828 allowed us to hypothesize that WTP 828 might constitute an appropriate treatment for MCH. The results indicated that the introduction of WTP 828 disinfection was successful in reducing the levels of *Legionella* in Building 2, but no significant change was observed in the levels recovered from Buildings 1 and 3. These results were associated with the fact that Building 2 was the first building to undergo WTP 828 treatment. Also, this building has never been refurbished or otherwise altered since it was built.

The conclusion of accommodation work and the completion of the final structures of Buildings 1 and 3 allowed us to increase the number of sampling sites, with a revised plan of monitoring *Legionella* contamination at 63 sampling points, representative of each building. To improve the efficiency of WTP 828 treatment, we compared the data obtained during WTP 1 with the data obtained in WTP 2 phase. We observed a reduction in the percentage of positive samples and mean *Legionella* levels in all buildings. A significant decrease of *Legionella* contamination was observed in Buildings 2 and 3, where we observed maintenance of *Legionella* control for the entire test period.

The observed differences in *Legionella* colonization between the buildings could probably be ascribed to the different uses and water consumption in these buildings. Risk factors not to be overlooked are, in fact, the scale of extension, the connection of existing pipes within the newly constructed branched networks, the presence of dead branches, pipe characteristics (e.g., materials, age), the treatment of the water system (e.g., water softening and disinfection), the intended utility, and maintenance procedures. In light of these considerations, we also investigated annual water consumption in each building, with respect to the building size and number of water outlets.

Building 1 covers an area of 18539.93 m^2, over six levels. It mainly comprises offices, surgeries, operating rooms, and a diagnostic room, some of which only require the use of sterile water, with limited water consumption. In this building, a whole floor (third floor) hosts a technical room for air treatment and water outlets are absent, and therefore some closed pipes are present. The evaluation of water consumption (1913 m^3/year) indicated a much lower use than in Building 2 (3017 m^3/year), suggesting lower water flushing from the outlets. It is evident that low use and stagnation of water may affect the activity of the disinfectant [46,47] and reduce the contact time between the microorganisms and the WTP 828 disinfectant. Our data revealed that, despite the reduction in the percentage of *Legionella*-positive sites and the mean *Legionella* levels, WTP 828 was not fully active in this building. Our results confirmed that, in the three buildings constituting MCH, *Legionella* control is most critical in Building 1, with continuous fluctuations in *Legionella* spp. colonization. The corrective measures that have since been put in place comprise two chemical shock treatments by raising the disinfectant dosage from 30 to 50 mg/l, in addition to increased flushing during the weekends. The long-term effects of our interventions resulted in the maintenance of *Legionella* contamination levels under the alert threshold prescribed by the Italian guidelines (101 and 1000 cfu/L, respectively) that limited the risk of exposure and preserved the health of patients and workers.

Building 2 water consumption, the presence of multiple outlets (336), and some facilities with high water consumption (e.g., cafe, restaurant, market) suggested that water flushing facilitated the circulation of the disinfectant in the plumbing system, reducing the number of bacterium-positive samples and the *Legionella* concentration, in accordance with a study by Douterelo et al. (2013). [46]

Building 3 is the smallest structure of MCH, covering an area of 1271.06 m^2. The total annual water consumption in this building is 589 m^3 per 129 outlets. The services and in-

patient rooms allow daily circulation of the disinfectant in the plumbing system, contributing to the effectiveness of WTP 828 in controlling *Legionella* spp. contamination levels in that building.

The serotyping and genotyping data revealed different colonization patterns in MCH buildings. We did not find an association between the presence of some *Legionella* strains in MCH buildings and building size, water composition, number of outlets, and their user.

The data suggested that, sometimes, changing the disinfection treatment (e.g., type of disinfectant) and dosage (e.g., shock treatment) differently affected the prevalent *Legionella* strains. During the study period, the increase of WTP 828 dosage (50 mg/L) during the shock treatment resulted in a decrease of *L. pneumophila* and increase of *L. species* (data not shown).

The absence of *P. aeruginosa* in the water samples was a good indicator of the effect of WTP 828 on the containment of these bacteria. The steps taken by the cleaning staff during weekly cleaning procedures (e.g., disinfecting the taps and showers) were important in preventing biofilm formation. Our organization of meetings with the stakeholders and hospital staff to inform them of the infection risk was also useful.

The presence of *Legionella* spp. was also associated with HPC, as an indirect indicator of water quality. often used to assess the efficacy of water treatment. Our results indicated a good performance of WTP 828 with respect to HPC containment during the entire study period, to below the limits admissible by legislation. [33]

The physical and chemical parameters were not affected by the WTP 828 treatment throughout the tested period. By controlling these parameters, it is possible to monitor the effect of the disinfectant on water pipes and take measures to preserve the water network damage. These results underline the role of the physical and chemical parameter monitoring

to assess changes in the water quality during disinfection. The “drinking water” requirement is an important feature to be taken into account when disinfection treatment is suggested.

It is often difficult to guarantee the absence of *Legionella* from water distribution systems, even if a disinfection system is in place. Our data revealed that differences in three buildings belonging to the same structure were linked to the building size, water consumption, number of outlets, and intended use. WTP 828 performed well in MCH in terms of the positive sites reduction and *Legionella* contamination level, but only a change in the study approach (adequate risk assessment plan, increase of water samples, and alteration of WTP 828 dosage in relation to the *Legionella* levels) facilitated the discovery of the differences in *Legionella* colonization and understanding of the disinfectant activity dynamics.

Further investigations are needed to elucidate how the dosage affects the presence of specific strains in each building and to generate a risk map with phylogenetic correlations between the strains. Evaluation of changes in colonization dynamics will be useful to control the level of the disinfectant.

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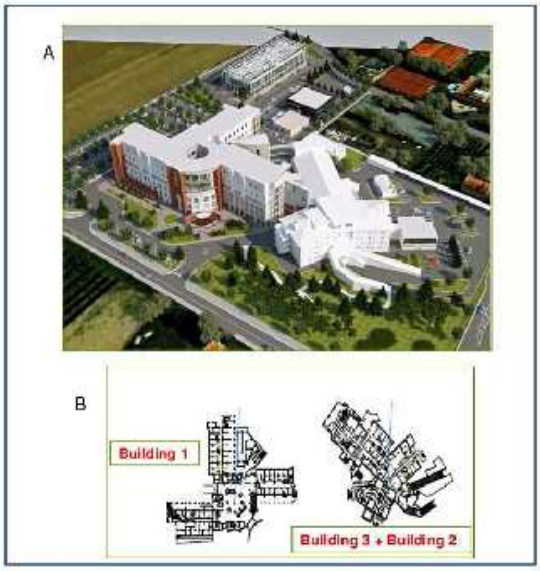


Figure 1. Site map of MCH, Cotignola (RA), Italy. A representative view of MCH and map of its three buildings: MCH picture (A) and MCH planimetry (B).

MCH characteristics

Area (m^2)	Number of total Water Outlets	Number of total in-patient rooms	Number of sampling points	Number of samples	Water consumption (m^3)/ year
27989,64	768	122	63	469	5519

Building 1

Area (m^2)	Floor/ building	Water Outlets	In-patient rooms	Sampling points	Water consumption (m^3)/ year
18539.93	Floor 0	50		4	1913
	Floor 1	25		4	
	Floor 2	119	27	5	
	Floor 3				
	Floor 4	73		4	
	Floor 5	36		4	
Total Water Outlets		303			
Total in-patient rooms			27		
Total sampling points				21	

Building 2

Area (m^2)	Floor/ building	Water Outlets	In-patient rooms	Sampling points	Water consumption (m^3)/ year
8178.68	Floor -1	4		1	3017
	Floor 0	65		5	
	Floor 1	92	25	5	
	Floor 2	104	26	5	
	Floor 3	66	18	3	
	Floor 4	5	1	2	
Total Water Outlets		336			
Total in-patient rooms			70		
Total sampling points				21	

Building 3

Area (m^2)	Floor/ building	Water Outlets	In-patient rooms	Sampling points	Water consumption (m^3)/ year
1271.06	Floor -1	7		2	589
	Floor 0	3		2	
	Floor 1	6		2	
	Floor 2	4		1	
	Floor 3	59	14	3	
	Floor 4	50	11	3	
Total Water Outlets		129			
Total in-patient rooms			25		
Total sampling points				13	

Figure 2. MCH structure and water outlet characteristics.The MCH buildings (Building 1 , 2 and 3) have different numbers of water outlets and different water consumption, which affects *Legionella* spp. colonization.

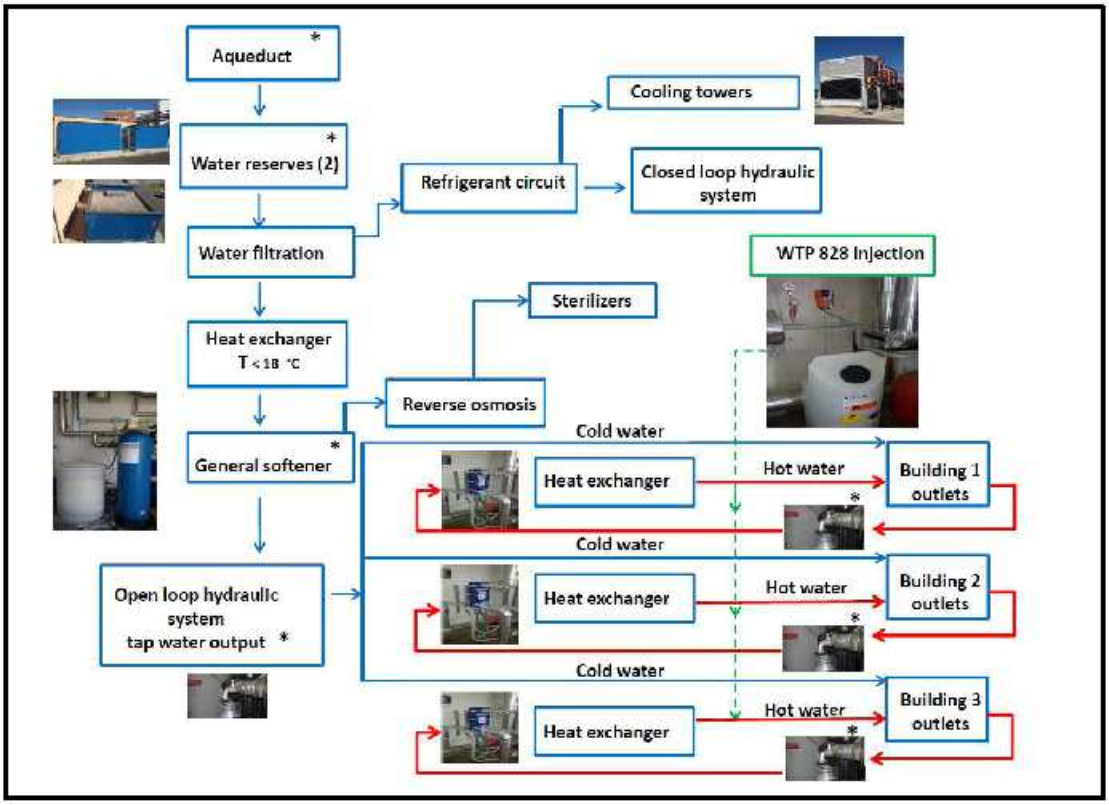


Figure 3. Plan of MCH water distribution network. A scheme of MCH water network with main sampling points in technical rooms (*).

Parameters	Methods	U.M.	Sampling points			
			Acqueduct	Hot water return line Building 1 (1a)	Hot water return line Building 2 (1b)	Hot water return line Building 3 (1c)
Temperature	EPA Method 170.1	°C	15.20 ± 3.40	49.03 ± 2.42	50.20 ± 0.61	53.87 ± 4.13
pH	APAT IRSA CNR 2060 Man 29 2003		7.82 ± 0.23	7.88 ± 0.24	7.87 ± 0.27	7.95 ± 0.19
Hardness	APAT IRSA CNR 2040 Man 29 2003	°F	12.10 ± 4.28	12.13 ± 3.14	12.63 ± 3.50	12.15 ± 2.44
Conductivity	APAT IRSA CNR 2030 Man 29 2003	µS/ cm	407.71 ± 35.20	416.53 ± 41.80	420.00 ± 40.70	423.44 ± 29.75
Turbidity	APAT IRSA CNR 2110 Man 29 2003	NTU	0.40 ± 0.09	0.39 ± 0.12	0.52 ± 0.25	0.88 ± 0.97
Total iron	APAT IRSA CNR 3160A Man 29 2003	mg/L	<0.04	0.04	<0.04	0.03
Total phosphorus	APAT IRSA CNR 4060 Man 29 2003	mg/L P_2O_5	<0.2	3.19 ± 1.31	3.40 ± 1.40	1.68 ± 0.85
Peroxide	Peroxide Test MQuant ™	mg/L	not detected	7.42 ± 2.71	8.46 ± 3.15	5.83 ± 3.84
Silver	EPA Method 272.2	µg/L	<3	<3	<3	<3

Table 1. Physical and chemical parameters of water in MCH hospital representative samples in technical room.

Buildings	Treatment - Study phase	Number of samples	Number of *L. spp*. positive samples	Mean *L.spp*. levels (log_{10} cfu/L) ± Standard Deviation
MCH	ClO_2 mixture	120	114 (95.0)	2.00 ± 0.86
	WTP 828 (WTP 1 phase)	53	32 (60.0)	2.12 ± 1.06
	WTP 828 (WTP 2 phase)	296	106 (35.8)	1.17 ± 0.77
Building 1	ClO_2 mixture	47	46 (98.0)	2.06 ± 0.82
	WTP 828 (WTP 1 phase)	25	16 (64.0)	2.59 ± 0.96
	WTP 828 (WTP 2 phase)	141	82 (58.1)	1.72 ± 0.74
Building 2	ClO_2 mixture	58	53 (91.3)	1.78 ± 0.55
	WTP 828 (WTP 1 phase)	23	13 (56.5)	1.39 ± 0.83
	WTP 828 (WTP 2 phase)	108	8 (7.0)	0.62 ± 0.28
Building 3	ClO_2 mixture	15	15 (100.0)	2.50 ± 1.49
	WTP 828 (WTP 1 phase)	5	3 (60.0)	2.39 ± 1.15
	WTP 828 (WTP 2 phase)	47	16 (34.0)	1.16 ± 0.76

Table 2. *Legionella* concentration in 3 buildings of MCH (mean value ± SD), numbers and percentage of positive samples [n(%)] for each study phases.

	Disinfectant	WTP 1	ClO_2 mixture
		p value	p value
Building 1	ClO_2 mixture	0.783	
	WTP 2	0.07	0.76
Building 2	ClO_2 mixture	0.046*	
	WTP 2	0.001*	0.0001*
Building 3	ClO_2 mixture	1	
	WTP 2	0.037*	0.045*

Table 3. Statistical analysis between WTP 828 (WTP 1 and WTP 2) and ClO2 mixture during study phases in MCH buildings; values are significant for $p<0.05$*.

Study phases	Odds Ratio (OR)	Intervals confidence 95%	P value
ClO_2 mixture *vs* WTP 1	3.5	0.44 - 41.04	0.15
ClO_2 mixture *vs* WTP 2	13.25	2.85 - 121.45	0.0001*

Study phases	Relative Risk (RR)	Intervals confidence 95%	P value
WTP 1 *vs* WTP 2	1.46	1.10 - 1.95	0.02*

Table 4. Odds Ratio and Relative Risk during study phases; values are significant for $p<0.05$*.

	Number of samples (WTP 1 + WTP 2)	Number of *L.* spp. positive samples	*L.* spp. serotyping °	*L.* spp. genotyping *§	Number of *L.* spp. isolates typing
Maria Cecilia Hospital	349	138 (39.0)	*L. pneumophila* Serogroup1	**ST 1** and/or **ST 104**	74 (53.6)
			L. species	***L. anisa*** and/or ***L. rubrilucens***	35 (25.3)
			L. pneumophila Serogroup1 + *L. species*	**ST 1** and/or **ST 104** + ***L. anisa*** and/or ***L. rubrilucens***	29 (21.0)

Table 5. MCH *Legionella* spp. contamination: numbers of positive samples [n (%)], serotyping° and genotyping*§ results.

Building	Positive Samples	Serotyping°	Genotyping*§	Isolates/ positive samples		Range of mean concentration log cfu/L (min-max)
Building 1	98	*L. pneumophila* SG1	**ST1 and ST104**	36/98		1.40 - 5.80
		L. species	*L. rubrilucens*	11/98		1.40 - 4.60
		L. species	*L. anisa*	20/98		1.40 - 3.77
		L. species	*L.rubrilucens* + *L.anisa*	2/98		2.83 - 3
		L. pneumophila SG1 + *L. species*	**ST1 and ST104** + *L.rubrilucens*	29/98	9/29	2 - 5.69
			ST1 and ST104 + *L.anisa*		20/29	1.70 - 3.53
Building 2	21	*L. pneumophila* SG1	**ST1 and ST104**	21/21		1.40 - 4.50
Building 3	19	*L. pneumophila* SG1	**ST1**	17/19		1.70 - 4.18
		L. species	*L. rubrilucens*	2/19	1/19	2.10
			L. anisa		1/19	1.40

Table 6. Serotyping (agglutination test°) ang genotyping (SBT* and mip gene sequencing§) of *Legionella* spp. isolates in MCH buildings.

CHAPTER 3

Application of *Sequence Based Typing* (*SBT*) technique to typing strains of *Legionella* spp.: development of an environmental risk map

INTRODUCTION

Legionnaires' disease (LD) is caused by *Legionella* spp., which are environmental Gram-negative bacteria that colonize and persist in moist environments, particularly water distribution systems. [1] Examples of water systems that might spread *Legionella* include hot tubs, hot water tanks and heaters, large plumbing systems, cooling towers (air-conditioning systems for large buildings), medical device (e.g. dental unit waterline), decorative fountain. [2] The infection is acquired through inhalation of aerosols from these sources contaminated with *Legionella*e.

In recent years, the increasing incidence of both nosocomial and community-acquired *Legionella* infections has been a major public health concern: in 2014, in Italy 1.497 cases were notified to the National Surveillance System (1,456 confirmed and 41 probable). Out of 1,497 cases reported, 62 (4.1%) had been admitted to hospital, 151 cases (10.1%) were travel-associated, 38 cases (2.5%) were living in day care centers and 5 cases (0.3%) had other risk factors. [3] Among the 59 *Legionella* species so far described , *L. pneumophila* serogroup 1 alone is responsible for ca. 90% of culture confirmed LD cases. [4] Nevertheless, the real risk still remains partially underestimated: for this reason it is essential to make a correct risk assessment in water systems, such as suggested by the guidelines,[5] in order to minimize the risk of colonization or its containment since the its eradication by the water networks is impossible, especially in the long term. [6]

Many factors can enhance the risk of *Legionella* infection, such as the design, construction and maintenance of the water distribution system; the presence of individuals who may be exposed and their vulnerability to infection; the degree of water system colonization (number of *Legionella* spp. cfu/L; the percentage of *Legionella* spp. positive samples) and properties of *Legionella* strains. [7] Each water system should be assessed individually, taking into account the proximity and susceptibility of the population and the modality of transmission from the water sources. This step can be realize understanding the characteristics of the water system, the risks and the maintenance procedures which affect water quality. [8]

Many studies have demonstrated the utility to perform the typing of *Legionella* strains in order to undertake the epidemiological investigation, useful to establish a link between the source of infection and cases to implement the appropriate risk control measures. However, for epidemiological investigations, two properties of the *Legionella* strains are usually determined: the serological groups (especially *L. pneumophila* SG 1) and the genotype. [7, 9]

Molecular techniques allow to obtain precise and rapid informations in terms of identification and genotyping of the various species and serogroups of *Legionella*. Currently there is no ideal genotyping method universally valid, since every organism appears better differentiated by a method rather than another. Different methods have been developed for molecular typing of *L. pneumpohila,* such a pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis and sequence based typing (SBT). These are useful tools during investigations of legionnaires' disease cases, clusters, or outbreaks. [10]

Scaturro et al, in 2005, compared PFGE, AFLP and SBT for molecular typing of isolates of *L. pneumophila*: the results showed as SBT was the most rapid and the easiest technique to perform providing unambiguous results. [11]

Sequence-based typing (SBT) is a variant of the classic *multilocus* sequence typing (MLST) schemes used to identify bacterial lineages and first described for *Streptococcus pneumoniae*

by Enright and Spratt, [12] who used seven housekeeping genes. The SBT is based on other schemes not using the same targets [13] but a combination of seven housekeeping and virulence genes (*flaA, pilE, asd, mip, mompS, proA and neuA*). The SBT is now considered as the 'gold standard' of genotyping and may be useful in identifying the source of infection, demonstrating the link between clinical and environmental isolates. [14]

SBT technique was developed by members of the European Legionnaires' Disease Surveillance Network (ELDSNet) and it has been described as a simple, rapid, discriminatory and portable method for typing *L. pneumophila* strains. [13]

OBJECTIVE

The purpose of this study is the application of SBT technique to strains of *Legionella pneumophila* previously isolated by standard culture method and identified by agglutination test, isolated from hot water samples collected during environmental monitoring in an Italian hospital, comparing them with the sequences stored in the database EWGLI; to assess the possibility to extend the same technique to the identification of *Legionella* non -*pneumophila* isolates (whose real risk comes not adequately considered, although the literature and international legislation suggest the research of *Legionella* spp. and not the only *L. pneumophila*); to study the phylogenetic relationship between the identified strains to create a map of environmental risk of the hospital that could support the management and maintenance assessment.

MATERIAL AND METHODS

Hospital characteristics

This study was conducted in an Italian hospital, Maria Cecilia Hospital (MCH), founded in 1973, which is located in Cotignola (RA) - Emilia-Romagna Region.

The structure of MCH is very complex and it consists of three separate buildings (*Building 1*, *2* and *3*) each with a different extension and location of the different services, covering a total area of 27989.64 m^2.

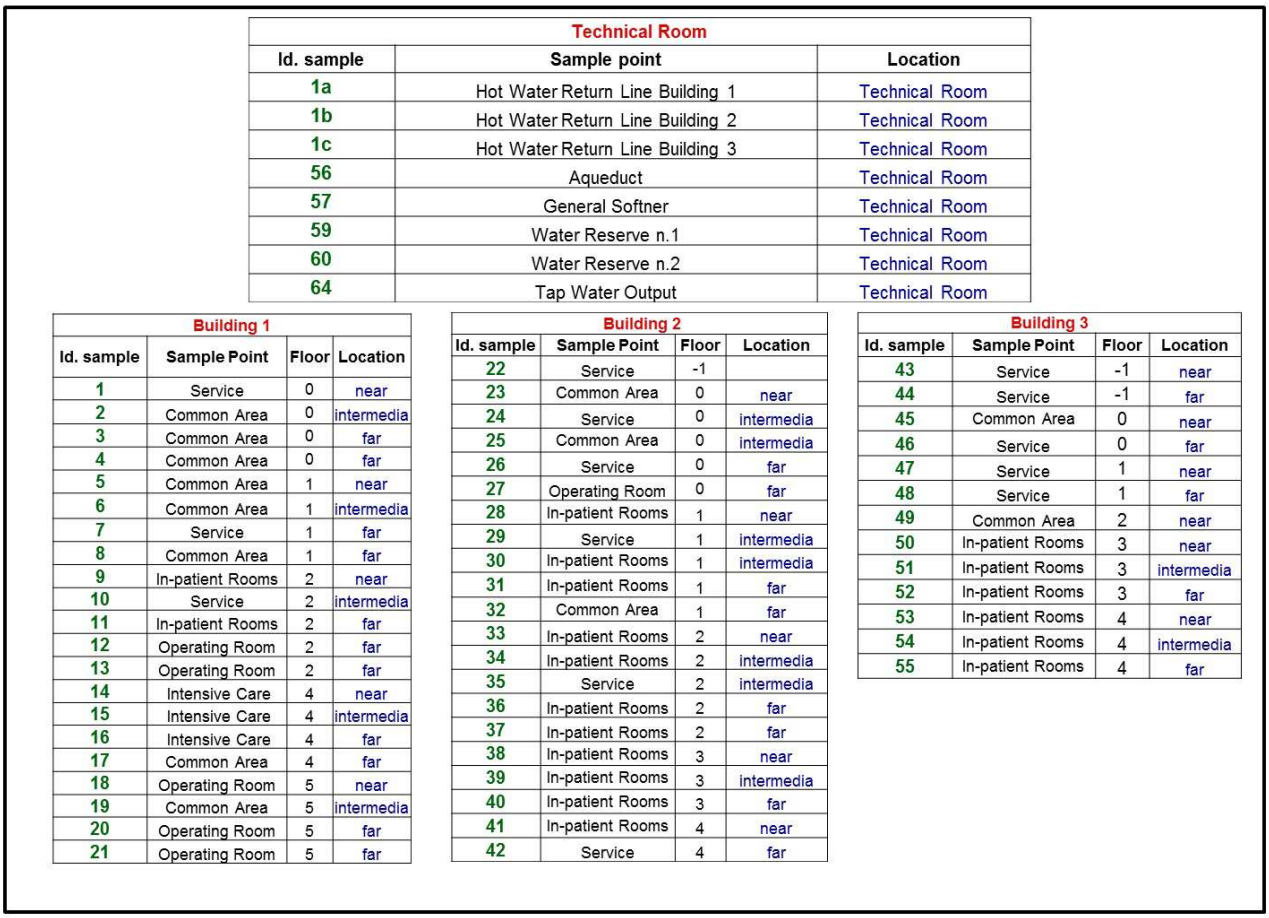
In October 2013 for controlling *Legionella* spp. contamination in hot water distribution networks of MCH, it was introduced a new disinfectant, the WTP 828, that is a multi-component oxidizing biocide formulated using a stabilized combination of H_2O_2 (34%, wt/wt) and Ag^+ salts (0.003%, wt/wt) in demineralized water to potentiate the power of disinfection[14].

The risk assessment plan to control *Legionella* contamination in all buildings consisted of 8 sampling points in the technical rooms (aqueduct, 2 cold water reserve, 1 after general softener, 3 hot water return lines) and 55 sampling points among common areas, diagnostic and operating room, offices, services or in-patient rooms (located variously in three buildings). All sampling points have been identified in three locations: near, intermedia and far from technical room.[15]

In *Building 1* were identified 21 sampling points and one hot water return line point. In *Building 2* are located the main part of in-patients room, and twenty-two sampling points were identified (21 plus 1 hot water return line point). *Building 3* has been recently expanded to the complete renovation in February 2015. It is the smallest of the buildings of MCH, indeed there were identified only 14 sampling points.

The hospital was subjected to a monthly monitoring for 35 months, during which the three buildings was alternatively sampled, as showed in figure 1.

The figure 1 shows the layout of the sampling points identified in the three buildings, the location in the floors and the respective location relative to the technical room.



Technical Room

Id. sample	Sample point	Location
1a	Hot Water Return Line Building 1	Technical Room
1b	Hot Water Return Line Building 2	Technical Room
1c	Hot Water Return Line Building 3	Technical Room
56	Aqueduct	Technical Room
57	General Softner	Technical Room
59	Water Reserve n.1	Technical Room
60	Water Reserve n.2	Technical Room
64	Tap Water Output	Technical Room

Building 1

Id. sample	Sample Point	Floor	Location
1	Service	0	near
2	Common Area	0	intermedia
3	Common Area	0	far
4	Common Area	0	far
5	Common Area	1	near
6	Common Area	1	intermedia
7	Service	1	far
8	Common Area	1	far
9	In-patient Rooms	2	near
10	Service	2	intermedia
11	In-patient Rooms	2	far
12	Operating Room	2	far
13	Operating Room	2	far
14	Intensive Care	4	near
15	Intensive Care	4	intermedia
16	Intensive Care	4	far
17	Common Area	4	far
18	Operating Room	5	near
19	Common Area	5	intermedia
20	Operating Room	5	far
21	Operating Room	5	far

Building 2

Id. sample	Sample Point	Floor	Location
22	Service	-1	
23	Common Area	0	near
24	Service	0	intermedia
25	Common Area	0	intermedia
26	Service	0	far
27	Operating Room	0	far
28	In-patient Rooms	1	near
29	Service	1	intermedia
30	In-patient Rooms	1	intermedia
31	In-patient Rooms	1	far
32	Common Area	1	far
33	In-patient Rooms	2	near
34	In-patient Rooms	2	intermedia
35	Service	2	intermedia
36	In-patient Rooms	2	far
37	In-patient Rooms	2	far
38	In-patient Rooms	3	near
39	In-patient Rooms	3	intermedia
40	In-patient Rooms	3	far
41	In-patient Rooms	4	near
42	Service	4	far

Building 3

Id. sample	Sample Point	Floor	Location
43	Service	-1	near
44	Service	-1	far
45	Common Area	0	near
46	Service	0	far
47	Service	1	near
48	Service	1	far
49	Common Area	2	near
50	In-patient Rooms	3	near
51	In-patient Rooms	3	intermedia
52	In-patient Rooms	3	far
53	In-patient Rooms	4	near
54	In-patient Rooms	4	intermedia
55	In-patient Rooms	4	far

Figure 1. Map of distribution in each building of 55 sampling points and 8 in technical room.

Disinfection treatment by H_2O_2/Ag^+ (WTP 828)

WTP 828 is a multi-component oxidizing biocide formulated using a stabilized combination of H_2O_2 (34%, wt/wt) and Ag^+ salts (0.003%, wt/wt) in demineralized water, for a highly effective disinfection solution. It is licensed by the European and Italian legislations [16] for its application in drinking water. The synergistic action of H_2O_2 and Ag^+ salts makes the biocide more powerful than H_2O_2 alone. [17, 18]

The WTP 828 is injected into mixed water (hot/cold) after hot water output downstream from the heat exchangers and dosed proportionally to the water supply.

WTP 828 was introduced for the first time in October 2013 in MCH Building 2, following the removal of a previous disinfection system that was based on a ClO_2 mixture and was not effective in controlling *Legionella* colonization. In subsequent years, the use of WTP 828 was also implemented in Building 1 and Building 3. The WTP 828 concentrations during the study were modulated according to the microbiological results for each building. The initial injection dosage was ~30 mg/L, which resulted in a residual concentration of WTP 828 at distal outlets of 5–10 mg/L. During shock treatment, the injection dosage was increased to 50 mg/L, which resulted in a 25–30 mg/L concentration at distal outlets.

Microbiological analysis

Hot water (2 L) was collected in post-flushing modality (running water for 1 min) in sterile 1 L polytetrafluoroethylene (PTFE) bottles containing sodium thiosulphate solution (10%, v/v). The samples were stored in coolers (at 0–4 °C), transported to the laboratory and processed on the day of sampling. All sampling phases, physical and chemical parameters (e.g., temperature and WTP 828 measure at distal outlets), maintenance procedures [e.g., disinfection procedure, thermostatic radiator valve (TRV) or faucet replacement and emergency servicing (shock treatment) of the hospital water system were performed by technical staff of the hospital, under the supervision of the authors. All adopted procedures were also recorded in a special register, as prescribed by the Italian Health Ministry guidelines. [15]

Microbiological analyses were performed in accordance with ISO11731-1998 method for the detection and enumeration of *Legionella.* [19] For each hot water sample (2 L), the sample was concentrated using 0.22 μm polycarbonate pre-sterilized filter membranes (Sartorius Stedim Biotech, Göttingen, Germany). The filter membrane was aseptically put in small bottles and shaken for 15 min with 10 mL of the original water sample to resuspend bacteria. The

concentrated samples (filtered, F) were heated (30 min at 50 °C) to inhibit the interfering microbiota (heated, H). A portion of the original sample (0.2 mL; untreated, UN) and 0.1 mL of each, F and H, sample were spread in duplicate onto GVPC agar plates (*Legionella* GVPC selective medium, ThermoFisher Scientific, Oxoid Ltd., Basingstoke, UK), and incubated at 35.5 °C in a humid (2.5% CO_2) environment. The plates were examined after 4, 8, and 14 d, and the presumptive colonies were enumerated and sub-cultured on BCYE agar, with and without cysteine. The data are expressed as mean concentration ± SD, $\log_{10}$ colony forming units (cfu) per liter of water ($\log_{10}$ cfu/L).

Serological identification

The identities of the isolates that grew on BCYE but failed to grow on the cysteine-free medium were verified by serological agglutination test with *Legionella* latex test kit (ThermoFisher Scientific, Oxoid Ltd) based on manufacturer instructions. The identification of species and serogroup is carried out on the basis of antigenic with serological tests that use monoclonal antibodies. This test allows separate identification of *Legionella pneumophila* serogroup 1 and serogroups 2-14 and detection of seven other *Legionella* species which have been implicated in human disease (*L. longbeachae* 1 & 2, *L. bozemanii* 1 & 2, *L. dumoffii*, *L. gormanii*, *L. jordani*, *L. micdadei*, *L. anisa*).

SBT typing and sequencing

Colonies identified by the agglutination test as belonging to the genus *Legionella* were subsequently analyzed by DNA sequencing. Genomic DNA was extracted from cultures using the InstaGene Purification Matrix (Bio-Rad, Hercules, CA) and DNA concentrations was determined using the Qubit fluorometer (Thermo Fisher Scientific, Paisley, UK). DNA extracts were kept at or below −20°C until use.

In particular, all strains identified as *L. pneumophila* and *Legionella species* were analyzed by Sequence-Based Typing (SBT) to determine the Sequence Type (ST); SBT using loci *flaA*, *pilE, asd, mip, mompS, proA* and *neuA* was performed according to the ELDSNet protocol (*Appendix 1*) (http://bioinforatics.phe.org.uk/*Legionella*/*Legionella*_sbt/php/sbt_homepage.php). Genotype analysis was based on the on sequencing of seven genes. PCR products were visualized by electrophoresis on 2% agarose gel and staining with ethidium bromide.

The amplicons obtained for each of the seven genes examined, after purification, were subjected to sequencing reaction cyclic (cycle sequencing at the Laboratory of Lab Gen-Genomic sequencing of the Department of Biomedical Sciences University of Modena and Reggio Emilia). Each of purified PCR fragment was subjected to two reactions linear polymerization of cyclic (one for sequencing the filament Fw and one for the filament Rv) using as primers the primers Fw M13 and M13. Following purification, product of cycle sequencing was subjected to capillary electrophoresis in an automated system to Fluorescence (ABI PRISM 3100 Genetic Analyzer - Applied Biosystems, Foster City, CA) provided with a laser beam capable of exciting the 4different fluorophores.

The nucleotide sequences obtained were confirmed by the SBT database available on the EWGLI website (*http:// www.ewgli.org/*), and the sequences were compared with those in the ELDSNet database from the website to assign the ST allelic profile (http://www.hpabioinformatics.org.uk/cgibin/*Legionella*/sbt/seq_assemble_*Legionella*1.cgi).

Mip **gene sequencing**

The strains serotyped by agglutination as *L. species* were then identified by analysis of *mip* gene sequence using bacterial DNA purified from isolated colony. The of *mip* gene amplification was performed by polymerase chain reaction (PCR) using degenerate primers, as described by Ratcliff et al. (1998), and modified by M13 tailing to avoid noise in the DNA

sequence. [20, 21] Gene amplification was carried out in a 50 μL reaction containing DreamTaq Green PCR Master Mix 2× (ThermoFisher Scientific) and 40 pmol of each primer; 100 ng of DNA extracted from the presumptive colonies of *Legionella* spp. was added as template. The protocol used for this purpose has been developed and standardized by the European Working Group (ESGLI) and the sequences obtained are comparable to those available in the database dedicated to this (http://www.hpa.org.uk/cfi/bioinformatics/ dbases.htm # EWGLI) and back to *Legionella species* isolates (*Appendix 2* – Ratcliff protocol). PCR products were visualized by electrophoresis on 2% agarose gel and staining with ethidium bromide. Following purification, they were sequenced using BigDye Chemistry and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Specifically, *mip* amplicons were sequenced using M13 forward and reverse primers (M13 FW, 5TGTAAAACGACGGCCAGT3'; M13 RW 3'CAGGAAACAGCTATGACC5') to obtain a complete coverage of the sequenced region of interest.

Raw sequencing data were assembled using CLC Main Workbench 7.6.4 software (https://www.qiagenbioinformatics.com/). The sequences were compared with sequences deposited in the *Legionella mip* gene sequence database using a similarity analysis tool (http://bioinformatics.phe.org.uk/cgi-bin/*Legionella*/mip/mip_id.cgi).

Phylogenetic and allelic diversity analysis

Phylogenetic analyses were performed on the Phylogeny.fr platform. Sequences were aligned with MUSCLE (v3.8.31) configured for highest accuracy. After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) using the following parameters:

- minimum length of a block after gap cleaning: 10

- no gap positions were allowed in the final alignment

- all segments with contiguous non conserved positions bigger than 8 were rejected

- minimum number of sequences for a flank position: 85%.

The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). The HKY85 substitution model was selected assuming an estimated proportion of invariant sites (of 0.538) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma=91.589). Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3). [22 - 28]

RESULTS

Microbiological results

From October 2013 to July 2016, 547 hot water samples were analyzed for detection and enumeration of *Legionella* spp.. All samples were subjected to analysis by culture method that has identified 205 positive samples (37%), variously distributed in three buildings: 142 in Building 1 (58%), 36 in Building 2 (18%) and 27 in Building 3 (26%) for the entire period of study. In Table 1 we show the results obtained during three periods: *period 1*, *period 2* and *period 3,* each of them characterized by a different approach in term of disinfectant dosage, implementation of buildings (e.g. area, changes in intended use,) and introduction of specific control measures (shock treatment).

In particular Table 1 shows a gradual decline in the trend of contamination in three periods in the buildings 1 and 3: in Building 1 the percentage of positive samples has a reduction from 64% to 56%; in Building 3 the percentage of positive samples has a reduction from 60% to 16%. Building 2 is an exception because after a statistically significant reduction between

period 1 and period 2 (data show in previous paper), it is observed an increase of contamination in period 3, also from microorganisms that never have been found (e.g. *L. anisa*).

	PERIOD 1	Positive sample (%)	PERIOD 2	Positive sample (%)	PERIOD 3	Positive sample (%)	Total	Total positive sample (%)
Building 1	**25**	**16 (64%)**	**141**	**82 (58%)**	**78**	**44 (56%)**	**244**	**142 (58%)**
Building 2	**23**	**13 (58%)**	**108**	**8 (7%)**	**69**	**15 (22%)**	**200**	**36 (18%)**
Building 3	**5**	**3 (60%)**	**47**	**16 (34%)**	**51**	**8 (16%)**	**103**	**27 (26%)**
Total	**53**	**32 (61%)**	**296**	**106 (35%)**	**198**	**61 (31%)**	**547**	**205 (37%)**

Table 1. Number of samples analyzed during study periods distributed in each building.

Legionella serotyping and genotyping results

According to the standard method (ISO 11731: 1998) at least three representative colonies of each colonial type of subculture were confirmed by agglutination test and identified *L. pneumophila* serogroup 1 (SG1) in 123 samples (60%) and *L. species* in 82 samples (40%).

SBT analysis performed on 205 positive samples assigned at 123 environmental isolates, identified as *L. pneumophila* SG1 by serotyping, two different STs: ST 1 and ST 104 (Table 2).

	Number of samples	Number of *L.* spp. positive samples	*L.* spp. serotyping °	*L.* spp. genotyping *§	Number of *L.* spp. isolates typing
MCH	547	205 (37%)	*L. pneumophila* Serogroup1	**ST 1** and/or **ST 104**	123 (60%)
			L. species	***L. anisa*** and/or ***L. rubrilucens***	82 (40)

Table 2. Serotyping° and genotyping*§ results of *Legionella* strains.

The SBT protocol was not applicable on *L. species* isolates. The experiments performed on these isolates showed as only some of the specific genes of SBT panel have been amplified.

The figure 2 show, in section A, two isolates of *L. rubrilucens* and *L. anisa* from the same sample (S13) with an non-specific bands that did not permit to perform sequencing and alleles assignment.

In section B of figure 2, two isolates belonging to *L.anisa*, from different samples (S17 and S4), showed a good amplification only for *mip* gene with non-specificity bands for other 6 genes.

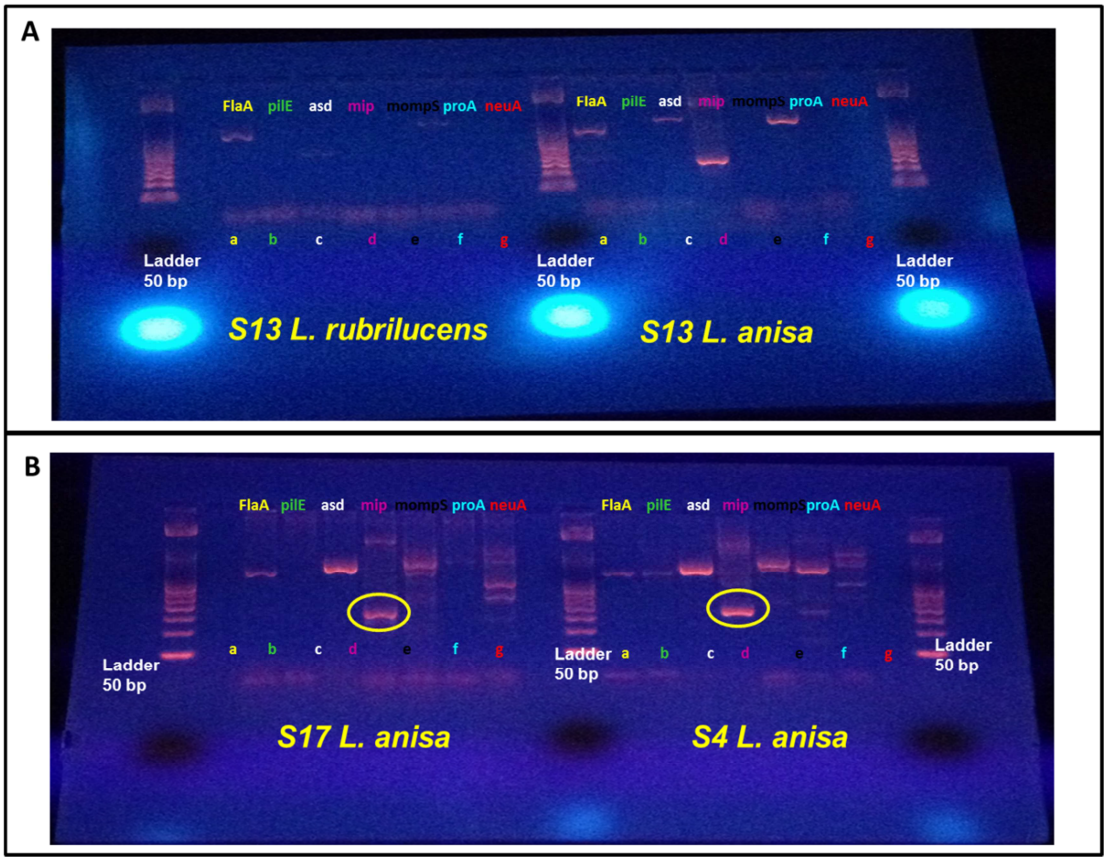


Figure 2. Application of SBTprotocol on *L.species* strains: section A *L. rubrilucens – L.anisa ;* section B *L. anisa*.

Mip amplification of *L.species*

As described in literature and in EWGLI guidelines[30], the identification of *L. species* has been performed by *mip* gene sequencing.

The *mip* gene sequencing identified inner *L. species*, *L. anisa* and *L. rubrilucens*, in 82/205 (40%) of *Legionella* positive samples, differentiating 7 (9%) *L. rubrilucens* and 75 (91%) *L. anisa*, both variously distributed in three buildings with the exception of the Building 2 where was isolated only *L. anisa*. Only 2 samples (S13 and S16, located in Building 1) showed the simultaneous presence of both species.

Table 3 shows the results of *Legionella* genotyping of the isolates. In particular, it is possible to observe the distribution of strains in three buildings with the percentages of positive samples in three periods of study.

	PERIOD 1		PERIOD 2		PERIOD 3		TOTAL	
	SEROPTYPE	**Positive sample (%)**	**SEROPTYPE**	**Positive sample (%)**	**SEROPTYPE**	**Positive sample (%)**	**SEROPTYPE**	**Positive sample (%)**
Building 1	*L. pneumophila* SG1	6/16 (**38%**)	*L. pneumophila* SG1	41/82 (**50%**)	*L. pneumophila* SG1	25/44 (**58%**)	*L. pneumophila* SG1	72/142 (**51%**)
	L. anisa	9/16 (**56%**)	*L. anisa*	40/82 (**48%**)	*L. anisa*	16/44 (**36%**)	*L. anisa*	65/142 (**45%**)
	L. rubrilucens	1/16 (**6%**)	*L. rubrilucens*	1/82 (**2%**)	*L. rubrilucens*	3/44 (**6%**)	*L. rubrilucens*	5/142 (**3%**)
Building 2	L. pneumophila SG1	10/13 (**77%**)	*L. pneumophila* SG1	8/8 (**100%**)	*L. pneumophila* SG1	14/15 (**93%**)	*L. pneumophila* SG1	32/36 (**89%**)
	L. anisa	3/13 (**23%**)	*L. anisa*	/	*L. anisa*	1/15 (**7%**)	*L. anisa*	4/36 (**11%**)
	L. rubrilucens	/	*L. rubrilucens*	/	*L. rubrilucens*	/	*L. rubrilucens*	/
Building 3	L. pneumophila SG1	3/3 (**100%**)	*L. pneumophila* SG1	14/16 (**87%**)	*L. pneumophila* SG1	2/8 (**25%**)	*L. pneumophila* SG1	19/27 (**70%**)
	L. anisa	/	*L. anisa*	/	*L. anisa*	6/8 (**75%**)	*L. anisa*	6/27 (**23%**)
	L. rubrilucens	/	*L. rubrilucens*	2/16 (**13%**)	*L. rubrilucens*	/	*L. rubrilucens*	2/27 (**7%**)

Table 3. Results of phenotypic identification and the percentage of positive samples in each building, in the three study period.

As concerning the geographic distribution of *Legionella* strains into each building, these data were presented in Figure 3.

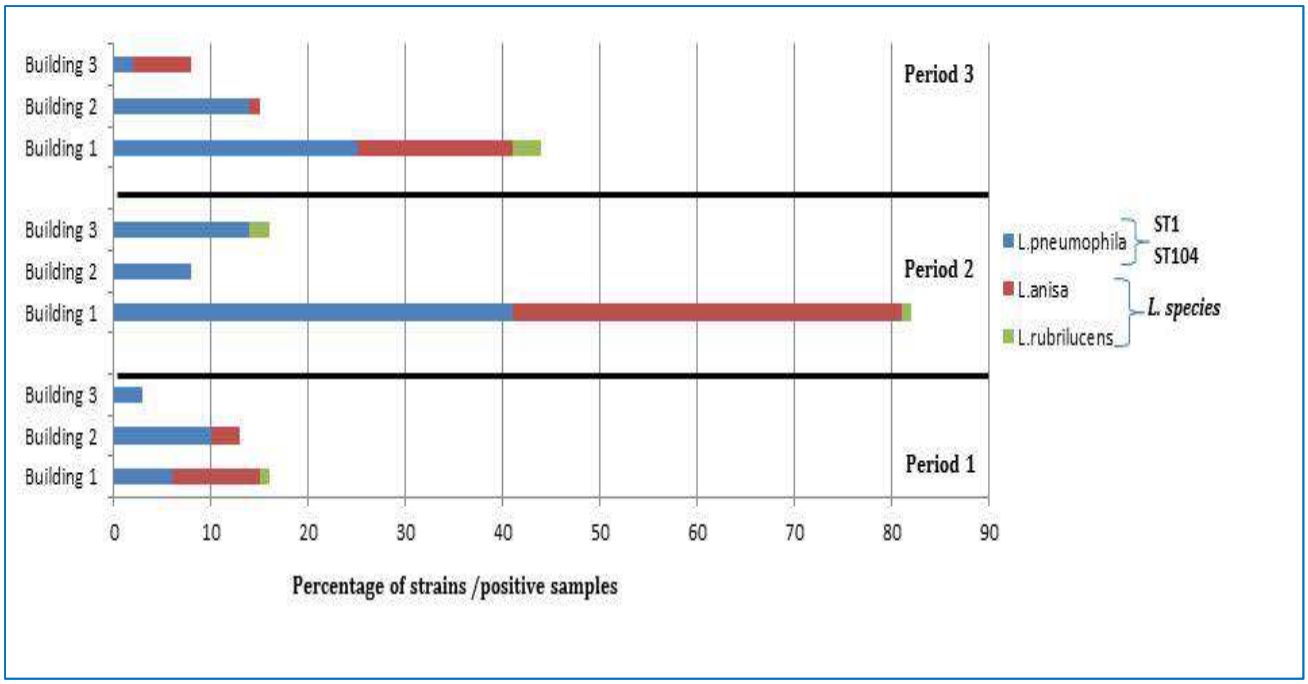


Figure 3. *Legionella* spp. distribution in three buildings during study periods

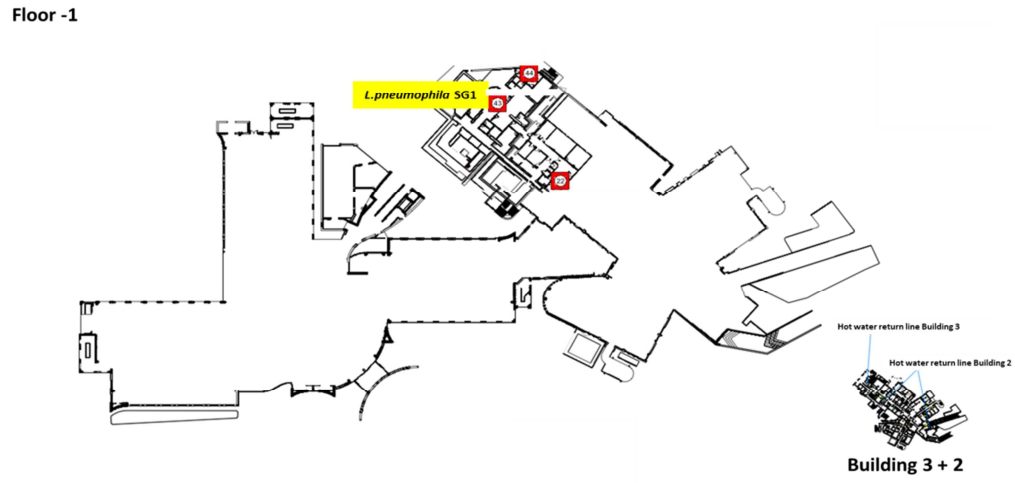
The data displayed as throughout all the study period, *L. pneumophila* SG1 (ST1-ST104) was the mostly found species in the hospital network. The analysis of strains distributions in each buildings *vs* periods displayed a cross contamination (*L. pneumophila*, *L. anisa* and *L. rubrilucens*) in Building 1, that is constant for all periods, with a change only about level of concentrations.

The Building 2 shows a contamination by a mixture of *L. pneumophila*/*L. anisa* in period 1 and period 3, by contrast in period 2 the contamination by *L. anisa* disappear.

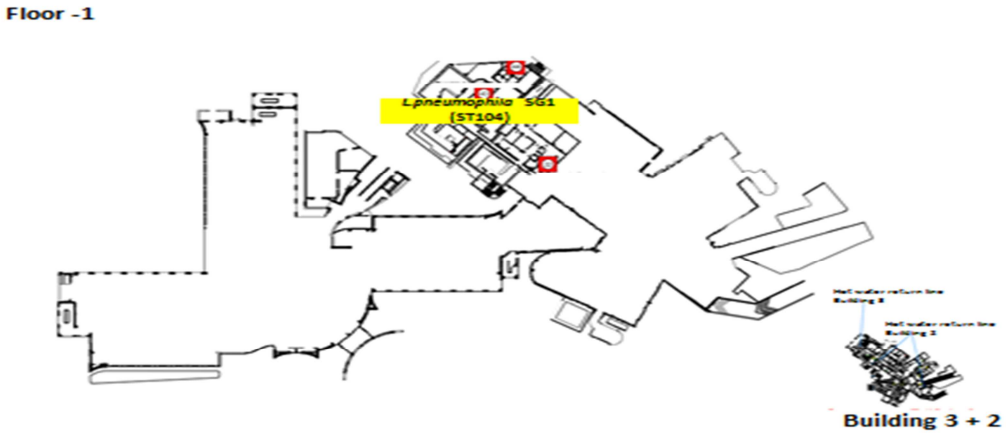
The Building 3 was the building with more changes in terms of dynamic of colonization, with the presence of *L. pneumophila* in period 1 and its gradual decrease in place of *L. rubrilucens* and *L. anisa* respectively in period 2 and 3.

In Table 4 to 10 we show the distribution of isolates for each sampling point in three study periods.

PERIOD 1



PERIOD 2



PERIOD 3

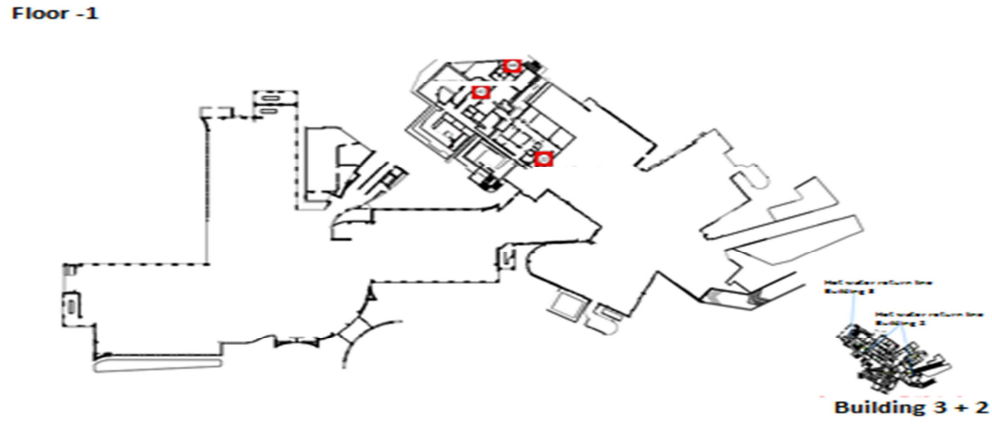
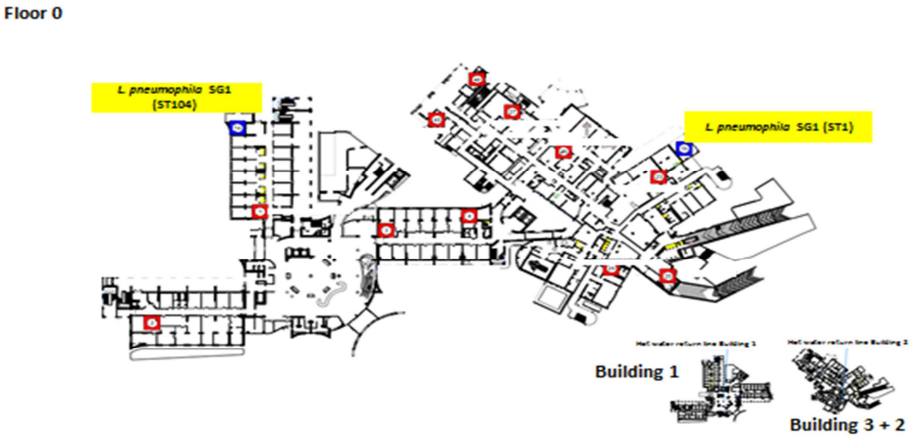


Table 4. Distribution of isolates in floor -1

PERIOD 1



PERIOD 2

PERIOD 3

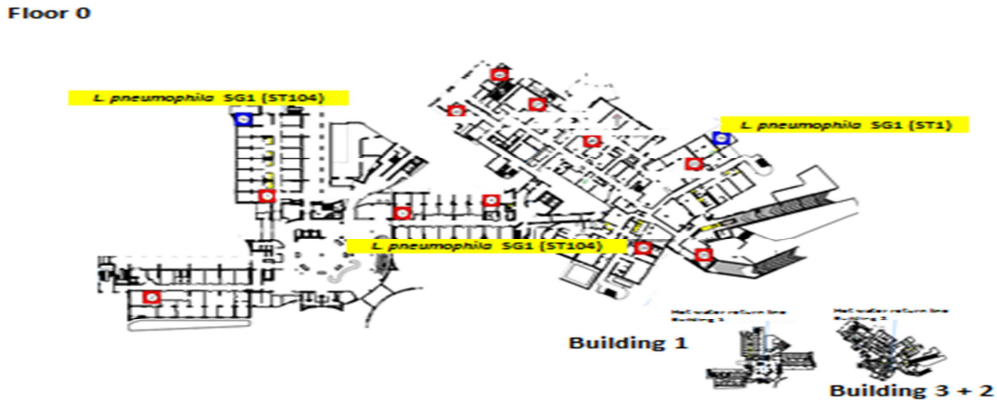
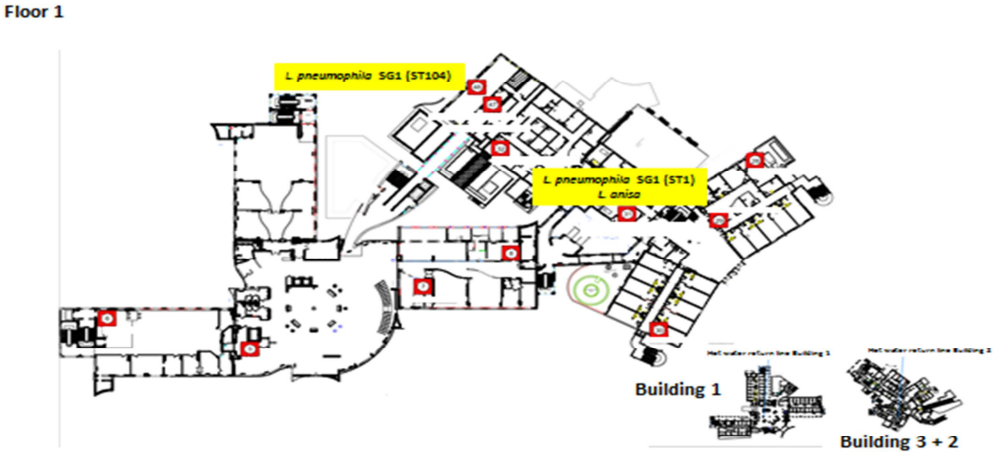
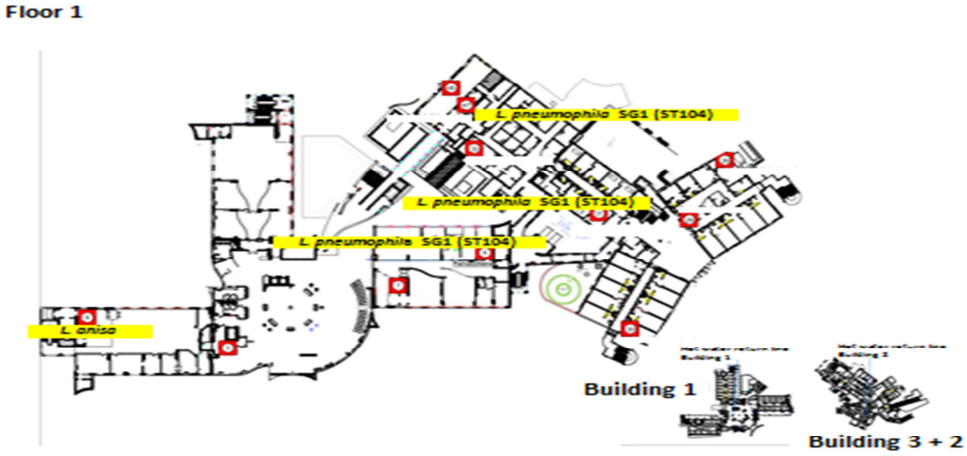


Table 5. Distribution of isolates in floor 0

PERIOD 1



PERIOD 2



PERIOD 3

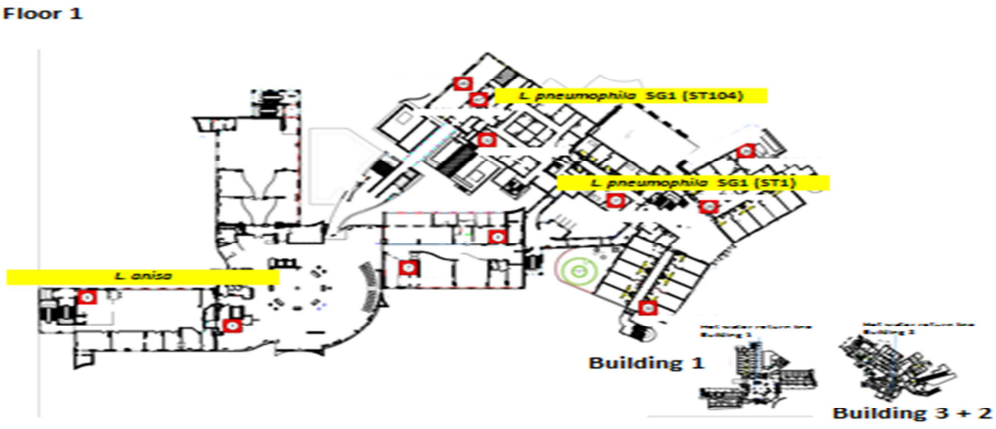
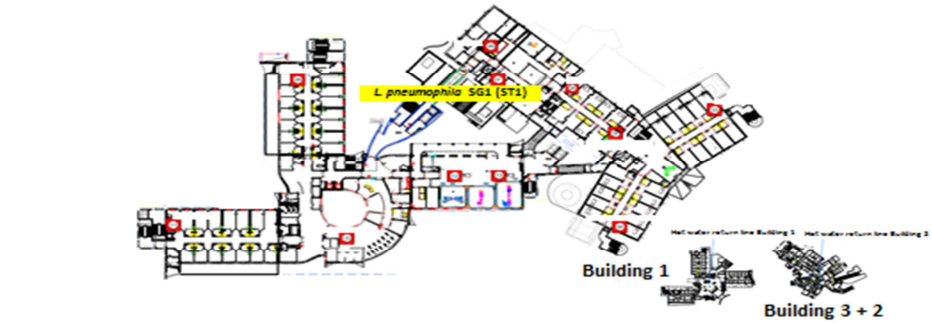


Table 6. Distribution of isolates in floor 1

PERIOD 1

Floor 2



PERIOD 2

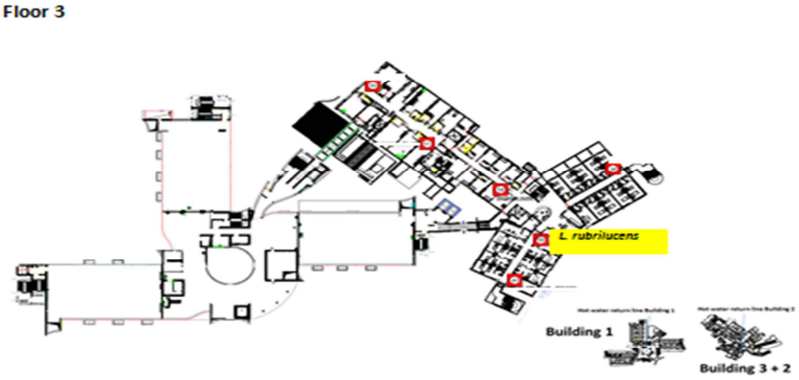
Floor 2

PERIOD 3

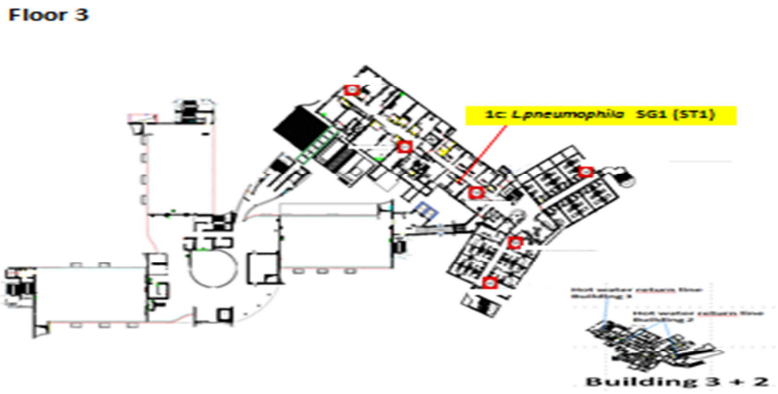
Floor 2

Table 7. Distribution of isolates in floor 2

PERIOD 1



PERIOD 2



PERIOD 3

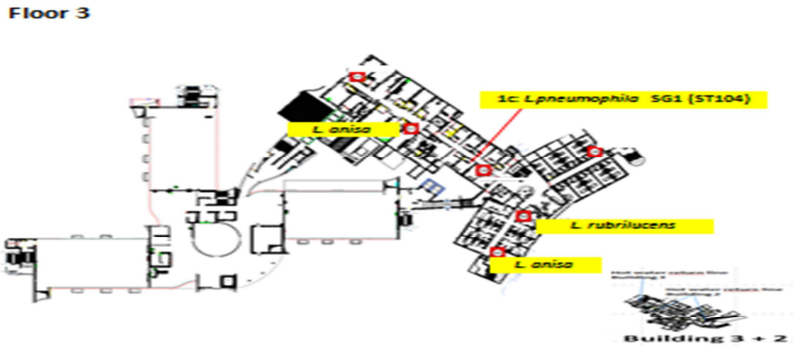
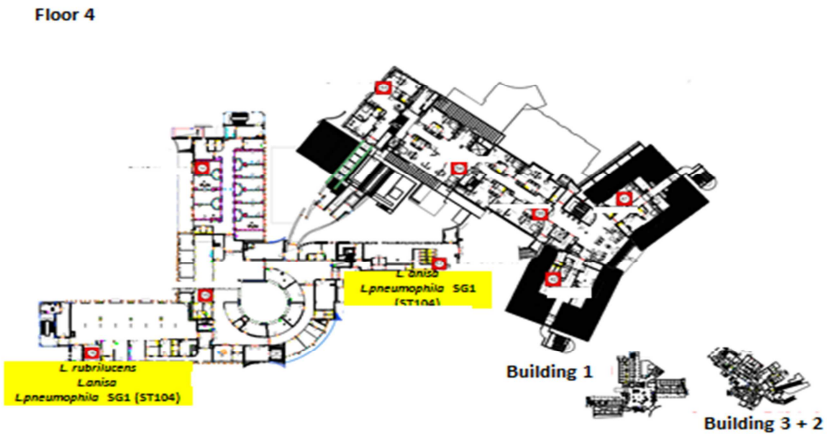
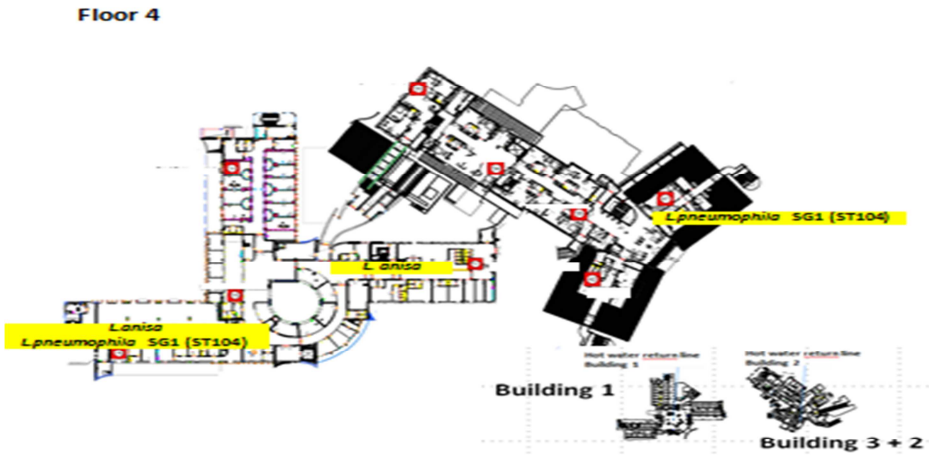


Table 8. Distribution of isolates in floor 3

PERIOD 1



PERIOD 2



PERIOD 3

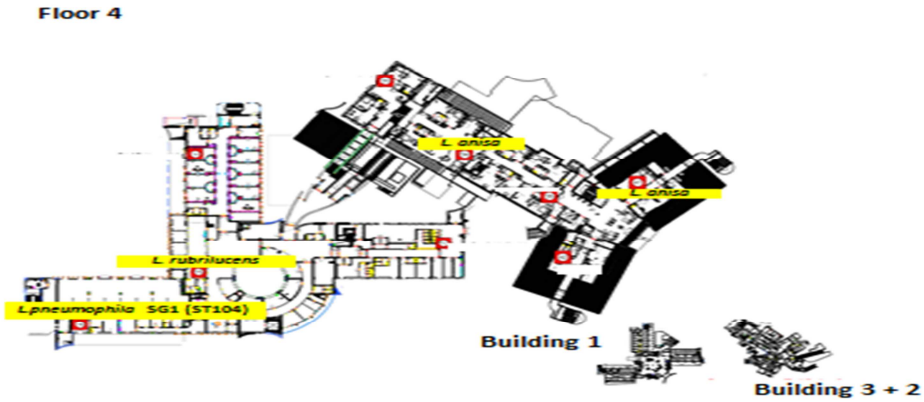
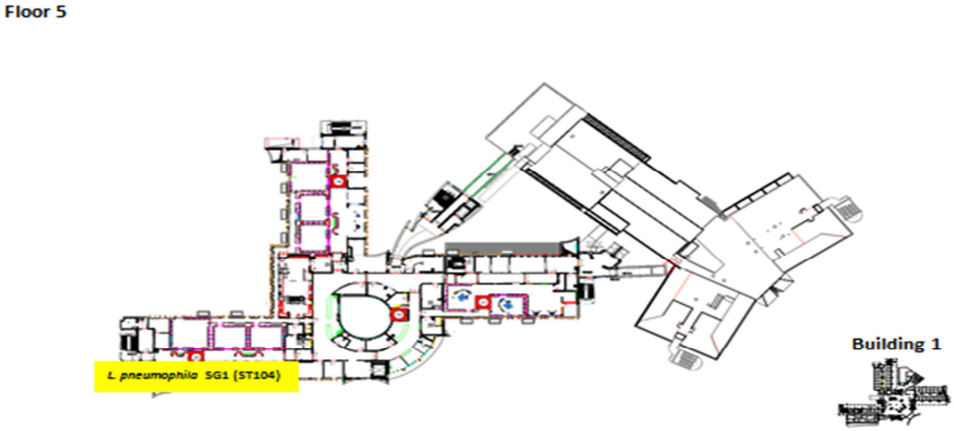
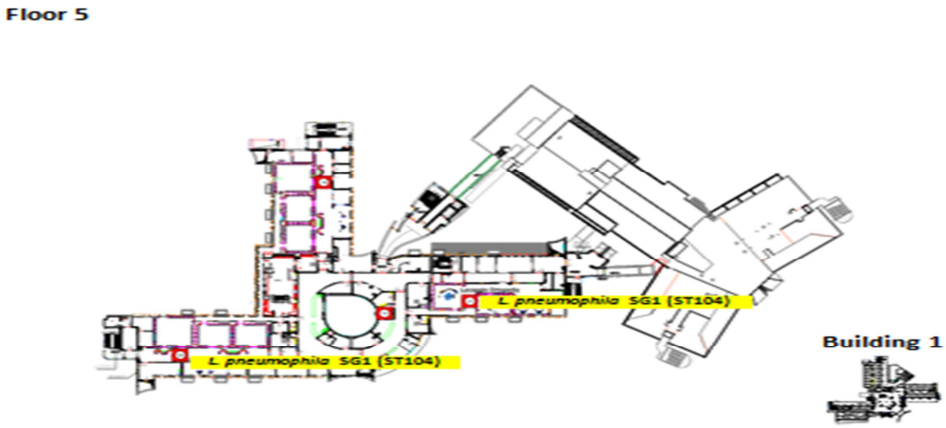


Table 9. Distribution of isolates in floor 4

PERIOD 1



PERIOD 2



PERIOD 3

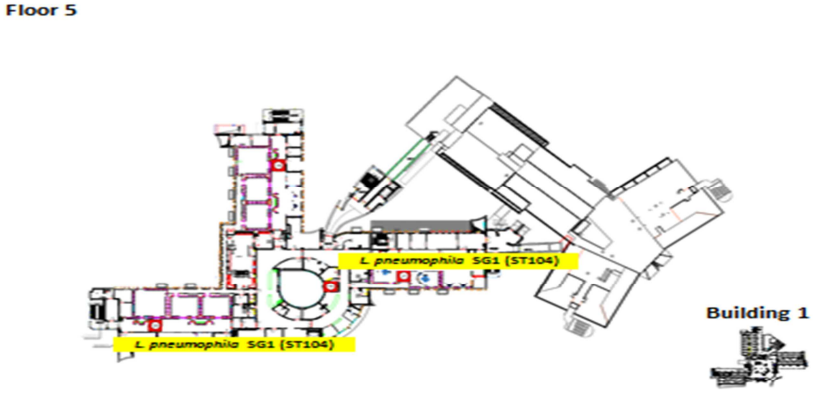


Table 10. Distribution of isolates in floor 5

Relationship between isolates - Phylogenetic analysis

Phylogenetic analysis was performed only on strains of *L. species* identified by the amplification of *mip* gene. (*L. rubrilucens* and *L. anisa*). The results obtained determined the creation of a tree in which the two groups form a cluster for each species, as shown in Figure 4.

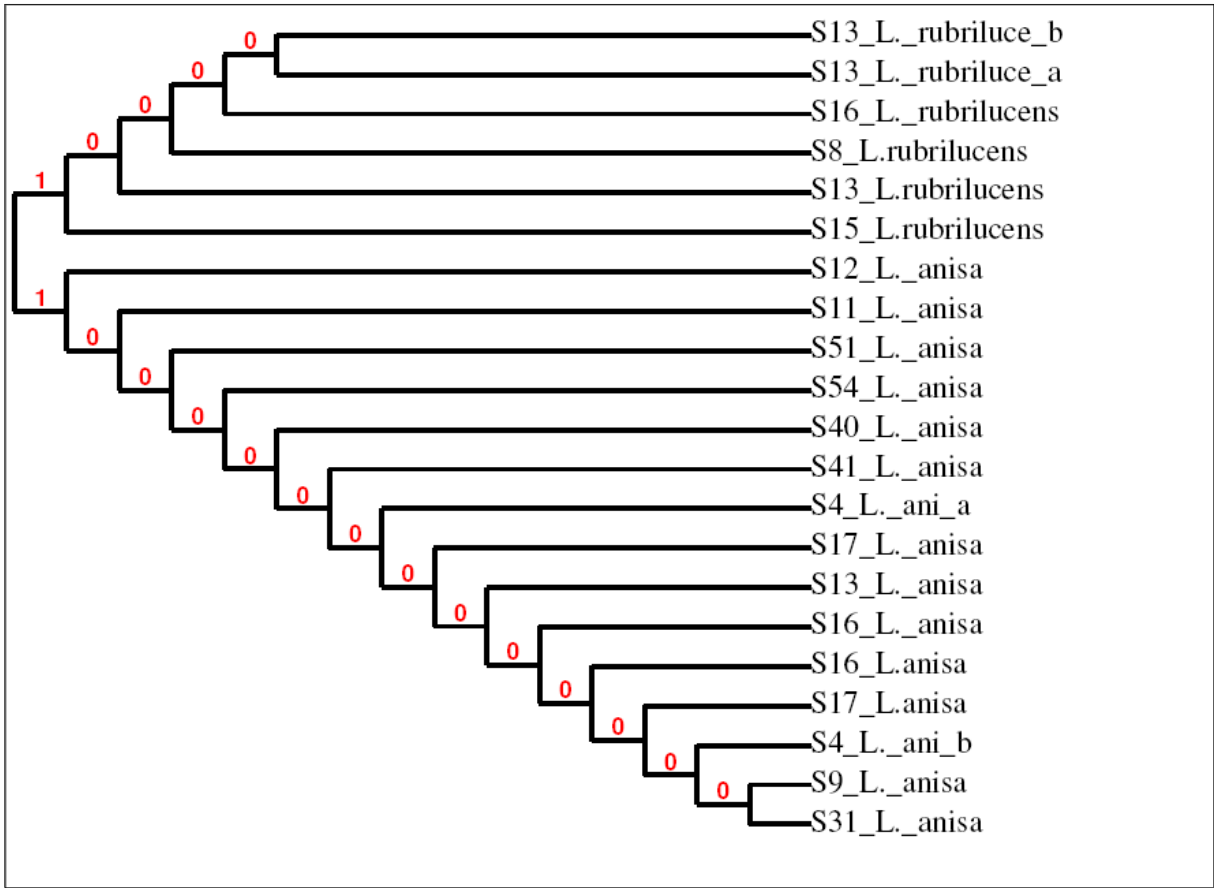


Figure 4. Phylogenetic tree of *L. species*

The strains subjected to SBT (*L. pneumophila*) resulting with ST identical have the same profile allelic for each of the 7 genes, with the 100% homology percentages. Therefore, phylogenetic analysis would not provide useful information regarding their correlation.

DISCUSSION

Our previous study (concerning the effectiveness of a new hot water disinfection method based on hydrogen peroxide + silver salts (WTP828), has suggested the possibility to study the contamination of hospital network through a genotypical approach. We tried to associate the culture standard method with genotyping techniques, obtaining innovative and interesting results especially as regards the analysis of environmental risk.

The culture analysis is still considered the gold standard for the enumeration of environmental *Legionella*, although only a presumptive method and it has several drawbacks including long incubation and poor sensitivity, causing delays in response times to outbreaks of Legionnaires' disease. Furthermore, it is unable to detect viable but nonculturable cells (VBNC) [29] and the discrepant results between culture method and PCR are most pronounced for *Legionella* non- *pneumophila.* [30]

The ability of public health organisms to respond rapidly to an outbreak of LD is thus delayed due to the time required for culture. The microbiological aspect of an epidemiological investigation is to seek evidence linking the source of the outbreak to the cases, by comparing *Legionella* isolates from environmental samples with those from patients. Accurate discrimination among *Legionella* isolates is important in order to identify cases with a common source of infection and the transmission routes of the microorganism. Then, faster quantification methods are required for both public health and routine sampling.

This study is focused on a new approach on environmental monitoring in a hospital of our region, for its peculiarity organization in three buildings.

The areas, the intended use and different water consumption, influenced the water characteristics in term of physical-chemical parameters (data show in previous paper) and

microbiological contaminations. The introduction of a new disinfectant (WTP 828) permitted to control the level and type of contamination in relation to disinfectant dosage.

Our data shown as the numbers of positive samples during study period changed in each building with differences in *Legionella* colonization. These results are linked to the changes performed during the whole study period as an increase of samples collected: from 53 samples analyzed in period 1 to 296 in period 2 and 198 in period 3.

The different numbers of samples can be explained with the different time of WTP828 introduction in three buildings; in period 2 it was analyzed the highest number of samples (296) as a result of the conclusion of accommodation works and the completion of the final structures of Buildings 1 and 3 and the elaboration of a new risk assessment plan and the extension of monitoring activity.

At the same time, we observed a gradual decline in the trend of contamination in three periods in the Buildings 1 and 3, with exception of Building 2.

These results could be explained with the failure of implementation and maintenance procedures other than structural changes (for example works of accommodation and reorganization) carried out without adequate increase of the building disinfectant level that about our experience are essential to contain microorganism growth.

Regarding the *Legionella* typing, our results confirmed the trend found in previous paper focused on the evaluation of WTP828 effectiveness, suggesting that the Building 1 is the most contaminated respect to others, showing a cross - contamination by *L. pneumophila* and *L. species* distributed heterogeneously in the building.

Our results showed that all strains (*L. pneumophila* SG1 ST1 and ST104, *L anisa* and *L. rubrilucens*) are conserved and casually distributed in three buildings, with a prevalence of *L. pneumophila* SG1 in all positive samples.

The main reason for typing *L. pneumophila* is to help identify environmental sources giving rise to cases of legionellosis. This allows control measures to be implemented and further cases to be prevented in a timely manner. Furthermore, typing methods, in particular sequence-based typing allow to infer the population structure of *L. pneumophila*, to study genetic diversity and clonal expansion and to undertake long-term epidemiological analyses of microbial populations. [31]

We conducted sequence-based typing (SBT) analysis on a large set of *L. pneumophila* isolates (n = 205) to investigate the sequence types (STs) distribution in order to identify dominant ST and to design a map of risk. SBT protocol permit to identify inside *L. pneumophila* SG1 isolates two STs – ST 1 e ST 104 – casually collocated in the hospital. The 100% of homology in 7 genes allelic profile did not permit to elaborate phylogenetic correlation between strains; it would be interesting the application of new DNA sequencing technique, as NGS approach, to evaluate differences in strains belonging to the same ST but it can present a different pathogenic patterns.

SBT protocol fails on *L. species* identification, suggesting the importance to develop a new protocol for them and the role that a similar approach could have when agglutination test for some *species* give false negative results.

The 16S rDNA and *mip* gene sequencing remain a valid alternative to correctly identify the *L. species* isolates and different protocols were developing with high discriminatory power. [31, 32]

The 16S rDNA gene is the most widely used molecular markers for phylogenetic studies of bacteria in general [33], although its use in *Legionella* fields has been widely discussed for its low heterogeneity and its presence in multiple copies of ribosomal operons. Different study showed as the sequencing of the 16S rDNA gave satisfactory results for identification of *Legionella*, [34] with a power of discrimination excellent at the genus level, but not high enough at the species level. Phylogenetic studies of other genes (including *mip*)[35] were gradually

introduced for their greater capacity than the 16S rDNA of discriminating *Legionella* at the species level. [36] The *mip* gene sequence has been extensively studied in numerous *L. species* and it is extremely useful in typing study for its presence in a single copy and its difference from one species to another, that give a unique identification. Due to the above mentioned considerations in this study we used *mip* gene sequencing.

The amplification of *mip* gene revealed a simultaneous presence of two different species of environmental *Legionella*e: *L. anisa* and *L. rubrilucens*, both of them presents in Buildings 1 and 3, by contrast only *L. anisa* was found in Building 2.

The amplified region of *mip* is not as polymorphic to allow to say that the *L. rubrilucens* or *L. anisa* are different from each other because the cut off to establish the diversity is determined by EWGLI and the % of homology is 97%. The sequences of *L. species* have undergone phylogenetic analysis, confirming the presence of two clusters , one formed by *L. rubrilucens* and one consisting of *L. anisa*. These results confirm the diversity of the two species as far as the *mip* gene and may be partially related to the degree of pathogenicity of two strains, indeed *L. anisa* is often connected to human disease, [30] while the scientific literature and epidemiological investigation did not report data about cases associated to *L. rubrilucens*.

Our data did not reveal a significant association between *Legionella* colonization in all buildings and specific STs but we observed a changing in dynamic of contamination not only in terms of positive samples and level of *Legionella* concentration, but also about the switch between strains.

In particular in relation to study period, when the shock treatment was performed – especially during period 2 - we observed a decrease of *L. pneumophila* strains and an increase of *L. species.* These observations might suggest as in environment water network there is a competition between the strain, and one, e.g. *L. pneumophila* prevails on *L. species*, suggesting a likely mechanism of resistance developed by *L. species* to disinfectants.

The approach used in this study is innovative from the point of view of environmental monitoring inasmuch the legislation requires to implement the corrective measure based on levels of contamination and type of risk. The results show as a disinfection treatment can select some species in a water distribution system; we clearly observed that when *L. pneumophila*, the species most abundant in water environments, breaking down after shock treatment, the *L. species* can found the absence of competition to growth.

During culture period, when the samples present a high *L. pneumophila* contaminations in term of cfu/L, often was not possible to estimate and enumerate *L. species* presence that usually growth at 7-10 day of culture. This issue could be underestimated the real risk of *Legionella* infection.

We want underline as the reference laboratories performed serological identification as routinely approach and genotyping approach was undertaken only in presence of one case or during epidemic events. The serological definition of *Legionella* has a limit of differentiate only between *L. pneumophila* serogroups or *L. species*, information that did not give support to study a dynamic of colonization and microbial diversity especially in presence of disinfection treatment. The knowledge of strains can help the Health authority, the engineers and technical staff to undertake the correct measures to perform the preventive control of *Legionella* contamination.

CONCLUSION

The study of environmental samples is essential to explore the population structure and genetic diversity of the species that could help the epidemiological investigations.

Our study confirms the role of SBT technique to study the distribution of *L. pneumophila* strains also in environmental monitoring in order to establish the epidemic sources of

infection to plan a long term prevention strategy. The develop of similar strategies for the standardized identification and typing of species of *Legionella* non-*pneumophila,* which are less known and associated with human disease, is the future goal.

The gap between clinical samples and environmental controls often determine a delay in diagnosis and confirmation of cases and this represent a failure of the quickly adoption of intervention strategies. The opportunity to have a genotypic map of environmental isolates could help to early undertake epidemiological investigation and correlate strains mutations in response to the disinfection treatment.

The increase of the disinfectant dosage is not adequate preventive approach because the water distribution systems do not tolerate long-term shock treatments and this type of intervention, in the long term, might compromise the quality of the water supplied, damage the network and the human health. Effective long-term control of *Legionella* contamination depends on the rigorous adherence to the control measures together with engineering modifications to the existing water systems as well as improvements in monitoring controls, knowledge of the distribution of microbiological genetic correlation between the isolates, a correct risk assessment plan and a continuous staff training.

The strategy to be followed for public health, for costs containment and energy saving is to identify the correct disinfection treatment and dosage in relation to the strains presence, its resistance to the treatment and their pathogenic characteristics.

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CHAPTER 4

Effect of Monochloramine Treatment on Colonization of a Hospital Water Distribution System by *Legionella* spp.: A 1 Year Experience Study

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Effect of Monochloramine Treatment on Colonization of a Hospital Water Distribution System by *Legionella* spp.: A 1 Year Experience Study

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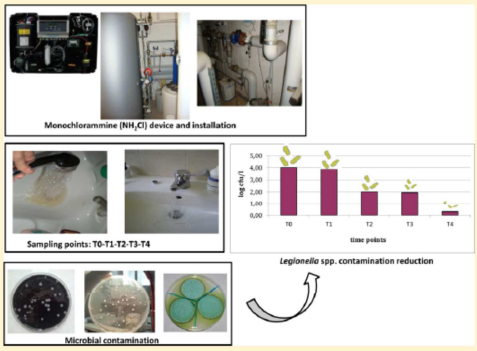
Supporting Information

ABSTRACT: Contamination of hot water distribution systems by *Legionella* represents a great challenge due to difficulties associated with inactivating microorganisms, preserving the water characteristics. The aim of this study was to examine over the course of 1 year in 11 fixed sites, the impact of monochloramine disinfection on *Legionella*, heterotrophic bacteria (36 °C), *Pseudomonas aeruginosa* contamination, and chemical parameters of a plumbing system in an Italian hospital. Three days after installation (T0), in the presence of monochloramine concentration between 1.5 and 2 mg/L, 10/11 sites (91%) were contaminated by *L. pneumophila* serogroups 3 and 10. After these results, the disinfectant dosage was increased to between 6 and 10 mg/L, reducing the level of *Legionella* by three logarithmic unit by 2 months postinstallation (T2) until 6 months later (T3). One year later (T4), there was a significant reduction ($p = 0.0002$) at 8/11 (73%) sites. Our data showed also a significant reduction of heterotrophic bacteria (36 °C) in 6/11 (55%) sites at T4 ($p = 0.0004$), by contrast the contamination of *P. aeruginosa* found at T0 in two sites persisted up until T4. The results of the present study show that monochloramine is a promising disinfectant that can prevent *Legionella* contamination of hospital water supplies.



INTRODUCTION

Legionella spp. are ubiquitous Gram-negative bacteria found in natural and artificial aquatic environments.[1,2] When *Legionella* colonize the water distribution systems of hospitals and health care facilities it increases the risk of legionellosis, particularly for immunocompromised individuals.[3] *Legionella* grow optimally at 35 °C (range 25−42 °C). They grow particularly well in the biofilm covering the inside of water pipes and tanks.[4,5] Several factors affect the survival of the microorganism in man-made systems, including temperature, pipe material, nutrient levels, and hydraulic conditions.[6] *Legionella* can then spread via aerosols created by artificial environments such as cooling towers, water boilers, whirlpools and spas, drinking water distribution networks, showerheads, instruments used for respiratory and dental treatments, and humidifiers.[7,8]

When a susceptible host inhales aerosolized *Legionella*-containing water particles (less than 5 μm in size),[9] they may suffer one of two clinical syndromes: Legionnaires' disease (LD), a severe form of pneumonia, or Pontiac fever, a self-limiting flu-like illness.[2]

Fortunately, LD does not appear to be transmitted from person to person. To date, 58 species of *Legionella* have been described (http://www.bacterio.net/legionella.html), with *Legionella pneumophila* (*Lp*) serogroup 1 (SG1) being the species most frequently isolated from infected humans.[10]

The National Institute of Health (ISS) in Italy began epidemiological surveillance for legionellosis in 1983 and registered an increase in the number of LD cases between 1990 and 2012 due to better data collection procedures and improved detection rates.[11]

In 2012, the ISS was notified of 1350 new LD cases, of which 72 (5.3%) were hospital-acquired infections, 137 (10.1%) were

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Table 1. Structural and Functional Characteristics of the Study Hospital

year of construction	year of renovation	number of floors	number of patients	building area (m^2)	plumbing materials	presence of water-softening system	water temperature at the sampling points (range, °C)	residual monochloramine at the sampling points (range, mg/L)	hardness of water at input	frequency of use of distal sites	Source for hot water heater
1950	1985 1995	2	100	8.5	zinc, polypropylene, multilayer polyethylene	yes	20.1−51.4	0.15−3.34	15 °F	daily/ weekly	2 electric tanks in parallel

travel-associated, 42 (3.1%) occurred at daycare centers, and 22 (1.6%) were associated with other risk factors. In all cases, the causative agent was *L. pneumophila*.[11] Unlike for other bacterial diseases of the respiratory system, there are currently no community-based measures (such as vaccination) that can protect against infection by *Legionella* spp. Therefore, maintaining control of aquatic environments is vitally important.

Contamination of hot water distribution systems is the most important risk factor for legionellosis. In addition, the risk of colonization is related to the age of the building, the complexity of the water system, and the type of hot water heater used.[12] Therefore, the best way to prevent infection is to design appropriate water networks and reduce the risk of *Legionella* contamination by applying appropriate control measures.

Several studies show that *Legionella* colonize the water systems of 60−80% of hospitals and that, once the bacteria have colonized a water system, they are almost impossible to eradicate.[13,14] Therefore, it is important to implement strict controls and prevent microbial regrowth[15] through appropriate water disinfection protocols, while at the same time maintaining the microbiological and chemical characteristics of the source water.

One of the most popular measures used to prevent colonization of water distribution systems focuses on increasing the temperature of the hot water supply along with the supplemental chlorination of drinking water.[16] The first documented method of water disinfection, the "superheat-and-flush" method, dates back to 1983 and involves raising the water temperature to 77 °C for 72 h.[17] Chlorine is the most common disinfectant added to drinking water, and it is the primary disinfectant of choice for the majority of water treatment plants.[18] The ability of a disinfectant to oxidize or rupture the bacterial cell wall and to diffuse into the cell (thereby interfering with cellular metabolism) is the primary factor that determines disinfection efficiency.[19]

During the 30 years since the introduction of superheat treatment, other methods such as hyper chlorination, ultraviolet light, ozone, copper−silver ionization, chlorine dioxide, point-of-use filters, and monochloramine have all been tested.[7] Although these measures may be effective at controlling the growth of *Legionella* spp. in drinking water systems, high water temperatures are associated with a risk of scalding, and supplemental chlorination may hasten corrosion and cause leaks in plumbing systems.[18] Moreover, disinfection byproducts (DBPs) pose a chronic and long-term risk to public health. Therefore, the optimal disinfection treatment should maximize protection against microbes while minimizing the levels of DBPs.

To date, no study has examined the long-term efficiency of "alternative" methods of controlling and eradicating *Legionella* spp. from hospital water systems.[7,20] Additionally, no standardized strategy has been proven to prevent community-acquired LD. Any such strategy should take into account the particular characteristics of the water plumbing system and both the type and level of contamination.[21]

A recent and novel approach to water treatment is based on the use of chloramines, which appear to be more stable than free chlorine and are more effective at controlling bacterial regrowth because they have a long residence time on the water distribution system.[22−25]

The reaction between ammonia and hypochlorous acid (NH_3 + HOCl) produces monochloramine (NH_2Cl) and H_2O. Even though little is known about the mechanism by which monochloramine kills bacterial cells, some studies suggest that disinfection of water systems with monochloramine may protect against outbreaks of hospital-acquired LD.[26]

Laboratory studies show that monochloramine inactivates bacterial cells by interacting with the cell envelope and creating DNA damage.[24] Indeed, monochloramine appears to inhibit bacterial transport, respiration, and substrate dehydrogenation.[27] The biofilm normally present in plumbing systems may protect *Legionella* and other bacteria from the action of disinfectants; however, some studies[7,28] show that monochloramine can penetrate biofilms,[29−32] thereby affecting the biological niche occupied by *Legionella* spp. (e.g., sessile *Legionella*).[33]

Berry et al. suggest that microbial diversity could influence both the effectiveness of disinfection and pathogen survival in drinking water distribution systems.[34] Their conclusions were based on the results of various studies, which show that microbial diversity is affected by the disinfection strategy;[35,36] however, the impact of biocides on the diversity and dynamics of a particular species is not well documented.

Therefore, the aim of the present study was to examine the impact of monochloramine on *Legionella* spp., heterotrophic bacteria, and *P. aeruginosa* in the plumbing system of a hospital in Emilia Romagna Region, Italy, after the removal of the previous disinfection system, which was based on a ClO_2 bioreactor.

MATERIALS AND METHODS

Hospital Characteristics and Monochloramine Treatment. The hospital in Emilia Romagna Region was built in the 1950s and subsequently restructured in 1985 and 1995. The structure spans two floors, with a total area of 8500 m^2. Two electric heaters run in parallel to generate hot water, and cold water flows through a water softening system. The building characteristics are listed in Table 1. The monochloramine disinfectant device was installed in July 2012 by Itaca s.r.l. and continuously produces and dispenses monochloramine. Batches of monochloramine (0.1% w/v) are produced and injected into the hot water return line (at about 1.5−2 mg/L) to maintain the residual concentration at distal outlets within 0.15−0.50 mg/L. The dose is proportional and based on the volume of the cold water supply compared with that of the hot water system. The residual monochloramine levels and water temperatures

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Table 2. Temperature Values and Residual Disinfectant Levels at Critical Sampling Points

sampling points	site	mean temperature °C (T0-T1-T2-T3-T4/site)		residual NH_2Cl levels during monitoring T0	T1	T2	T3	T4	mean NH_2Cl residue level/site (mg/L)
1	hot tap water output	52		0.43	0.43	2.48	3.21	2.13	1.74
2	hot water return line	45.2		0.44	0.42	2.96	3.34	2.43	1.92
3	medical office (washbasin)	45.8		0.44	0.44	2.22	2.15	2.05	1.46
4	room, diagnostics (washbasin)	48.7		0.45	0.45	2.31	2.25	1.92	1.48
5	public wc (washbasin)	44.4		0.47	0.47	2.36	2.40	2.75	1.69
6	changing room (washbasin)	43.3		0.50	0.50	2.94	2.81	3.14	1.98
7	hospital room (shower)	48.5		0.40	0.33	3.01	2.56	2.83	1.83
8	hospital room (washbasin)	47.4		0.34	0.39	2.40	2.39	2.50	1.60
9	hospital room (washbasin)	49.7		0.45	0.41	2.76	2.41	2.71	1.75
10	room, nurses (washbasin)	35.7		0.15	0.18	2.32	2.31	2.42	1.48
11	hospital room (shower)	37.9		0.15	0.20	2.70	2.73	2.53	1.66
	mean temperature (°C)	**45.3**	**mean NH_2Cl residue (mg/L)**	**0.38**	**0.38**	**2.59**	**2.60**	**2.49**	

Table 3. Physicochemical Parametres (Mean Values) at All Critical Sampling Points Mesured during the Test Period

paremeter	U.M.	mean value sampling points/test period	uncertainty	method
hydrazine	mg/L	<0.1		ML24.4.12 LEBSC
trihalomethanes	mg/L	<0.1		ML24.4.12 LEBSC
ketones	mg/L	<0.1		ML24.4.12 LEBSC
pH		7.33		APAT CNR IRSA 2060 A Man 29 2003
conductivity	μS/cm	970	±0.01	APAT CNR IRSA 2030 A Man 29 2003
ammonia nitrogen (NH_4^+)	mg N/L	0.35	±0.1	APAT CNR IRSA 4030 Man 29 2003
nitrous nitrogen (NO_2^-)	mg N/L	1.8	±0.1	APAT CNR IRSA 4050 Man 29 2003
nitrate nitrogen (NO_3^-)	mg N/L	<0.01		APAT CNR IRSA 4040 Man 29 2003
chloride	mg Cl^-/L	8.8	±0.1	APAT CNR IRSA 4020 Man 29 2003

measured at each site during the study period are shown in Table 2.

Water Sample Collection. Hot water was run for 2 min and samples collected in accordance with a risk assessment plan set up by the Local Health Authority. A total of 55 samples were collected at five different time points from July 2012 to July 2013: T0 (3 days after monochloramine device installation), T1 (15 days after installation), T2 (2 months after installation), T3 (6 months after installation), and T4 (1 year after installation).

"Sentinel sites" were defined as proximal or distal sites located in different areas of the hospital in which washbasins and showerheads were used daily (or at least once per week). Such sites included offices, consulting and diagnostic rooms, locker rooms, and patients' rooms. In particular, hot water samples were collected from hot tap water output and hot water return line points, while other samples were taken from points at different distances from the heater room (two showerheads and seven washbasin faucets) (Table 2).

To detect *Legionella* contamination, 2 L of water were collected in two sterile 1 L glass bottles containing 10% sodium thiosulfate (1 mL/l) (to neutralize any residual disinfectant). At the same time, another 1 L sample was taken to monitor the physicochemical properties of the water. Microbiological samples were refrigerated, protected from heat and sunlight during transport to the laboratory, and analyzed within 3 h of collection.

Physical and Chemical Analyses. During the test period, the following parameters were recorded at each sampling point: temperature, pH, and residual monochloramine levels (measured with a Hach photometer; Hach method 10172). Hydrazine, trihalomethanes, aldehydes, ketones, electrical conductivity, and secondary products of monochloramine reduction. DBP were measured by ion-exchange chromatography (APAT CNR IRSA 2060/2030/4030/4020/4040/4050 Man 29 2003)[44] (Table 3).

Microbiological Analysis. All water samples were examined for total heterotrophic bacteria (36 °C), *P. aeruginosa* and *Legionella* spp. Total heterotrophic bacteria were isolated using the standard plate method (culture on Tryptic glucose yeast agar; Biolife, Milan, Italy) according to UNI EN ISO 6222:2001,[37] and the results expressed as $\log_{10}$ colony forming units (cfu) per milliliter of water ($\log_{10}$ cfu/mL).

P. aeruginosa, which is a strong competitor of *Legionella* spp. in drinking water,[38] was isolated using the standard Membrane Filter Technique (filters cultured on Pseudomonas selective agar; Biolife, Milan, Italy) according to UNI EN ISO 16266:2008,[39] and the results expressed as $\log_{10}$ cfu/100 mL. Colonies growing on selective agar were subcultured and identified using a Crystal Enteric/Non-Fermenter ID kit

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(Crystal E/NF; Becton Dickinson, Cockeysville, MD) according to the manufacturer's instructions.[40]

Legionella was isolated by culture according to ISO 11731-1998[41] as previously described.[42,43] Isolates were identified on the basis of culture (lack of growth on CYE agar and blood agar), biochemical (fluorescence, oxidase activity, catalase activity, the ability to hydrolyze hippurate, and β-lactamase activity), and serological characteristics (*Legionella* latex test kit; Oxoid, Basingstoke, UK). Serogroups were also identified by agglutination tests using commercial antisera (Polyclonal latex reagents; Biolife, Milan Italy). The results were expressed as $\log_{10}$ cfu/L.

Hospital Legionnaires Disease Surveillance. Throughout the whole period of monitoring, the hospital object of our study performed actively a Legionellosis surveillance. In presence of symptoms of acute infection of the lower airways with clinical and/or radiological signs of focal pneumonia the preliminary diagnosis is routinely confirmed by an urine antigen test (*Legionella* Urine Antigen EIA, Biotest, Italy) and the serological immunofluorescence test (*L. pneumophila* IFA, Meridian Diagnostic, Europe).

Statistical Analysis. Bacteriological data were converted into $\log_{10}(x + 1)$ values to normalize non-normal distributions. The results were then analyzed using Student's t test (Stata 10 Data Analysis and Statistical Software; StataCorp LP, Texas, USA). Any p values <0.05 were considered significant.

RESULTS

Physical and Chemical Analyses. The results of the physical and chemical analyses are shown in Table 3. The data are expressed as mean concentrations at each sampling point at different times during the test period. Introducing monochloramine into the hospital hot water circuit did not change the water temperature, pH, or conductivity. Also, no DBPs were detected in cold water samples (according to Italian regulations related to drinking water, D. Lgs 31/2001).[45]

Microbiological Analysis. We compared the levels of contamination in the hot water line at T0 and T4 and also monitored the mean levels of contamination at T1, T2, and T3. Figure 1 shows the results for *Legionella* during the test period.

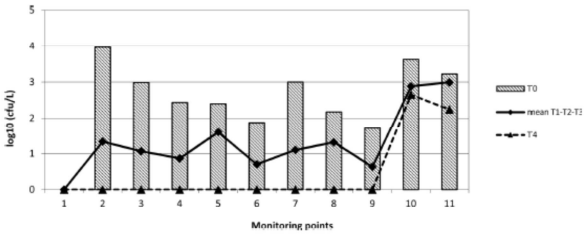

Figure 1. *Legionella* spp.levels at the 11 selected sites.

At T0, when the residual monochloramine level at distal outlets was 0.15−0.50 mg/L, only the hot tap water output (site 1) was free of contamination. Indeed, this site remained free of *Legionella* throughout the study period.

Regarding the other test sites, the majority of points in the water distribution system (10/11; 91%) were contaminated with *L. pneumophila* (serogroups 3 and 10) at concentrations between 1.72 and 3.97 $\log_{10}$ cfu/L. The highest level (3.97 $\log_{10}$ cfu/L) was measured in a hot water return line (point 2). The concentration of *Legionella* temporarily increased at all sites (range 1.90−4.00 $\log_{10}$ cfu/L) at 15 days postdevice installation (T1). After examining the results from the first sampling phase (T0−T1), we decided (in accordance with the Local Heath Authority) to increase the concentration of the disinfectant to between 6 and 10 mg/L.

The concentrations of ammonia and DBPs at distal outlets remained within the limits suggested by D. Lgs 31/2001.[45]

At T2 (about 2 months after installation of the monochloramine device), the level of *Legionella* contamination at 64% (7/11) of the monitoring sites fell below levels detectable by the assay (<25 cfu/L). The remaining sites showed contamination levels between 1.70 and 1.88 $\log_{10}$ cfu/L (sampling points 5, 8, and 10); the highest level of contamination (3.30 $\log_{10}$ cfu/L) was detected at site 11. This trend was repeated at T3 (6 months after installation). We hypothesize that the higher concentration of disinfectant and the longer residence time within the system efficiently prevented colonization of all sites except 10 and 11, which showed contamination levels of 2.88 and 2.18 $\log_{10}$ cfu/L, respectively. Only 2/11 (18%) sites were contaminated at T4 (1 year after installation). The level of contamination at this time point was significantly lower than that at T0 (p = 0.0002); however, contamination was detected at sites 10 and 11 (2.65 and 2.24 $\log_{10}$ cfu/L, respectively) (Table S1, Supporting Information).

The data for heterotrophic bacteria (36 °C) and *P. aeruginosa* showed a different trend. For heterotrophic bacteria at 36 °C (Figure 2), 100% (11/11) of all sampling points were

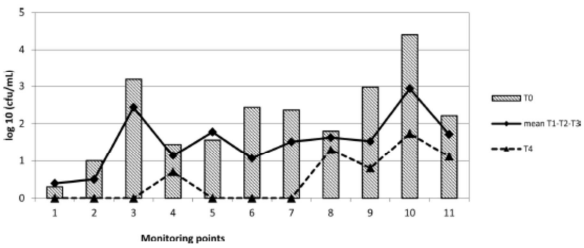

Figure 2. Total heterotrophic bacteria (36 °C) levels at the 11 selected sites.

contaminated at T0 (range, 0.30−4.40 $\log_{10}$ cfu/mL). After 15 days (T1), all points showed an increase in contamination (range, 0.40−4.60 $\log_{10}$ cfu/mL); however, there was a reduction in contamination levels at all sites at T2 (range, 0.30−1.72 $\log_{10}$ cfu/mL), which persisted at T3. This trend led to a significant reduction in contamination at T4. There was a 100% reduction in contamination at 6/11 (54%) sites and a decrease of contamination level at the remaining sites (range 28−73%). Overall, the reduction in contamination level between T0 and T4 was statistically significant at all sites (p = 0.0004) (Table S2, Supporting Information).

The results for *P. aeruginosa* (Figure 3) showed yet another trend. At the beginning of the test period (T0), 18% (2/11) of sites were contaminated (range, 2.67−4.20 $\log_{10}$ cfu/100 mL); however, from T1 to T3, we found punctiform contamination at five different sites (four washbasin faucets and one showerhead), which only disappeared at T4. Sites 8 and 10 were still contaminated at T4, with values of 3.00 and 3.92 $\log_{10}$cfu/100 mL, respectively) (Table S3, Supporting Information).

Figure 4 and Table S4 (Table S4, Supporting Information) compare the *Legionella* data obtained from the present study

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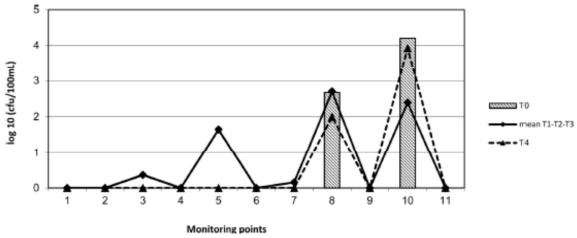

Figure 3. *Pseudomonas aeruginosa* levels at the 11 selected sites.

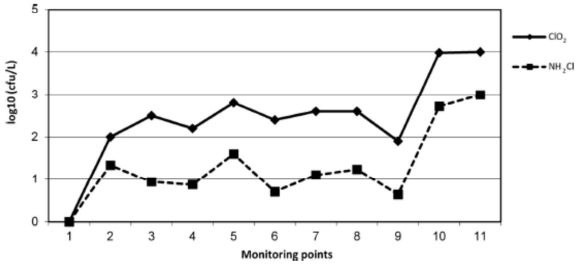

Figure 4. Effect of disinfection treatment (NH_2Cl vs ClO_2) on *Legionella* spp. levels at the 11 selected sites.

(test period) with those during the period when ClO_2 was used as the disinfectant. The results clearly show that monochloramine was significantly better than ClO_2 at reducing *Legionella* contamination ($p = 0.011$).

Hospital Legionnaires Disease Surveillance. No cases of nosocomial legionellosis have been reported by the Hospital Infections Committee during the study.

DISCUSSION

The present study examined the effectiveness of monochloramine at reducing *Legionella* colonization in a hot water distribution system over a period of 1 year. The aim was to understand how monochloramine impacts the ecology of *Legionella* and other water-associated pathogens. The object of the study was an old, large, and complex hospital distribution system; a typical reservoir for *Legionella* spp.

Monochloramine has been used as a residual disinfectant in drinking water since 1916, and several studies show that it has a better capacity to penetrate biofilms than chlorine.[22,24]

In addition, it kills sessile biofilm bacteria such as *Legionella* spp. and *Pseudomonas* spp.[29,30] The major benefits of chloramines are derived from their persistence within the water distribution system, their tendency not to form trihalomethanes and other DBPs, and their lack of taste and odor.[7,32,46]

On the basis of a literature review and our previous data, we hypothesized that changing the residual disinfectant in a hospital water plumbing system (from ClO_2 to monochloramine) might reduce the level of *Legionella* colonization. Our data showed that the previous ClO_2-based system did control the level of bacterial contamination because the level of *Legionella* increased to 10^4 cfu/L at only two sites.

The decision by the Local Health Authority to switch to monochloramine was based on several complex factors, such as the persistence of colonization at critical points, damage to the water distribution pipes, and changes in drinking water quality. It is well documented that ClO_2 is rapidly converted to chlorite and chlorate after reacting with organic materials and corrosion/scale within pipes.[47] These byproducts may pose a health risks for hospitalized individuals.[48] Therefore, corrosion of galvanized pipes can lead to the breakdown and loss of ClO_2 from the system.[47]

The present study (conducted between July 2012 and July 2013) comprised five sampling times (T0 to T4) and acquired 55 samples. At the beginning of the experiment (T0), all sites sampled were contaminated by *Legionella* spp. (*Lp* isolate serogroups 3 and 10). Contamination was particularly high at site 2 (a hot water return line) and at two distal sites (10 and 11: a washbasin faucet and showerhead, respectively). The only site free from contamination was the hot tap water output (site 1). These data suggest that the contamination was punctiform and localized at distal outlets.

Examination of the water system management protocols revealed that the two heaters were emptied and disinfected once a year. No contamination of the heater (data not shown) and hot tap water output was detected during the study period. These results are in line with data collected during the last monitoring period performed before the ClO_2 device was removed (data not shown).

During the second sampling period (T1), we observed a significant increase in the levels of all microbiological parameters, indicating an increase in contamination of the hospital water network. We hypothesized that this was due to the fact that monochloramine penetrates the biofilm; therefore, bacterial colonies would be released into the system, resulting in an initial increase in the number of colonies detected. This hypothesis is supported by the fact that an increase in the disinfectant concentration from a mean of 0.38 mg/L to a mean of 2.59 mg/L, coupled with an increased residence time within the system, led to a reduction in *Legionella* spp. contamination of up to three logarithmic units at T2 (2 months after monochloramine installation). This ultimately led to a significant reduction in bacterial contamination at almost all sample points examined at T4 (1 year after installation).

The persistence of contamination at critical sites (points 10 and 11) may be attributed to the low temperatures recorded during the sampling phase (Table 2). A detailed analysis by the unit manufacturers and plumbers revealed that a washbasin faucet (site 10) and a showerhead (site 11) were sited in two rarely used staff rooms. A second inspection of both outlets revealed that a thermostatic radiator valve (TRV) was not working; thus low usage and a fall in the water temperature may have allowed *Legionella* spp. to multiply. Accordingly, we recommended that new TRVs be installed to permit an increase in temperature and that all such utilities should be flushed at least once per week.

The data relating to heterotrophic bacteria at 36 °C (which are microbial indicators of water quality) showed that monochloramine had an important and significant effect on these species. At T1, we found an increase in the number of heterotrophic bacteria. Again, these results can be explained by the penetrative action of monochloramine into biofilms. At T2, there was a significant reduction in the number of heterotrophic bacteria at almost all sample points, which persisted until T4. This suggests that monochloramine may be effective against microorganisms other than *Legionella* spp. The data related to sampling points 10 and 11 are similar to those for *Legionella* spp., confirming that the monochloramine disinfection system stabilized contamination at low levels. Legislation related to drinking water quality is more tolerant of these micro-

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organisms, which are considered autochthonous components of water; however, we suggest that the levels of heterotrophic bacteria are tested once a month as they provide a useful readout for monitoring changes in water quality.

Again, a different trend was observed for *P. aeruginosa*, which it is important to note, is not predicted by classical indicators of water contamination. *Pseudomonadaceae*, especially *P. aeruginosa*, are ubiquitous in biofilms; therefore, their presence in water loops, which are often located at the terminals of water distribution facilities (e.g., faucets, filters, showerheads, and tap nozzles), is a public health concern.

Our data showed that at T0, most sites were free of the microorganism, that the contamination was punctiform, that this species was not recovered from the hot tap water output or from hot water return line, and that they were detected at high levels in only two washbasin faucets. As mentioned above, these findings may be explained by a fall in temperature and a lack of adequate cleaning and flushing protocols. To test this idea, we removed tap nozzles before collecting samples and increased the flushing time. No contamination was detected after these measures were taken. Therefore, we suggested that all fitting outlets be cleaned one per week as follows: tap nozzles or shower heads should be removed, descaled, and cleaned by immersion in a descaler followed by a disinfectant solution (e.g., sodium hypochlorite).[14]

The monitoring of *P. aeruginosa* during the study period shows that monochloramine was not able to eradicate these bacteria from all sampling sites in the absence of an adequate daily or weekly cleaning protocol. Our experience of environmental monitoring also suggests that the simultaneous presence of *P. aeruginosa* and *Legionella* may have affected the growth of *Legionella* growth in in vitro culture, leading us to underestimate actual *Legionella* contamination levels. Therefore, these data suggest that *Pseudomonas* spp. monitoring has an important role to play during environmental sampling and risk assessment.

The present study shows that converting to a monochloramine disinfection system (2−3 mg/L) had a positive impact on the levels of microbial contamination in a public potable water system. Therefore, monochloramine disinfection appears to be a promising approach to reducing colonization by *Legionella* spp; however, further investigations are needed to ascertain both its efficiency over the time and how it affects the microbiome present in the water system.

The risks of nosocomial *Legionella* infection of hospitalized populations are likely to increase as water systems age; thus, interest in novel water treatment systems will continue to grow. According to WHO guidelines on Drinking Water Quality,[49] hospitals, nursing homes, and other healthcare facilities are considering high-risk environments, both because of the complex nature of their drinking water systems and because of the sensitivity of their occupants. The CDC recommends that environmental cultures for *Legionella* spp. should only be performed if a case of hospital-acquired legionellosis occurs.[50] However, experts in the field of waterborne disease and governmental agencies are considering legislation that will require healthcare institutions to develop *Legionella* control plans that include regular water cultures. By contrast, Italian and European guidelines and several research studies favor a preventive approach, indicating that periodic culture of hospital water samples is as an important strategy for preventing legionellosis cases and outbreaks.[51−54] Finally, we believe that water monitoring should be performed to test the efficiency of disinfection treatments and to prevent the occurrence of LD cases, thereby saving public money and avoiding legal problems.

■ ASSOCIATED CONTENT

Supporting Information

Statistical analysis for each microbial contaminant analyzed. The data are expressed as percentage of decrease in T0 vs T4 with relative *t*-student and *p*-values. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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CHAPTER 5

Effect of Earthworms and Plants on Efficiency of Vertical Flow Systems Treating University Wastewater

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1. Introduction

Constructed wetlands (CW) are engineered systems that mimic processes occurring in natural wetlands and make use of them in wastewater treatment. They are particularly good choice for small to medium communities (Wu et al, 2013) since their area requirements can be quite high. What makes them so suitable is that their costs are lower compared to the conventional wastewater treatment plants (Nivala et al, 2012) and they can be well integrated into the environment. Additionally, the staff operating them does not need specific training (Li et al, 2011; Nivala et al, 2012; Wu et al, 2013). Even though use of CWs has spread in the past few decades, and some important advances have been made (Meng et al, 2014), they still face certain difficulties such as clogging and poor nutrient removal (Babatunde et al, 2008).

Even though CWs can be anaerobic, earthworms have been found in these kinds of systems before (Nuengjamnong, 2010) and Chen et al (2016) stated that vertical flow CWs (VFCWs) can be a viable habitat for these invertebrates. Earthworms prefer dark, humid environments, with abundance of organic matter and presence of oxygen (Li et al, 2011). They ingest a mixture of organic matter and sand, the latter one having a role in breaking organic material (Li et al, 2011). They also consume microorganisms present in the soil and due to the favourable conditions inside the earthworms' gut, these microorganisms rapidly multiply and are later excreted back to the environment (Le Bayon and Milleret, 2009). It has been established that the addition of earthworms can increase plant growth and crop yields.

Moreover, due to the formation of channels containing more available nutrients than the surrounding soil they can also increase the root growth (Edwards and Bater, 1992).

One of the most common species is *Eisenia fetida*. It is a tough earthworm and in mixed cultures it usually becomes dominant as it can out-compete other species (Edwards and Bater, 1992). It lives for 2-10 years in natural conditions or up to 15 years if artificially fed (Wu et al, 2013). Li et al (2011) have reported that this species lives in the top layer of the CW. It consumes accumulated organic solids and loosens the compacted substrate and therefore increases the air presence in the bed. Earthworms are poikilotherm organisms and their body temperature is highly dependent on the air temperature. Temperatures higher or lower than their optimal range (25-30°C) affect them and could even cause their death (Arora and Kazmi, 2015). Edwards and Bater (1992) have established that the temperature range tolerated by Eisenia fetida is 0-35°C, while their maximum growth occurs at 30°C and the moisture content of 85%.

Earthworms have previously been successfully applied for composting and stabilisation of organic material (vermicompost) and the final product of this process (humic substances) have a high potential for adsorption of metals (Matos and Arruda, 2003). Moreover, they were used for sludge dewatering, stabilisation and reduction (Tamis et al, 2011; Chen et al, 2016) and were reported that can prevent clogging or restore already clogged VFCWs (Li et al, 2011; Nivala et al, 2012). Even though there are some studies that have examined influence of earthworms on the different aspects of the CW or filter operation (Taylor et al (2003); Li et al (2011); Nuengjamnong et al (2011); Xu et al (2012); Xu et al (2013)a; Xu et al (2013)b; Xu et al (2013)c; Wu et al (2013); Arora and Cazmi (2015); Chen et al (2016)), none of them provided longer term results from an open air system or compared effect of different seasons in temperate climate. Therefore, objectives of this research were to: (1) evaluate the effect that earthworms and their combination with *Phragmites australis* have on vertical flow filter

(VFF) performance during the whole year and the different seasons, (2) to compare the growth of the earthworms in CWs and in the natural environment and (3) to assess the effect that these organisms have on the CWs' substrate.

2. Materials and methods

2.1. Effect of earthworms and plants on CW efficiency

2.1.1. Experimental set-up

The pilot plant used in this research was located at the Department of Civil, Chemical, Environmental and Materials Engineering (DICAM) of the University of Bologna (Italy). It consisted of a septic tank, followed by an inflow tank that provides wastewater for four vertical flow systems (Ø 0.48 m, 0.55m depth): vertical flow filter (VFF), vertical flow constructed wetland with earthworms (VFCWw), vertical flow constructed wetland (VFCW) and vertical flow filter with earthworms (VFFw). The VFCW effluent was further treated by two horizontal flow systems: horizontal flow filter (HFF) and horizontal flow constructed wetland (HFCW) (Figure 1a).

<Figure 1 – Pilot plant during the construction phase (a)
and water flow in the system (b)>

The substrate used in all the vertical systems was 12 cm of gravel at the bottom (Ø 0.63–5 cm) covered with 33 cm of sand (Ø 2-4 mm). Each system had two aeration tubes open to the atmosphere in order to increase concentration of oxygen in the substrate, but no artificial aeration was applied. Water was distributed by means of plastic pipes placed on the top of the substrate. During warm (April-October) period of the year systems were receiving 15 L of

water 3 times a week and the retention time was 24 h. Conversely, during cold (October-April) period of the year they it was 15 L two times a week and retention time of 48 h.

The plants used in the CWs (*Phragmites australis*) were taken from the botanical garden of the University of Bologna and were planted in April 2015. At first, the earthworms were obtained from the area near the pilot plant. However, since July 2016 it was not possible due to the increased air temperatures, and the earthworms were purchased in a fishing equipment shop. They were kept in a mixture of sand and organic matter in order to prevent the effect a sudden change of environment could have on them. Unfortunately, it was not possible to determine the exact species, but the authors believe that the majority of them were *Eisenia fetida* that is usually the dominant species (Edwards and Bater, 1992). Every 3-4 weeks an average of 3.5 g of the earthworms were added to the VFCWw and VFFw.

2.1.2. Experimental design and analytical methods

The influent and effluents were monitored in the period October 2015-October 2016. They were analysed for pH by electrometric method, chemical oxygen demand (COD) spectrophotometrically with COD Vario cuvette kit (Aqualytic, Germany) and total suspended solids (TSS) with gravimetric method. Total nitrogen (TN) and total phosphorus (TP) were analysed by digestion by the persulfate method followed by measurements of NO_3^--N (ultraviolet spectrophotometric screening method) and PO_4^{3-}-P (vanadomolybdophosphoric acid colorimetric method), respectively. Finally, different ions such as nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{3-}), chloride (Cl^-), bromide (Br^-), and sulphate (SO_4^{2-}) were analysed by ion chromatography (DX-120, Dionex Corporation, USA). These analysis were performed in the laboratories of DICAM according to methods from APHA (2005) unless stated otherwise. The microbiological parameters (*E. coli*, Total coliforms and *Enterococcus*) were analysed at the Department of Biological, Geological, and Environmental Sciences, by membrane filter technique followed by incubation and enumeration using Chromogenic

Coliform Agar for *E. coli* and Total coliforms (ISO 9308-1, 2014), and Slanetz Bartley Agar for *Enterococcus* (ISO 7899-2, 2000).

The plants were harvested at the beginning of summer (7th June 2016) and at the end of the experimental period (6th October 2016). Their dry weights were measured after drying for at least 48 hours at 105°C. Before the second harvesting, the length of above-ground part of longest plant from both VFCW and VFCWw was measured.

The results obtained were compared on the average level for the whole experimental period, and also for the different seasons. The year was divided in four seasons by the authors according to the mean daily temperature (Table 1). The temperatures were recorded by the DICAM instruments in the vicinity to the pilot plant.

<Table 1 – Division of the research period to seasons>

2.2. Earthworms growth and effect on CW substrate

2.2.1. Experimental set-up

A separate experiment was conducted in order to assess effect of the earthworms on the substrate and estimate possibility for them to live and grow in constructed wetlands. For that, an earthworm-reactor was installed and it was monitored during the period May-July 2016. It consisted of three small vertical flow filters: filter (SVF), filter with earthworms (SVFw) and a control system that contained soil, organic matter and earthworms (SFC). Each of them had dimensions of 20x25x20 cm. The substrate of the two filters (SVF and SVFw) was the same as the sand used in the VF systems (Ø: 2-4 mm). In order to increase organic matter content of the substrate, it was covered with raw wastewater for 2 weeks before addition of earthworms. The water charge schedule was the same as one of the vertical flow systems, with the

difference that they received 1 L instead of 15 L of wastewater at each feeding. The control system received 1 L of tap water once a week.

2.2.2. Experimental design and analytical methods

The earthworms were taken out three times (19th May, 7th June, 14th July), counted, weighed and put back to the filters. The substrate samples were taken at the beginning and the end of the experiment (19th May and 14th July) and stored at -20°C for a few weeks. They were analysed for the organic matter content as loss on ignition at 550°C after drying to constant weight at 80°C (Tanner and Sukias, 1995).

2.3. Data analysis

Differences between the effluents from the four systems were tested using SigmaPlot 11 software for each of the parameters analysed. The data were first checked for normality and equal variance by using Shapiro-Wilk and Levene Median Test, respectively. If the assumptions were met, the differences were tested by one-way ANOVA test. If the assumptions were not met, the values were $\log_{10}$ transformed and tested by one-way ANOVA in the case that transformation helped to meet normality assumptions, or Kruskal-Wallis test if the assumptions could not be met even after the transformation.

3. Results and Discussion

Results obtained after the whole experimental period are given in Table 2. Influent was high strength wastewater as it originated at the university, and there were no showers or washing machines that usually dilute domestic wastewater. However, it differed a lot throughout the year depending on the number of people present in the building. For example, maximum inflow concentration of COD was 1398 mg L^{-1} on 9th November 2015, while minimal one was

474 mg L^{-1} on 20th June 2016 when the lectures were over and many students were not coming to the university on a daily basis. Similarly, TN was in the range 32-119 mg L^{-1} and E. coli 69000-1028000 UFC 100 mL^{-1}. Seasonal differences were also visible and while influent TP concentration during the winter was 16 mg L^{-1}, during the spring it was much lower and it amounted to 10 mg L^{-1}.

<insert: Table 2 - Performance of the pilot plant during the period October 2015-October 2016>

Organic matter removal measured through COD and TSS was similar in all four systems and it was never below 65 and 80%, respectively (Table 2). Effluent pH values were increased compared to the influent while both TN and TP removal were in the range 43-50% (Table 2). Nitrogen removal is on the level given by Vymazal (2007) for VFCWs in different world countries (45%), while phosphorus removal was lower than the one given by the same author (60%). NO_2^--N and NO_3^--N effluent results show that these ions were both produced or converted to other compounds during the retention time. Overall, it can be said that the VFCW had the best removal efficiencies regarding chemical parameters and VFFw regarding microbiological ones.

3.1. Effect of earthworms on wastewater treatment

Statistical data analysis showed that the effect of the earthworms was not significantly important regarding any of the parameters considered for both filters and CWs. Similar conclusions were also found by Nuengjamnong et al (2011), who conducted a study based on

the application of earthworms in VFCWs treating swine wastewater. However, some differences existed between the systems containing these invertebrates and the systems that do not. For example, if VFF and VFFw on one side, and VFCW and VFCWw on the other one are compared, it can be seen that the COD removal was slightly lower in the systems containing earthworms. That was probably a consequence of the production of organic waste material as part of the earthworm's digestive cycle but it might also be connected to the decomposition of the dead individuals. As mentioned previously, earthworms live for 2-10 years but as, to the authors best knowledge, there are no long-term studies that focused on their growth in CWs, it cannot be said for how long they can live in this kind of systems.

TN removal was somewhat higher in the VFF compared to the VFFw (Table 2), probably due to the faster flow through the aerated zone in the latter one. Wu et al (2013) found that the most earthworms in the VFCW studied were present in the top 5 cm of the substrate, and that their number decreased with the depth. Since aerated zone is closer to the surface and it is also living environment for the earthworms, it can be presumed that due to their burrows water flow through that part is faster. Therefore, wastewater in the VFFw was exposed to the oxygen for shorter period of time before reaching anoxic zone on the bottom. Similarly, Taylor et al (2003) found that wastewater oxygen concentration increased after passing through the first 10 cm of the filter bed and attributed it partly to the presence of earthworms and their casts and burrows. The same difference cannot be seen between VFCW and VFCWw probably due to the fact that the plants oxygenate the substrate much more than the earthworms.

3.1.1. Effect of earthworms on plant growth

After the first harvesting, dry weights of above-ground biomass were 312 g and 426 g for VFCW and VFCWw, respectively. In the end of the experimental period, the above-ground dry biomass and longest plant were 360.5 g and 130 cm for VFCW and 505.5 g and 151 cm

for VFCWw. That is in accordance with Xu et al (2012) who found that the introduction of earthworms into VFCWs improved *Iris pseudacorus* growth and increased its dry weight. Moreover, Xu et al (2013a,b,c) also reported similar results and stated that it can probably be attributed to the better substrate for root growth. Although some authors have found different results, the majority of experiments conducted in this purpose have reported positive correlation between the plant growth and presence of earthworms (Nuengjamnong et al, 2011). Since the soil that has passed through the earthworm´s organism has more organic matter and available nutrients than the soil that did not go through this process (Le Bayon and Milleret, 2009), and given the results obtained by this study, it can be said that the presence of earthworms in VFCWw has improved the plant growth.

3.2. Effect of plants on wastewater treatment

Similarly to effect of earthworms, no significant statistical difference was found between planted and unplanted systems. This is in accordance with Ciria et al (2005) who has also found similar results and reported that COD and TSS removal occurs mostly due to physical processes rather than biological ones. As for TP removal, since it is mainly a result of adsorption to the substrate (Vymazal, 2007), plants usually do not have any effect on this process. However, presence of plants improves nitrogen removal in CWs due to the transport of oxygen from the atmosphere to the root system (Xu et al, 2013a) and in general can enhance CWs performance (Kouki et al, 2009).

When results of the two filters and the two CWs are compared, it can be seen that the addition of plants did slightly reduce effluent concentrations of COD and TN (Table 2). Białowiec et al (2012) stated that the plants can improve these removals by microbial activity and increased oxygen concentrations. Another reason are the plants themselves, since for their growth they consume organic matter and nutrients.

On the other hand, the two filters had higher removal of *E. coli* and Total coliforms than the CWs (Table 2). That can be explained by the fact that the root system presents suitable environment for the development of different microorganisms and microbial activity (Bialowiec et al, 2012). Conversely, Ciria et al (2005) found better removal of Total coliforms in planted than in unplanted bed. In this study removal of *E. coli* and Total coliforms was higher in the VFFw than in the VFF (Table 2) probably due to the predation by the earthworms or their different excrete that were unfavourable to the growth of these pathogens.

3.3. Seasonal differences

No significant statistical difference was found between the four systems for any of the four seasons. However, slight differences did occur. COD removal was, lowest during the spring and highest during the summer (Figure 2). It was expected for VFFw and VFCWw since summer temperatures are optimal ones for earthworms. Furthermore, those temperatures are also optimum ones for different microorganisms (Arora and Kazmi, 2015) and therefore the highest organic matter decomposition rate. Similar situation can also be observed for TSS (Figure 2).

<insert: Figure 2 – Seasonal removal percentages of COD, TSS and E. coli in the vertical flow systems>

E. coli removal was positively affected by the presence of earthworms during the summer, as was also observed by Arora and Kazmi (2015). It is very likely to be a consequence of increased earthworm activity at their optimum temperatures. For all four systems, summer removal was higher than the spring removal and that also corresponds to the results obtained by Arora and Kazmi (2015). Contrary to some other studies (Molleda et al, 2008), *E. coli*

removal was noticeably higher during the winter time. However, retention time of the systems used in this study was 48 hours during this period, double the time during the other three seasons. Hence increased removal of *E. coli*.

TN removal for all the systems was higher during the warmer part of the year (spring and summer) (Figure 3) as growth of nitrifying bacteria is higher in the higher temperatures. Moreover, TN removal was slightly higher in the planted systems compared to the unplanted ones during the growing season (spring and summer) and that can be attributed to the plant uptake. Finally, higher TN removal in VFCWw than in VFCW during the warmer part of the year can be explained by higher plant growth due to the presence of earthworms and consequently higher nitrogen uptake. From the Figure 3 it can be seen that the phosphorus removal was highest in the spring and lowest in the winter, and, since phosphorus removal is mostly a physical process (Kadlec and Wallis, 2009), that is probably a consequence of a big difference in inflow concentration as previously explained.

<insert: Figure 3 – Seasonal removal percentages of TN and TP in the vertical flow systems>

3.4. Growth of earthworms in CWs

Mass and number of earthworms have reduced for the both systems (control one and SVFw) during the experimental period (Table 3). It can probably be attributed to the lack of the organic matter due to the limited space and short time of operation. However, reduction was much lower for SVFw, since the control systems' substrate partly consisted of organic matter and consequently it had conditions more favourable for the earthworms' growth. Therefore, it can be said that filters treating wastewater are not the environment in which earthworms can

live for a long time without constant addition of the new individuals as was done in the VFFw and VFCWw.

<Table 3 – Growth of earthworms in the reactor and their effect on the substrate >

Moreover, the presence of earthworms did not affect organic matter content of the substrate that was 1.05% and 1.00% for SVF and SVFw, respectively (Table 3). Some other studies agree on the fact that earthworms can reduce or prevent clogging problems that CWs could have as they translocate subsurface clog matter towards the surface. Therefore, application of these invertebrates seems to be the cheapest solution for the restoration of the clogged systems (Nivala et al, 2012). Different results obtained by this study could be explained by the fact that organic matter content of the substrate was not very high as the filters did not operate for a long time before addition of earthworms. Results might have been different if the systems were functional for some time before and therefore substrate organic matter content would have been higher.

4. Conclusions

Although small differences were found between the four systems during one year of operation, statistical analysis did not confirm any significant difference. Therefore, it can be concluded that neither plants neither earthworms affected the treatment. However, the presence of earthworms positively influenced plant growth in CWs. Further experiments also showed that the filters treating wastewater are not a suitable living environment for the earthworms, and they did not reduce organic matter content of the filter substrate.

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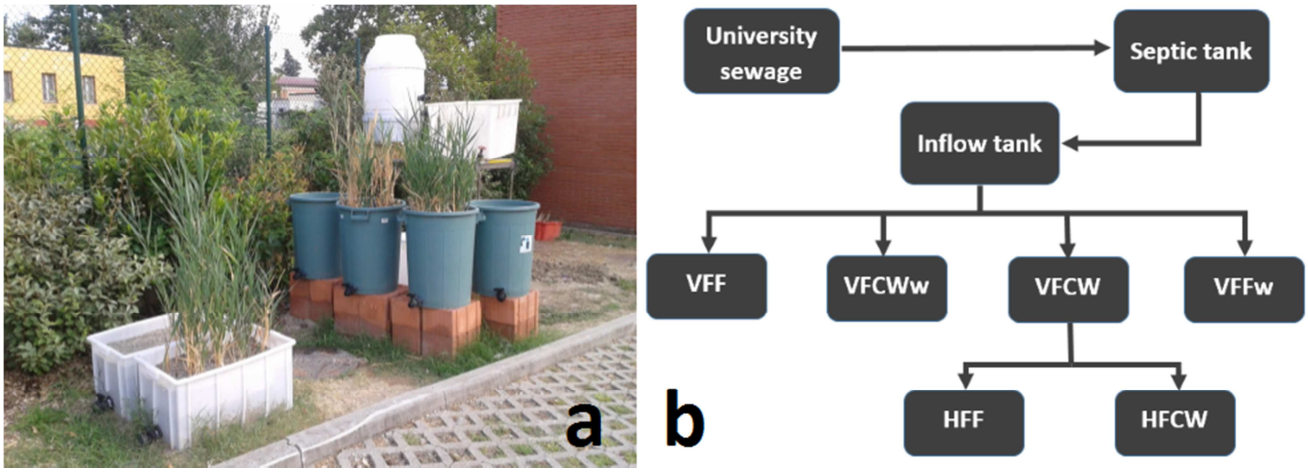

Figure 1 – Pilot plant during the construction phase (a) and water flow in the system (b)

Table 1 – Experimental periods (the temperature values are given in the form: mean±st. error (n))

	Beginning date	Ending date	Average air temperature (°C)
Autumn	20th October 2015	20th November 2015	12.5±0.3 (32)
Winter	21st November 2015	17th March 2016	6.9±0.2 (115)
Spring	18th March 2016	19th June 2016	18.3±0.4 (94)
Summer	20th June 2015	14th September 2016	27.0±0.3 (87)

Table 2 - Performance of the pilot plant during the period October 2015-October 2016 (except for pH, the values are given in the form: mean±st. error (n)).

	Influent	VFF		VFFw		VFCWw		VFCW	
		Value	Re (%)	Value	Re (%)	Value	Re (%)	Value	Re(%)
COD (mg L^{-1})	886±74 (13)	300±42 (15)	*66*	308±39 (14)	*65*	283±39 (15)	*68*	263±32 (15)	*68*
TSS (mg L^{-1})	168±28 (10)	31±4 (18)	*81*	31±5 (16)	*82*	31±4 (19)	*81*	33±4 (18)	*80*
pH	6.65 (7)	7.40 (11)	-	7.45 (11)	-	7.12(11)	-	7.18 (12)	-
TN (mg L^{-1})	65±6 (18)	34±6 (16)	*48*	36±6 (16)	*45*	33±7 (17)	*50*	33±6 (17)	*49*
NO_3^--N (mg L^{-1})	0.85±0.12 (19)	0.99±0.41 (17)	*-14*	0.42±0.12 (17)	*51*	0.39±0.08 (18)	*54*	1.16±0.60 (18)	*-36*
NO_2^--N (mg L^{-1})	0.03±0.03 (19)	0.02±0.02 (17)	*24*	0.13±0.13 (17)	*-77*	0 (18)	*-54*	0 (18)	*-24*
TP (mg L^{-1})	12.32±2.83 (17)	6.83±1.17 (15)	*45*	7.04±1.43 (14)	*43*	6.66±1.14 (15)	*46*	6.50±1.18 (15)	*47*
PO_4^{3-}-P (mg L^{-1})	7.63±1.43 (17)	5.55±0.79 (14)	*26*	5.48±0.90 (15)	*27*	5.36±0.93 (15)	*28*	4.93±0.83 (15)	*34*
Cl^- (mg L^{-1})	78±5 (19)	81±11 (17)	*-4*	76±7 (17)	*2*	79±7 (18)	*-1*	77±7 (18)	*1*
Br^- (mg L^{-1})	2.56±0.35 (19)	1.68±0.27 (17)	*34*	4.51±2.50 (17)	*-76*	6.32±4.09 (18)	*-59*	1.72±0.26 (18)	*33*
SO_4^{2-} (mg L^{-1})	69±5 (19)	53±8 (17)	*23*	66±9 (16)	*3*	59±9 (18)	*14*	66±9 (18)	*3*
E. coli (10^4 CFU 100 mL^{-1})	48.69±10.71 (10)	10.31±3.11 (10)	*79*	7.27±1.75 (9)	*85*	12.46±3.66 (10)	*74*	12.34±3.67 (10)	*75*
Total coliforms (10^4 CFU 100 mL^{-1})	70.18±64.95 (4)	5.24±2.08 (10)	*93*	3.92±1.33 (9)	*94*	6.78±2.97 (10)	*90*	6.51±2.53 (10)	*91*
Enterococcus (10^4 CFU 100 mL^{-1})	32.15±3.92 (10)	13.75±4.72 (10)	*57*	15.89±6.05 (9)	*51*	15.78±5.61 (10)	*51*	16.04±5.79 (10)	*50*

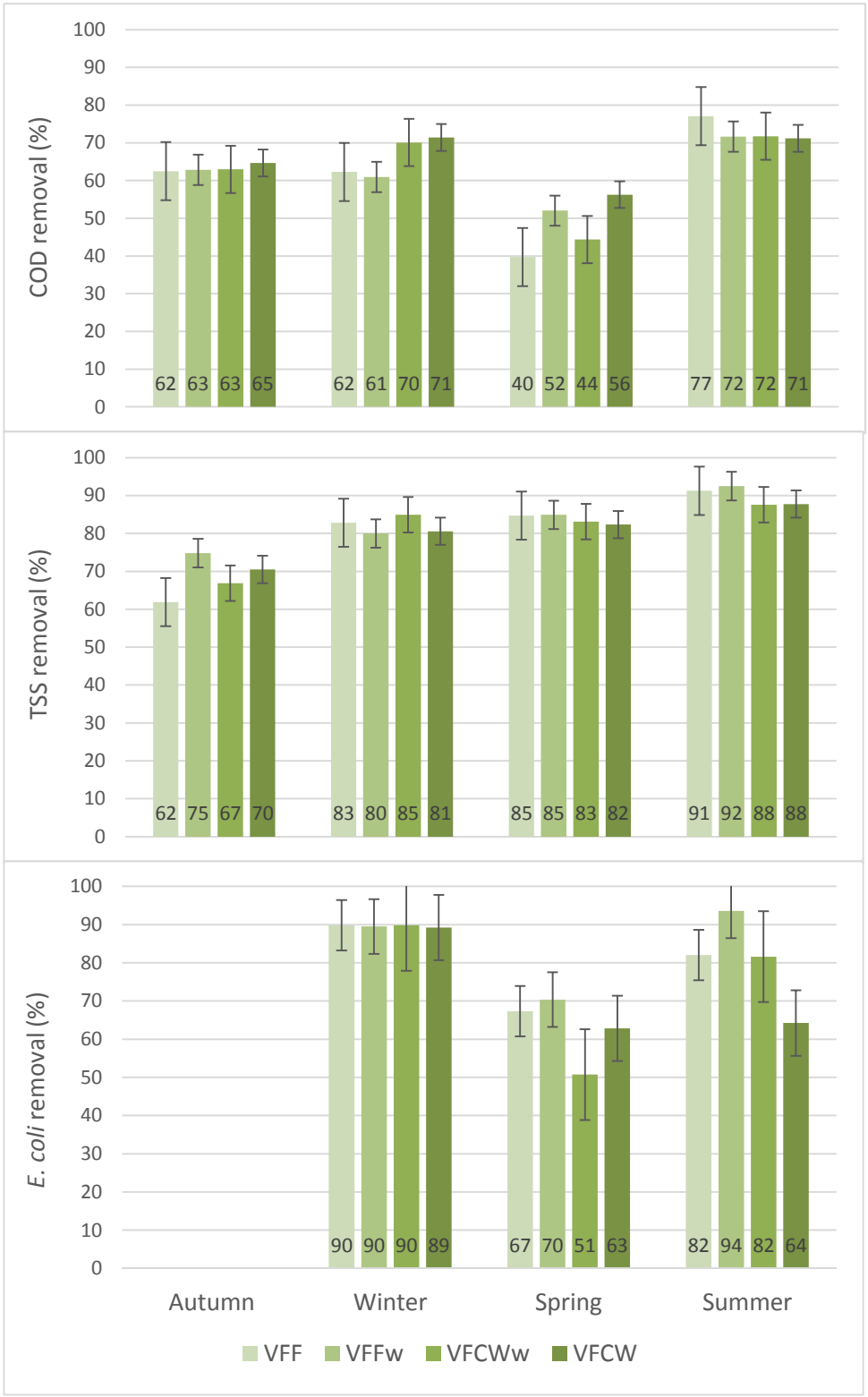

Figure 2 – Seasonal removal percentages of COD, TSS and *E. coli* in the vertical flow systems (autumn values for *E. coli* are missing due to technical problems).

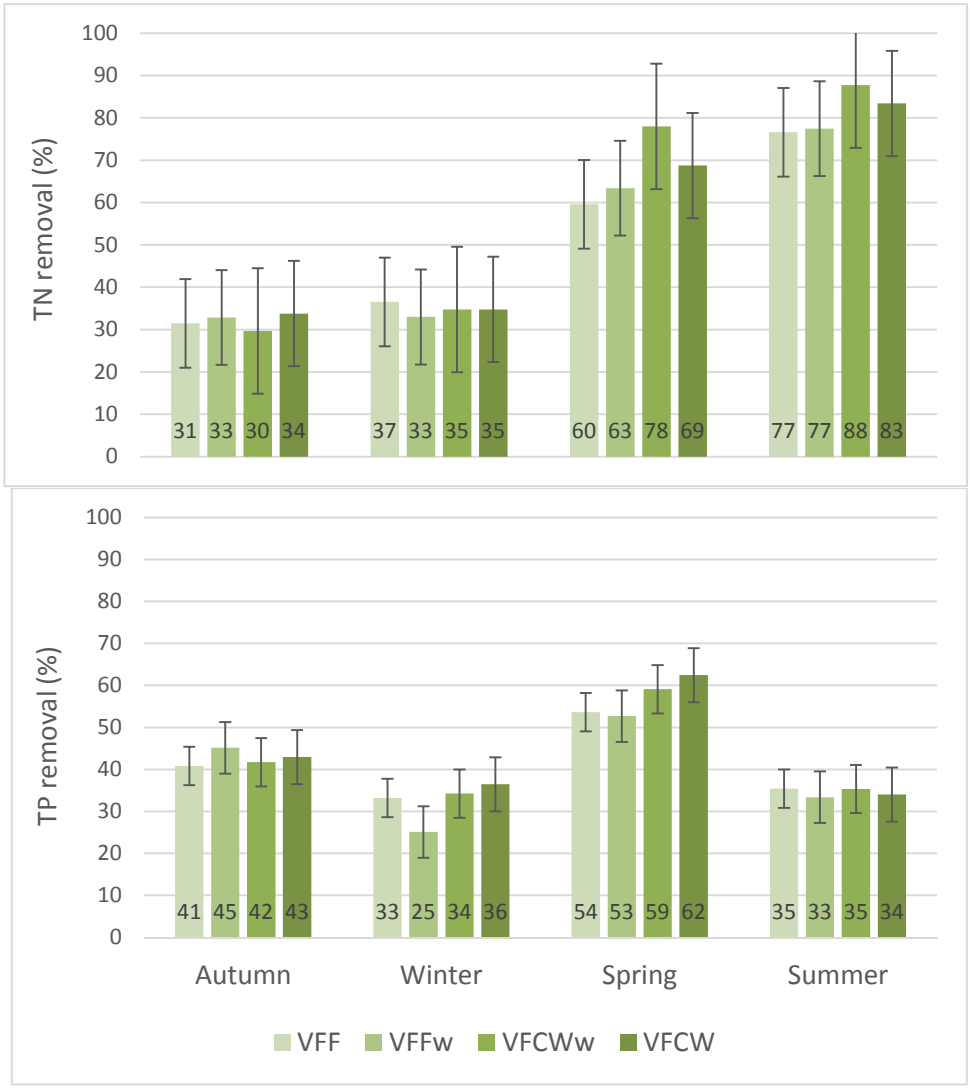

Figure 3 – Seasonal removal percentages of TN and TP in the vertical flow systems

Table 3 – Growth of earthworms in the worm reactor and their effect on the substrate

		5th May	19th May	7th June	14th July
SVF	Earthworm number	-	-	-	-
	Earthworm mass (g)	-	-	-	-
	OMC (%)	1.08	-	-	1.05
SVFw	Earthworm number	17	13	6	1
	Earthworm mass (g)	3.051	2.451	0.990	0.155
	OMC (%)	1.08	-	-	1.00
Control system	Earthworm number	17	16	17	7
	Earthworm mass (g)	3.041	2.086	2.107	1.072
	OMC (%)	-	-	-	-
Temperature (°C)	mean±st. error (n)	-	18.2±0.4 (15) *	22.0±0.4 (19)	26.0±0.6 (37)

*Average daily temperature in the period 5th May-19th May; SVF - Small Vertical Flow Filter; SVFw - Small Vertical Flow Filter with earthworms; OMC – Organic Matter Content

CHAPTER 6

APPENDICES

Appendix 1

European Working Group for *Legionella* Infections Sequence-Based Typing (SBT) protocol for epidemiological typing of *Legionella pneumophila*

European Working Group for *Legionella* Infections

Sequence-Based Typing (SBT) protocol for epidemiological typing of *Legionella pneumophila*

Version 4.2

SUMMARY

This procedure describes the European Working Group for Legionella Infections (EWGLI) method for Sequence-Based Typing of *Legionella pneumophila*. Genomic DNA is extracted then amplified using primers targeting seven specific gene loci (i.e, *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*). Following purification, amplicons are sequenced directly with forward and reverse primers, and the resulting consensus sequences trimmed and compared to previously assigned allele numbers using the online database. Using a pre-determined order (i.e., *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*), the combination of alleles is defined as a 7-digit allelic profile (e.g. 1,4,3,1,1,1,1) and a sequence type represented by a number (e.g., ST1). Putative new allele types can be submitted following the instructions on the website. This method can be used in the epidemiological typing of *L. pneumophila*.

DNA extraction:

The following methods have been found to be reliable and the first two are rapid:

(i) BIO-RAD InstaGene Matrix (Catalogue no. 732-6030), using 5-10 μl supernatant as template DNA in the primary PCR amplification,

(ii) Heating emulsified colonies in 0.5 ml sterile water at 100°C for 8 minutes, using 5-10 μl lysate as template DNA in the primary PCR amplification,

(iii) Nucleon BACC2 DNA extraction kit (Amersham Pharmacia Biotech), using 10-100 ng DNA as template DNA in the primary PCR amplification

SBT targets:

We recommend that all 7 loci (i.e. *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*) are determined if possible.

Amplification primers:

The positions of the primers with respect to the GenBank accession number of the reference sequence are shown below:

Gene	Primer name[1]	Position[2]	Primer sequence (5'-3')	Annealing temperature
flaA	flaA-587F	568-587	GCG TAT TGC TCA AAA TAC TG	55 °C
	flaA-960R	981-960	CCA TTA ATC GTT AAG TTG TAG G	
pilE	pilE-35F	12-35	CAC AAT CGG ATG GAA CAC AAA CTA	55 °C
	pilE-453R	471-453	GCT GGC GCA CTC GGT ATC T	
asd	asd-511F	487-511	CCC TAA TTG CTC TAC CAT TCA GAT G	55 °C
	asd-1039R	1062-1039	CGA ATG TTA TCT GCG ACT ATC CAC	
mip	mip-74F	58-74	GCT GCA ACC GAT GCC AC	55 °C
	mip-595R	616-595	CAT ATG CAA GAC CTG AGG GAA C	
mompS	mompS-450F	430-450	TTG ACC ATG AGT GGG ATT GG	55 °C
	momp-1126R	1140-1126	TGG ATA AAT TAT CCA GCC GGA CTT C	
proA	proA-1107F	1090-1107	GAT CGC CAA TGC AAT TAG	55 °C
	proA-1553R	1570-1553	ACC ATA ACA TCA AAA GCC	
neuA	neuA-196F	176-196	CCG TTC AAT ATG GGG CTT CAG	55 °C
	neuA-611R	634-611	CGA TGT CGA TGG ATT CAC TAA TAC	

[1] the number in the primer name is the position in the reference sequence where the 3'-terminus of the oligonucleotide binds

[2] shows the binding positions of the primer with respect to the reference sequence on the website and table

Sequencing primers:

As above except that the *mompS*-1015R primer is used for the reverse sequencing reaction of *mompS*.

Primer name[1]	Position[2]	Primer sequence (5'-3')
mompS-1015R	1032-1015	CAG AAG CTG CGA AAT CAG

Please note that the *mompS* forward primer (mompS-450F) is a new standard primer and **MUST** be used for the primary amplification and sequencing of the *mompS* target:

for primary amplification:
mompS-450F: 5'-TTG ACC ATG AGT GGG ATTG G-3'
mompS-1126R: 5'-TGG ATA AAT TAT CCA GCC GGA CTT C-3'

for sequencing:
mompS-450F: 5'-TTG ACC ATG AGT GGG ATT GG-3'
mompS-1015R: 5'-CAG AAG CTG CGA AAT CAG-3'

Fragment sizes of amplified products and regions used for allele assignment

Gene	Fragment size of amplified product (bp)	Size in nucleotides of region used to determine allele type	Region used for allele assignment	GenBank accession number of reference sequence
flaA	394	182	653-749	X83232
pilE	459	333	103-435	AF048690
asd	575	473	538-1010	AF034213
mip	558	402	117-518	AJ496265
mompS	710	352	523-1010	AF078136
proA	480	405	1134-1230	M31884
neuA	459	354	229-583	A6017354

Oligonucleotide storage and handling recommendations:

Appropriate storage of oligonucleotides will extend their shelf life. Most oligonucleotides are supplied dry (lyophilised) and manufacturers (e.g., MWG Biotech AG, Germany) usually supply detailed information on appropriate resuspension and storage conditions. Information on data-sheets and instructions from the manufacturers provided with the oligonucleotides should be read, noted and followed. To maximise the shelf-life of oligonucleotides it is recommended that concentrated "stock solutions" (e.g., 100pmol/µl) are made in 1x nuclease-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5-8.0) and stored below -20°C, and dilute "working solutions" (e'g', 10 pmol/µl) made in nuclease-free 10 mM Tris pH 7.5-8.0. Working solutions are frozen in 50 µl aliquots and should **NOT** be subjected to repeat freeze-thaw cycles and must be kept for **no longer than one week at 4°C** before discarding.

Primary amplification:

PCR amplification is performed in a total volume of 50 µl. Place PCR plate or tubes on a cold block. Start with adding 10 µl of Taq DNA polymerase (2.5 units/reaction) to each PCR tube or 96 well plate, then add 35 µl of the master mix, finally add the DNA template (5 µl /tube or /well). Gently spin tubes/plates briefly in an appropriate centrifuge before placing them in a thermal cycler heating block.

PCR reaction mix

Reagent	Stock concentration	Final concentration	Volume/reaction (µl)
PCR buffer	10 x	1 x	5.0
$MgCl_2$	50 mM	2.5 mM	2.5
Primer 1	10 pmol/µl	10 pmoles	1.0
Primer 2	10 pmol/µl	10 pmoles	1.0
dNTPs	5 mM	200µM	2.0
Nuclease-free water			23.5
Volume/reaction			35.0

Prepare *Taq* DNA polymerase by adding:

Nuclease-free water	9.0 µl
PCR buffer (x10)	0.5 µl
Taq DNA polymerase (5U/µl)	0.5 µl
Volume/reaction	10.0 µl

Controls

For each PCR run include a negative (5 µl of nuclease-free water) and a positive control (5 µl of known DNA template). Each sample is amplified in duplicate. Pipette solutions in the order of: negative control, test samples, positive control.

Thermal cycler parameters

Step	Temperature (ºC)	Time		No. cycles
1	95	5 min	Initial denaturation	1
2	95	30 sec	Denaturation	35
	55	30 sec	Annealing	
	72	40 sec	Extension	
3	72	10 min	Final extension	1
4	12	∞	Hold	

Analysis of purified PCR products by E-Gel electrophoresis

Run PCR products (2 µl) + 1x BlueJuice (Invitrogen) (18 µl). Also run E-Gel low range quantitative DNA ladder (Invitrogen) (10 µl) + nuclease free water (10 µl). After 20 minutes of run, the E-Gel is analysed using the UVP gel imaging system. If positive and negative controls as well as the test sample results are satisfactory, proceed with the DNA sequencing reaction.

DNA Sequencing using the CEQ 8000 Genetic Analysis System (Beckman Coulter)

Primary PCR amplicon purification

PCR products are purified using: Montage PCR_{96} filter plates (Millipore); Qiaquick PCR Purification Kit (Qiagen); Wizard PCR Preps Purification System (Promega) or equivalent.

DNA Sequencing materials

Dye Terminator Cycle Sequencing is undertaken using the DTCS Quick Start Kit (Beckman Coulter). Follow the manufactures instructions carefully. The Sample Loading Solution contains formamide. Formamide waste (i.e., sequencing reactions and plates) should **NOT** be autoclaved and must be disposed for direct incineration.

The CEQ DTCS Quick Start Kit contains the following reagents (sufficient for 100 reactions):

1) Quick Start Mix (800 µl)
   dATP, dCTP, dTTP, dITP
   ddUTP, ddGTP, ddCTP, ddATP (WellRED label)
   Tris-HCl, $MgCl_2$, reaction buffer - pH 8.9
   Thermo Sequenase DNA Polymerase
   Pyrophosphatase
2) 47 Sequencing Primer (240 µl)
3) pUC18 Control Template (20 µl)
4) Glycogen (110 µl)
5) Mineral Oil (5 ml)
6) Sample Loading Solution (SLS) 6 ml

DNA Sequencing procedure

DNA sequencing reactions are prepared **following the manufacturers instructions** by combining the following:

1) PCR-grade water (e.g., Nuclease-Free Water, W4502, Sigma)
2) DNA template
3) User supplied sequencing primer or (-) 47 Sequencing Primer (1.6 pmol/µl or 1.6µM)
4) DTCS Premix (Beckman Coulter)

N.B. Can run half-volume reactions (total of 10µl/reaction).

Thermocycler parameters (for DNA sequencing):

Step	Temperature (°C)	Time		No. cycles
1	96	20 sec	Denaturation	30
	50	20 sec	Annealing	
	60	4 min	Extension	
2	4	∞	Hold	

N.B. The above parameters are those recommended for the CEQ 8000 Genetic Analysis System (Beckman Coulter). For alternative sequencing platforms the relevant manufacturer's recommendations should be followed.

Ethanol precipitation is carried out as per manufacturer's instructions using a suitable centrifuge. For ethanol plate precipitation in a CEQ sample plate, please refer to the "Dye terminator cycle sequencing chemistry protocol", page 13. For ethanol precipitation in 1.5 ml eppendorf tubes, please refer to the manufacturer's instructions provided with the DTCS Quick Start Kit (Beckman Coulter).

N.B. for half volume reactions add 10 µl of H2O, before proceeding to ethanol precipitation.

Sample preparation for loading into the CEQ

Add 55 µl of Sample Loading Solution to each well, leave on plate-shaker for 10 minutes. Overlay each re-suspended sample with one-drop of mineral oil.

From this point follow the instructions provided by the manufacturer of the Sequencing Instrument.

Sequence analysis:

EWGLI Sequence Quality Tool

Forward and reverse sequence trace files (.scf or .abi) of all seven targets are submitted to the "**Sequence Quality Tool**" which can be accessed from the EWGLI website (www.ewgli.org). This tool assembles contig(s) from the traces, finds start and end (reference) positions in the contig, trims the contig using these positions and finally matches the trimmed sequence against those in the SBT database. The tool identifies individual alleles as well as an allelic profile and a sequence type (ST). Sequences with <100% match are identified as -1, and the position of mismatches are also indicated. Sequences of poor quality should either be repeated or further analysed using a DNA analysis software e.g., BioNumerics (Applied Maths).

EWGLI SBT Database

- Data from both forward and reverse sequencing reactions are combined and aligned to produce a consensus sequence. Reference sequences trimmed to the correct length can be downloaded from the website in order to aid contig assembly. Consensus sequences trimmed to the correct length are submitted to the SBT database as flat text file. The database returns an allele type eg., 1, when submitted sequence shows a 100% match to a pre-designated allele type. Sequences with <100% match are identified as the closest match to a pre-existing allele type with the number of mismatches specified. The SBT database also returns an alignment with mismatches highlighted.

- For each isolate, the combination of alleles at each of the loci is defined as the allelic profile using a pre-determined order, i.e., *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*. For example, for strain EUL no. 120, the allelic profile is 4,7,11,3,11,12,9.

- If an individual allele number has not been determined, a zero is entered into the allelic profile, thus maintaining its integrity. For example, if the *proA* allele number was not determined for the examples above, the profile would be 4,7,11,3,11,0,9 and if the *mompS* allele was not determined, it would be 4,7,11,3,0,12,9.

Instructions for accessing the EWGLI SBT Website

- Go to (www.ewgli.org)
- Click the "**Typing and identification schemes**" button:

Follow the link for the EWGLI Sequence-Based Typing (SBT) Database for *Legionella pneumophila*

Instructions for submitting sequence data to the EWGLI Sequence Quality Tool

- Under Query Functions, click on the **"Sequence Quality Tool"**
- Choose the number of sequence trace files to be uploaded.
- Click on the **"Browse"** button to download sequence files.
- Forward and reverse sequences must be downloaded for each allele in standard file format (*.scf) or ABI trace file format (*.abi).
- Click the **"Analyse Trace Files"** button.
- The tool also produces a sequence quality report for each uploaded contig and generates a seven-figure allelic profile (e.g., 3,4,1,1,14,9,1), as well as indicating whether the obtained allelic profile is of a novel combination. The tool also identifies a single designated sequence type (ST) for a pre-existing allelic profile.
- You can print a summary sheet by clicking the link: "**Print Friendly Summary**"
- For further details on the sequence quality tool please click on this link: "**explanation and frequently asked questions**"

- Putative novel alleles must be submitted for verification following the link **"New Allele Submission"**.
- To submit a novel allelic profile click on the link **"Strain Data Submission"**.

Instructions for submitting sequence data to the EWGLI SBT Database

- Under Query Functions, click on the **"Check a sequence for it's allele number"**
- Before pasting in a query sequence the user must ensure that the consensus sequence is trimmed to the correct length.
- Reference sequences trimmed to the correct position and length can be downloaded to aid contig assembly.
- Consensus sequences of the correct length can be submitted for more than one target, alternatively you can submit consensus sequences one at a time before hitting the **"upload sequence"** button.
- Please note that sequences of incorrect length will be rejected.
- Sequences of correct length and with a 100% match to a sequence in the database will return an allele number e.g., "1".
- Sequences of correct length and with <100% match to a sequence in the database will return an alignment with the closest matches showing where differences are.
- To enter consensus sequences for another strain, click the "**Reset form**" button and proceed as before.

N.B. The website is under constant development, so please see the website for any changes, new tools and to ensure you are using the latest version of this protocol etc.

References

Ratzow S, Gaia V, Helbig JH, Fry NK, Lück PC (2007). Addition of *neuA*, the gene encoding N-acylneuraminate cytidylyl transferase, increases the discriminatory ability of the consensus sequence-based scheme for typing *Legionella pneumophila* serogroup 1 strains. *J Clin Microbiol* **45**,1965-1968.

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Gaia V, Fry NK, Harrison TG, Peduzzi R (2003). Sequence-based typing of *Legionella pneumophila* serogroup 1 offers the potential for true portability in legionellosis outbreak investigation. *J Clin Microbiol* **41**, 1491-1502.

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Appendix 2

Sequence-based identification of *Legionella* using the macrophage infectivity potentiator (*mip*) gene

Sequence-based identification of *Legionella* using the macrophage infectivity potentiator (*mip*) gene (Version 2.0)

(adapted from Ratcliff *et al* 1998, J. Clin. Microbiol. **36**:1560-67)

Bacterial culture conditions. Cultures were grown on buffered charcoal yeast extract agar in humidified conditions at 37°C for 4 to 7 days. Genomic DNA was extracted using a commercial kit or by boiling for 5 min and using 2 µl of crude extract for amplification.

Strategy

Oligonucleotide primers targeting the ribosomal binding site or open reading frame region and the PPIase site of the *mip* gene (Legmip_f and Legmip_r) were designed to amplify *c*. 661 to 715 bp product. These primers require redundancies to account for variation at the third codon site. Following amplification the PCR product is purified and sequenced directly using the forward sequencing primer (Legmip_fs). The sequence obtained is compared to the Legionella *mip* gene sequence database which contains a complete set of available quality controlled sequences.

Oligonucleotide primers

Three primers are required, two for amplification and one for sequencing. Parentheses indicate sites with mixed bases and the sequence using IUB nomenclature is given underneath.

1. Forward amplification primer

Legmip_f (27-mer)

5'-GGG (AG)AT T(ACG)T TTA TGA AGA TGA (AG)A(CT) TGG

5'-GGG RAT TVT TTA TGA AGA TGA RAY TGG

2. Reverse amplification primer

Legmip_r (23-mer)

5'-TC(AG) TT(ATCG) GG(ATG) CC(ATG) AT(ATCG) GG(ATCG) CC(ATG) CC

5'-TCR TTN GGD CCD ATN GGN CCD CC

3. Forward sequencing primer

Legmip_fs (26-mer)

5'-TTT ATG AAG ATG A(AG)A (CT)TG GTC (AG)CT GC

5'-TTT ATG AAG ATG ARA YTG GTC RCT GC

PCR amplification and sequencing.

Reaction mixtures were in a total volume of 50 µl and contain 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM each deoxynucleotide, 20 pmol each primer (Applied Biosystems/MWG Biotech Ltd/Operon), and 2.5 U *Taq* polymerase (Applied Biosystems/Life Technologies). Template DNA (*c.* 10-100 ng) was added and reaction mixtures with no added DNA served as negative controls. Amplification was performed in a thermocycler (MJ DNA Engine, MJ Research) under the following conditions: pre-denaturation for 3 min at 96°C, then 35 cycles consisting of denaturation for 1 min at 94°C, annealing for 2 min at 58°C, extension for 2 min at 72°C, followed by a final extension for 5 min at 72°C. Reaction mixtures were then held at 4°C.

Analysis of PCR products

5µl of the total 50µl PCR reaction mix was analysed by gel electrophoresis in 2% agarose gels stained with 0.5 to 1.0 µg/ml ethidium bromide and photographed under UV transllumination using Polaroid film.

Purification of PCR products

Amplification products were purified using QIAquick PCR purification kit (QIAGEN)/Wizard PCR Preps purification system (Promega Corp.) according to the manufacturers' instructions.

Sequence determination

Nucleotide sequence was determined by cycle sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (PE Applied Biosystems)/ Dye Terminator Cycle Sequencing kit (Beckman Coulter).