UNIVERSITA' DEGLI STUDI DI ROMA

"LA SAPIENZA"

DOTTORATO DI RICERCA

SCIENZE FARMACEUTICHE

XXIII Ciclo

"Evaluation of the effectiveness of some disinfectants against microbiological contamination and biofilm in hospital water systems"

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Anno accademico 2019/2020

The water cycle and the life cycle are one

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Preface

Water quality has a direct link to the protection of human health.

The awareness of the importance of the quality of water intended for human consumption has been affirmed thanks to the development of principles of risk assessment related to the presence of natural or anthropogenic substances (such as disinfection byproducts); in fact, water intended for human consumption is healthy, safe, not contaminated by microorganisms, parasites and substances in concentrations high enough to represent a danger to the health of users.

Safeguarding the quality of drinking water is absolutely essential, especially in particularly critical situations, such as in hospitals and in other healthcare facilities. In fact, these facilities represent a very delicate environment, both from the structural and architectural point of view and for the type of users served, more susceptible to environmental risk factors. In these facilities, in fact, a contamination of the water may represent a health hazard and a potential risk for exposed people, already characterized by conditions of immunosuppression.

A water network that is not protected from possible microbiological contaminations, either because it is not subjected to appropriate disinfection and maintenance interventions, or because of its intrinsic structural characteristics, can therefore determine the exposure to a water lacking the quality requirements.

The bacterial species *Legionella* spp. has been frequently found in drinking water distribution networks and, in particular, in the domestic hot water (HWS) distribution networks of hospitals.

Environmental surveillance of *Legionella* spp., therefore, remains one of the most effective strategies for the prevention and control of the risk of legionellosis for the protection of public health. In fact, it makes it possible to monitor contamination levels over time and to apply the most appropriate remediation measures from time to time.

The object of this study was to compare biocidal efficacy of two disinfectants, currently used in hospitals and hotels, monochloramine (NH_2Cl) and silver ion hydrogen

peroxide (H_2O_2-Ag^+) against the presence of *Legionella pneumophila* species in hot-water system and in biofilm formed in the same networks of two hospital facilities, named H1 and H2, in Italy.

CHAPTER 1
INTRODUCTION

1.1 Water Distribution Systems

1.1.1 Drinking Water Distribution Systems (DWDS)

Water is the essential component for life and therefore its safety is of fundamental importance. The concept of wholesome drinking water dates back to the 19th century, but it was not until the last century that adequate criteria for defining its quality were established. Water for human consumption must possess specific organoleptic characteristics and, above all, its use must not constitute a health risk. Water must therefore be free of pathogens and dangerous chemical substances. There are two main requirements to define the quality of a water: assess that the water is free from hazardous substances or dangerous microorganisms and verify that the water is healthy during its distribution in the network up to the tap. **(Bonadonna 2008)**

A water distribution system is a set of structures aiming at distributing water from one or several sources to consumers. From the point of view of a developed country, a distribution system is considered to work properly if it provides drinking water, with sufficient flow conditions, at any location of the system and at any time. However, ensuring such a service can be complex because it is highly dependent on water source(s), number of consumers to supply and on their geographical locations from the source(s) in terms of elevation and distance. **(Coron 2014)**

Materials used for treatment, supply and distribution of water intended for human consumption must not, over time, alter the quality water in contact with them, giving them harmful characteristics for health or modify their organoleptic, physical, chemical and microbiological qualities. **(Bonadonna 2008)**

Drinking water distribution networks (DWDNs), fig 1, is a complex infrastructures, consisting of hundreds of kilometers of pipes, storage tanks, pumps, valves and other important elements necessary for perfect operation **(Tsitsifli 2018)**.

Fig 1: Drinking Water Distribution Sysytem

The quality of the drinking water is conditioned by the structure and the state of the water networks used for distribution.

It is known that in all drinking water distribution systems (DWDS) the inner walls of pipes, storage tanks, sediments and all surfaces in contact with water are colonized by microorganisms, which can survive, grow and detach depending on the conditions. The release of microorganisms from the surfaces of the distribution systems into the water can be one of the causes of microbial contamination, responsible for the deterioration of the hygienic quality of drinking water **(Bonadonna 2009)**.

The integrity of well managed distribution systems is one of the most important barriers protecting drinking water from contamination **(WHS 2014)**.

It was noted that inadequate management of drinking water distribution systems have been associated with disease outbreaks. The causes of these outbreaks are related to the presence of chemical and microbial risks sometimes present in the network.

Drinking water distribution systems (DWDSs) can act as reservoirs for opportunistic pathogenic microorganisms, especially important in healthcare facilities.

In fact, although water disinfection processes are effective, it can contain low concentrations of several potentially pathogenic microorganisms of water origin. The water that passes through the water system maintaining a continuous flow does not allow microbial proliferation, but when the pressure of the primary system decreases to reach the domestic water network, the problem arises because often the structural

complexity of distribution networks, the time of construction of the plants and the type of pipes, fig 2, are not always optimal.

Fig 2: Water distribution network pipe

Moreover, the lack of or insufficient maintenance and disinfection could facilitate the colonization of microbial contaminants.

Under favorable environmental conditions, microorganisms multiply or remain viable for long periods of time within the biofilm that lines the inside of DWDS pipes. Many of these organisms are associated with infections occurring among susceptible patient populations. These organisms are transmitted by direct contact, ingestion of water, indirect contact (medical treatment devices), inhalation of aerosols generated by water and aspiration of contaminated water **(Williams 2013)**.

In aquatic environments, microorganisms present have the ability to adhere to solid surfaces and form biofilm **(Mulamattathil 2014)**.

In fact, microorganisms can grow in two different ways: in the form of single cells, floating freely in the culture medium (planktonic form) or as sessile aggregates closely adhering to biotic or abiotic surfaces (biofilm form) **(Brancatisano)**.

Microbial adhesion within drinking water distribution systems (DWD_S) reduces the quality of the water itself, increasing the corrosion rate of distribution networks and reducing the microbiological safety of the water product **(Simòes 2007)**.

The presence of biofilms in the pipelines of the water distribution networks is one of the currently recognized dangers that affect the microbiological quality of drinking water **(Codony 2002)**, also causing changes in the organoleptic characteristics.

1.1.2 Hospital Water Distribution Networks

Hospitals, nursing homes, hotels and other recreational facilities are buildings with large and complex water supply systems, including large hot water tanks. Stagnation of water and favorable temperatures lead to a strong proliferation of bacteria and *Legionella*. For this reason, such buildings are at greater risk of microbial contamination **(Preventing *Legionellae* in an Austrian hospital through a new water installation that complies with Austrian standard Önorm B5019)**.

In heath facilities, water is an essential element for hygiene and health of patients; depending on the activities that take place, the structure requires a significant amount of water every day for different purposes (human use, rehabilitation pools, sterilizers, equipment for dialysis, and/or irrigation). Factors that have the greatest impact on quantity of water used in hospitals are: number of beds, number and type of wards and units, and general services present within the structure. Several literature studies report that the amount of water used in hospitals is between a minimum of 200 and a maximum of 1200 L per bed per day **(D'Alessandro 2016)**.

Water safety in hospitals is a top priority and a constant challenge for healthcare **(Decker 2014)**.

Water is vitally important for both clinical and other areas of hospitals.

The study and management of problems relating to water quality in health facilities is a topic of great interest: in fact, the contamination of water intended for human consumption may represent a health hazard and a potential risk for users with chronic debilitating conditions and immunosuppression **(Bianchi 2013)**.

Hospitals represent critical environments for the coexistence of particular architectural, functional typologies complexity, distribution network deterioration (presence of "dead" branches, limestone deposits and biofilm) and the typology of users: a deficient population, suffering from health problems and sometimes with reduced immune defenses.

Water therefore represent one of the most effective vehicles to transfer pathogens.

In hospital, hot and cold water systems, fig 3, are the main sources of infection. In these structures the control of the water system by means of chemical and physical disinfection means assumes enormous importance which, although effective in timely interventions, do not guarantee a lasting effect over time.

Moreover, factors as temperature, configuration and age of the distribution systems, physicochemical constituent of the water and plumbing materials encourage microbial growth. Old components of pipeline systems, areas of stagnation, or low flow, dead-legs and storage tanks allow their survival and development. In addition, respiratory devices and nebulizers may also be the source of nosocomial disease **(Borella 2016)**.

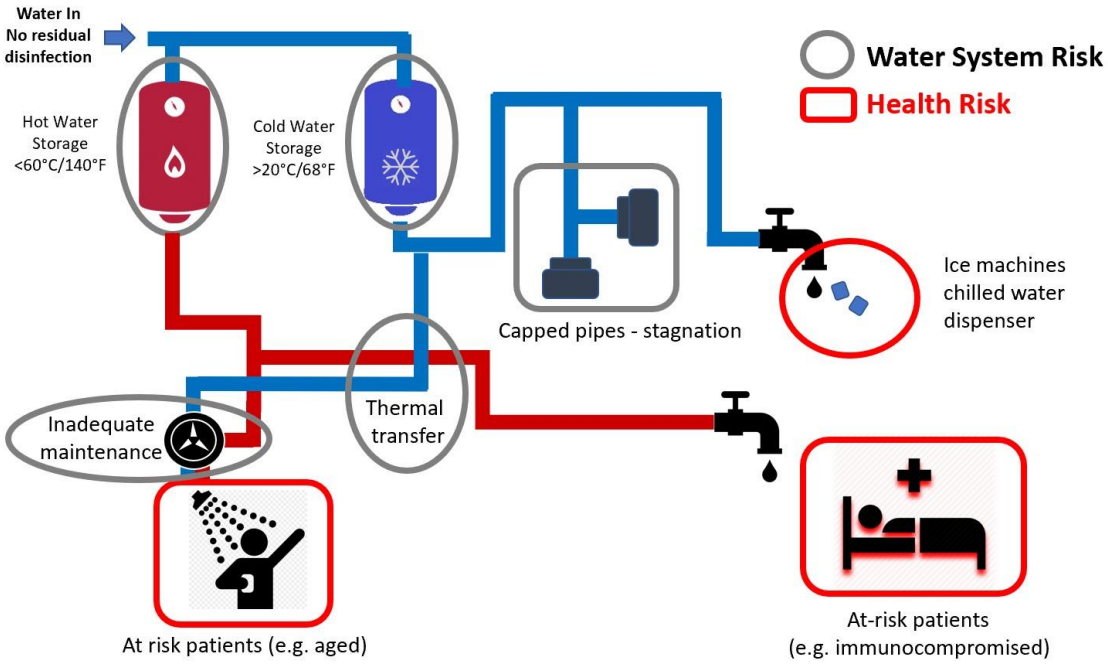


Fig 3: Hot and cold water systems

The water system within the hospital is the most frequent source of cases or outbreaks where patients may be at a higher risk for a severe infection. Contamination of the hospital water supply with potentially pathogenic organisms (*Legionella*, *Pseudomonas aeruginosa and* NTM) is very common worldwide, and is a well-known

risk factor for nosocomial infection. Water at the point of use (taps and showers) can be a source of transmission of water-based microorganisms.

The main cause of poor water quality is the accumulation, within the water network, of biofilms, of corrosion, the age of the system and stagnant water **(Shareef 2008)**.

The transmission of microorganisms responsible for nosocomial infections is a very frequent problem, affecting many countries, both developed and developing. Each year, approximately 2 million of nosocomial infections cause approximately 90,000 deaths **(Capelletti 2016)**.

1.1.3 Sanitary water distribution systems in hospitals

In the sanitary distribution systems, hot and cold water systems should be designed in such a way as to minimise or prevent conditions which permit the growth of microorganisms and biofilm formation and also to allow easy cleaning and disinfection.

To this end, certain principles must be respected.

Units or wards design is important to minimise the opportunity for water distribution system contamination (clinical hand wash sinkseasily accessible, splash containment, showers should be installed in a manner that encourages more frequent showering and in cabins that allow for adequate cleaning of surfaces and avoid water accumulation).

All materials, fixtures and fittings used in those systems should be certified.

Cleanliness is a prerequisite to precede subsequent disinfection. The frequency and method of routine cleaning should be identified during the risk assessment (cleaning of showerheads and hoses to remove scale and other deposits should be done at least quarterly or more frequently if necessary. Water storage tanks and hot water heaters shall be cleaned annually).

The use of disposable filters at the point of use provide a barrier to the passage of waterborne organisms.

Appropriate disinfection treatments. The effects of disinfection on planktonic bacteria differ significantly to the effects on sessile bacteria contained within biofilms. It has been estimated that 95% of all microbial cells present in drinking water distribution as biofilms that are adherent to pipe surfaces. Only 5% exist in the water phase. Ideal disinfection should determine :

1. Inactivate microorganisms in circulating water
2. Control/prevent/remove biofilm and inactivate associated biofilm microorganisms
3. Have minimal adverse effects on the water distribution network and be safe for human contact.

There are different types of disinfectants that are selected on the basis of certain defined requirements, as shown in Table 1.

Table 1: Factors that influence selection of the appropriate disinfection method for healthcare facilities

Age of the building
Layout of the water distribution system within the building
Pipework and materials used in the water distribution system
Uses of the building
Patient risk groups and high risk units within the facility
Source water characteristics
Degree of existing contamination
Need for systemic or focal disinfection
Availability of sufficient technical support and maintenance capacity
Availability and performance of dosing and monitoring equipment
Cost-effectiveness

In addition, disinfection treatments can be carried out in different ways, as reported in Table 2.

Table 2: Disinfection methods applied to healthcare facility water distribution systems

Method	**Disinfectant**
Systemic continuous	Temperature control regime
	Chlorine dioxide
	Monochloramines
	Copper-silver ionisation
	Electrochemically activated water
	Thermal disinfection (Superheat and flush)
Systemic intermittent	Shock hyperchlorination
	Shock chlorine dioxide

Focal continuous	Silver catalysed hydrogen peroxide UV Ozone

Systemic disinfection methods aim to disinfect the entire distribution system including distal outlets. Focal disinfection methods disinfect only a portion of the distribution system acting at the point of application with no residual effect.

Continuous secondary disinfection methods that may be employed in healthcare facilities may not respond effectively to sudden unanticipated significant contamination of the incoming water supply due to major disruptions or repairs.

Hot water system (HWS) is essential for major domestic sanitation services. This water can be produced by two different types of appliances:

1. Instant production systems (in which water is heated at the same time as the user's request occurs)
2. Storage systems (in which hot water is stored in special containers and released at the time of demand. These types of appliances, like boilers, enable large volumes of water to be heated. However, in storage systems, some fenomena such as calcareous precipitation, can compromise the good functioning of the system and then determine proliferation of dangerous bacterial species such as *Legionella pneumophila*)

A further distinction between instantaneous and storage systems lies in the mode of heat exchange:

1. Direct-type heat exchange (in which water is heated by the heat source, as natural gas, LPG, sun)
2. Indirect heat exchange (in which the water is heated indirectly by the energy produced by the heat source through a heat transfer fluid, such as coil boilers).

Sanitary water distribution systems must have separate lines for cold water (CWS) and hot water (HWS), adequately separated from each other and from other heat sources and, as pointed out by the Guidelines, and must be thermally insulated.

In the cold sanitary network the water temperature should remain, according to the indications of the Guidelines, below 20°C to avoid the proliferation of *Legionella* bacteria and to keep the water temperature below 20°C. It is also essential to evaluate the surrounding insulation thickness.

The insulation of the hot and cold water piping lines is aimed at limiting the dispersion of heat to the outside (in the case of hot water distribution) and at avoiding the temperature increase of the fluid (in the case of distribution of cold water) **(Idraulica 2017)**.

The protection of the HWS network from *Legionella* can be provided by following the indications of the Guidelines, first of all for an adequate temperature and by carrying out chemical or physical disinfection treatments.

“All new hot water systems (HWS) must provide heated water, at a temperature not exceeding 45°C for centers such as early childhood schools, primary and secondary schools and nursing homes or similar facilities for young people, the elderly, the sick or people with disabilities; and HWS at a temperature not exceeding 50°C in all the others buildings."

Inside the systems they are also installed systems to verify that hot water is maintained at an adequate level (> 60°C) to avoid the proliferation of *Legionella* in the storage tanks, and that the delivery temperature to the user does not exceed 50°C.

However there are some limits, in fact maintaining the temperature at high temperatures as well as presenting the risk of burns for users, needs of a high energy requirement **(Cloutman-Green 2019)**.

Chemical disinfection treatments, on the other hand, tend to modify the chemical characteristics of the water and could make it aggressive and non-compliant with the quality requirements of current regulations on water intended for human consumption.

Physical or thermal treatments, on the other hand, act only by modifying the water temperature and can be of two types, either thermal shock (temporary disinfection) or thermal disinfection (preventive and systemic measure) **(Idraulica 2017)**.

1.2 Waterborne and nosocomial infection

1.2.1 Waterborne infections

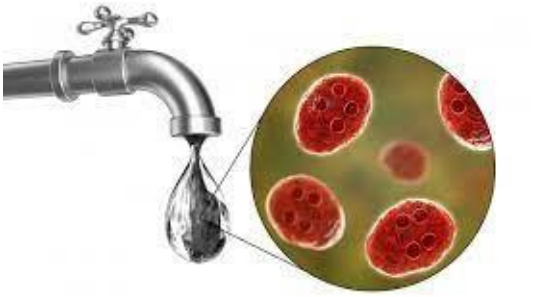


Fig 4: Microorganisms present in drinking water

Waterborne diseases are infections caused by the ingestion of (or, more generally, contact with) water contaminated with pathogenic organisms, fig 4, which are micro- (protozoa, bacteria, viruses, algae) and macro-parasites (helminths such as flatworms and roundworms).

They still pose a serious threat to the human health, especially in developing countries. Cholera and typhoid fever are among the best known examples of life-threatening waterborne diseases. In addition, diarrhea, commonly associated with waterborne pathogens are responsible for the deaths of approximately 1.5 million people each year, mostly infants and children in developing countries. Unsafe water supplies, lack of sanitation and poor sanitation, are crucial factors in the spread of waterborne infections **(Mari 2019)**.

Water quality problems are related to microbiological and chemical contaminants that may result from contamination occurring at the water source or due to deterioration in the water system. For these reasons, it is important optimize technical solutions aimed at improving water quality, as well as monitoring programs they must be performed regularly to prevent and limit any dangerous situations **(Bigoni 2014)**.

Waterborne pathogens of concern to humans have the following characteristics:

- are widespread in the environment in high concentrations, or are highly contagious to humans or even animals a low doses (such as protozoan cysts);
- they can survive and remain infectious in the environment for long periods, or they are highly resistant to water treatment;
- some types can multiply outside a host in favorable environmental conditions **(Funari 2012)**.

Potable WDSs contain a diverse microbial community of bacteria, protozoa and fungi. Investigations on bacterial populations in the North American and European WDS have shown that most systems contain a variety of *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Bacteroidetes* and *Planctomycetes*, most of which are considered non-pathogenic and unregulated. In these communities are also present several opportunistic pathogens, such as *Sphingomonas paucimobilus*, *Methylobacterium mesophilicum* and *M. extorquens* in α-proteobacteria, *Ralstonia pickettii*, *R. mannitolytica* and *Burkholderia cepacia* complex in β-proteobacteria, *Legionella* spp., *Pseudomonas* spp. and *Stenotrophomonas maltophilia* in γ-proteobacteria and environmental NTM in *Actinobacteria* **(Williams 2013)**.

Many epidemiological studies have shown that reduced water quality in domestic water systems is a cause in the transmission of waterborne diseases.

In the absence of adequate residual disinfectant, all water systems are vulnerable to bacterial proliferation.

In addition, other factors such as water temperature, the age of tanks and associated distribution pipes, the type of material used, the hydrodynamics of the system, and the chemical and microbial components of the water itself all influence the potential for microbial growth within water systems **(Campos 2003)**.

The most commonly occurring microorganisms in healthcare facility water systems which may result in clinical illness include: *Legionella* spp and *Pseudomonas aeruginosa* **(HPSC-Guidelines 2015)**.

1.2.3 Nosocomial waterborne infections

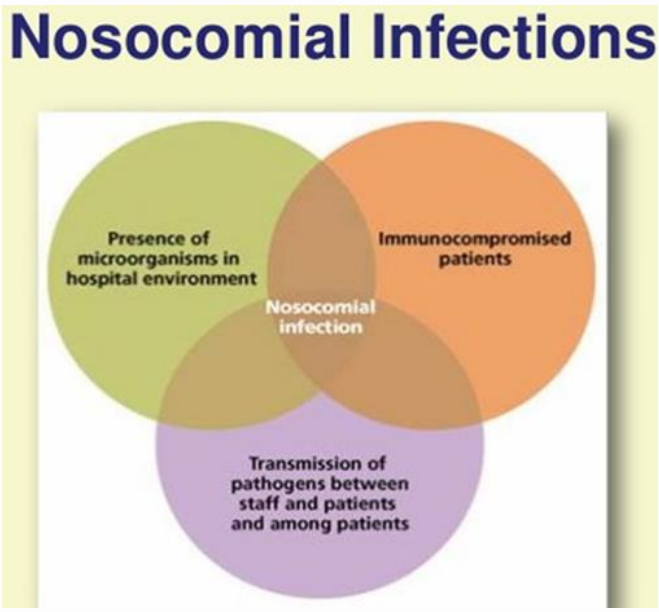


Fig 5: Factors influencing the spread of nosocomial infections

The term "nosocomial" refers to any infections contracted during a hospitalization: they are infections that occur during hospitalization or after discharge, depending on the incubation time. In general, infections that occur within 48 hours of hospitalization are considered nosocomial.

Many factors, as shown in figure 5 and 6, promote the phenomena of transmission of infection among hospitalized patients: first of all a reduced immunity of the patients themselves; the growing variety of medical procedures and invasive techniques that can represent potential routes of entry for a infection; and antibiotic resistance. Nosocomial infections occur all over the world and affect developed countries and developing countries. Infections acquired in health care facilities are among the leading causes of death and increased morbidity among hospitalized patients **(Prevention of hospital-acquired infections, WHO 2002)**.

Bacteriologically, almost any organism have the potential to cause nosocomial infection but only limited number of organisms are frequently responsible for diseases acquired in hospitals. Ninety percent of the nosocomial infections is caused by bacteria, while mycobacterial, viral, fungal or protozoan agents are less commonly involved.

Bacteria that commonly cause nosocomial infections include *Pseudomonas aeruginosa*, *Legionella* non-tuberculous mycobacteria. But also *Staphylococcus aureus, Streptococcus* spp., *Bacillus cereus, Acinetobacter* spp., coagulase negative staphylococci, enterococci, , and members of the family such as *Escherichia coli, Proteus mirabilis, Salmonella* spp., *Serratia marcescens* and *Klebsiella pneumoniae*.

Usually these are localized or systemic infections resulting from an adverse reaction to infectious agents or its own toxins. The most common nosocomial infections are those affecting the urinary tract (usually catheter associated) (31%), followed by surgical site infections (17%), primary bloodstream infections (14%), and pneumonia (usually associated with ventilation) (13%).

According to estimates reported by the World Health Organization (WHO), about 15% of all hospitalized patients contract this type of infections, with a frequency of overall infections in low-income countries three times higher than in high-income countries **(Tolera 2018)**.

Higher rates of nosocomial infections were recorded in hospitals Eastern Mediterranean and Southeast Asian countries (with values of 11.8 and 10.0% respectively).

A study conducted by the WHO, also showed that the highest prevalence of nosocomial infections occurs in intensive care units and in surgical departments. The likelihood of getting the infection is higher among patients who have a chronic susceptibility or a greater age. Hospital-acquired infections add to the patient's emotional distress and can, in some cases, determining disabling conditions that reduce the patient's quality of life; furthermore these infections are also a leading cause of death. To all this is added a significant increase in the economic costs **(WHO 2002)** incurred by the structure and the health system (increase the length of hospital stay for infected patients and increased consumption of drugs and materials).

Different are factors influencing the development of nosocomial infections:

- The microbial agent. The patient is exposed to a variety of microorganisms during hospitalization. The contact between the patient and a microorganism does not necessarily lead to the development of a clinical disease. The likelihood that exposure leads to infection depends on the characteristics of the microorganisms (antibiotic resistance), their intrinsic virulence and the amount of infectious material the patient comes into contact with. depending on how the infection is taken, we can talk about infections caused by microorganisms acquired from others people in hospital (cross infections) or infections caused by the patient's own bacterial flora (endogenous infections) or by infections caused by present on contaminated medical objects or devices (environmental infections) **(WHO 2002)**.
- Patients susceptibility. Important factors for the patient that can influence the acquisition of one infection include age, immune status, children and the elderly are considered to be the most vulnerable categories associated with infections. Patients with chronic diseases such as malignant tumors, leukemia, diabetes mellitus, renal insufficiency, o acquired immunodeficiency syndrome (AIDS) have an increased susceptibility to infections from opportunistic pathogens. Skin or mucosal lesions can also reduce the body's natural defense mechanisms; but malnutrition is also a risk factor **(WHO 2002)**.
- Environmental factors. In healthcare facilities, patients with infections or carriers of pathogenic microorganisms are potential sources of infection for other patients and for the staff themselves working in these facilities. In addition, the crowded conditions inside the hospital, the frequent transfers of patients from one ward to another, the presence of numerous patients highly susceptible to infections in specific wards (newborns, burn patients, ICU patients) contribute to the development of nosocomial infections. Infections, indeed, can be transmitted over short distances from large droplets and at greater distances from droplets smaller ones (such as those generated by coughing and sneezing). The latter can remain suspended in the air for long periods and can spread widely in

a sensitive environment such as a hospital ward or an operating room where they can be acquired by patients directly or indirectly. In addition, cleaning activities such as sweeping, using dry cloths to remove dust, or shaking the laundry, can cause aerosolization of particles containing microorganisms.

Similarly, *Legionella pneumophila*, the bacterium responsible for legionellosis (Legionnaires' disease; Pontiac fever), it can become airborne during evaporation of water droplets from the cooling towers of the air conditioning or can be dispersed in the air with aerosols produced by patient showers and cause high risk exposures. To all this are added new infections associated with the presence of other bacteria carried by water (atypical mycobacteria, and *Pseudomonas*) and / or viruses and parasites that continue to be detected in these structures. The concentration of microorganisms present in the air of hospitalization rooms of health facilities depends on several factors, on the number of people who occupy the room, the activities carried out in it and the air exchange rate. Fresh air, properly circulated, will do dilute airborne bacterial contamination. obviously the ventilation rates expressed in the form of air changes must vary according to the type of area considered. The ventilation systems used in these facilities require proper design and maintenance to minimize microbial contamination. All external air vents must be positioned as high above ground level as possible. Also, to minimize the presence of airborne particles, the air must be circulated around the room with a speed of at least 0.25 m / sec and a high efficiency air filter (HEPA) must be filtered. In this way the air enters the room will be essentially clean and free of bacterial contaminants **(WHO 2002)**.

- Bacterial resistance. The use of antibiotics promotes the emergence of multi-resistant bacterial strains that can become an endemic element especially in hospitals. Continued, inappropriate and uncontrolled use of antimicrobial substances due to excessive prescriptions, administration of suboptimal doses insufficient treatment durations, and misdiagnoses that they lead to inappropriate

choices of drug, have contributed to the development of drug-resistant bacteria, which can lead to increased morbidity and mortality, in particular in health structures where the presence of patients suffering from debilitating chronic diseases is greater **(WHO 2002)**.

Bacteremia they can also arise from bacteria entering the central venous catheter. But inhalation of contaminated aerosols (e.g. showers, fans, nebulizers, hydrotherapy pools and splashes from sinks) are also routes of exposure that can cause infections of the respiratory system.

In addition to infections, too pseudo-infections often occur in healthcare settings; these occur when patients are colonized without showing disease manifestation. Pseudo-infections can have a detrimental effect on patients, leading to misdiagnosis and inappropriate treatment **(Williams 2013)**.

The risk of becoming infected with hydro diffusing pathogens depends on a dynamic interaction between the germ and the host. Theoretically, the risk of acquiring such an infection has been simplified as follows:

Infectious risk = Microbial load x Virulence of the pathogen / Immune status of the host

Focal points of nosocomial infection should be identified and promptly analyzed in order to limit transmission episodes and to be able make an improvement in patient care. Early identification of an outbreak is therefore crucial.

The prevention of nosocomial infections requires a great responsibility on the part of health facilities.

To reduce the risk of infection for patients and staff working in these facilities checks are needed for staff providing direct patient care, good management of the plants, suitable supply of materials and products for healthcare professionals **(WHO 2002)**.

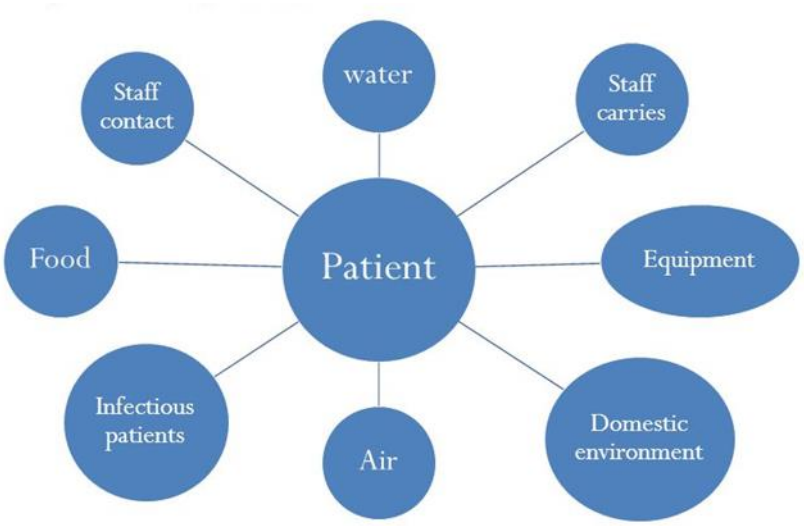


Fig 6: Hospital acquired infection

Hospital water safety is a major priority and constant challenge for healthcare epidemiologists, safety officers, engineers, and administrators. Waterborne infections incur significant morbidity and mortality, and some are preventable. Waterborne infections can occur from proximal (central pipes) or distal (points of use) contamination of the hospital water supply. Municipal and hospital tap water are not free of pathogens, but thoose are undergoes routine microbiological surveillance to assure safe levels of pathogens **(Decker 2014)**.

The epidemiological link between presence of *Legionella pneumophila* in the hospital drinking water and the occurrence of hospital-acquired legionellosis was first made in the early 1980s by Tobin and Stout **(Lin 2011)**.

1.2.4 Major pathogens in hospital water systems

- ***Pseudomonas aeruginosa***

Fig 7: ***Pseudomonas aeruginosa***

Pseudomonas aeruginosa, fig 7, bacterium of high clinical importance and classified as pathogen with critical priority 1 by WHO in 2017 **(Sommer 2019)**.

This opportunistic pathogen, that colonized immunocompromised patients **(Dèziel 2001)**, is responsible for over 11% of all nosocomial infections and has been implicated in a variety of infections including chronic wounds, cystic fibrosis, chronic obstructive pulmonary disease (COPD), urinary tract infections; the persistence of this bacterium in these infections is permitted by its wide metabolic diversity and its ability to form biofilms that allow it to thrive in avariety of stressful environments.

Pseudomonas aeruginosa is one of six bacterial pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp., which are commonly associated with antimicrobials resistance and are denoted by the acronym ESKAPE **(Ciofu 2019)**.

It is a ubiquitous organism widespread in various environments, such as soil, water, plants, animals and humans.

P. aeruginosa is observed in a wide range of environments, such as soil, water and vegetation.

The ability of this species to resist in various environments depends on its extensive metabolic versatility, its simple nutritional requirements, its tolerance to various

physical conditions **(Integration of hygienically relevant bacteria in drinking water biofilms grown on domestic plumbing materials 2011)** and its numerous factors of virulence and survival associated **(Dèziel 2001)**.

According to some authors, the ability of *P. aeruginosa* to adapt and survive in a wide variety of environments can be attributed to its large genome of 6.3 Mbps **(Maunders 2017)**.

A series of superficial and extracellular components synthesized by *P. aeruginosa* contribute to its virulence, and facilitate it the ability to adhere to inanimate and biological surfaces, an important stage in infectivity **(Cappello 2006)**.

Pseudomonas aeruginosa can be found in low-nutrient or oligotrophic environments (saline solutions) as well as in high-nutrient (copiotrophic) environments. Adaptability to low-nutrient concentrations makes its growth in water not directly linked to the level of organic matter content **(Bédard 2016)**.

They have rather simple nutritional characteristics nutritional; although it prefers organic and fatty acids as sources of carbon, it can use a wide range of other carbon sources (over 75 organic compounds), even in minute concentrations (<100 μg/L) and can survive for months in deionized or distilled water. Similarly, nitrogen can be obtained from multiple sources, but amino acids, organic acids, and DNA are the preferred sources **(Bédard 2016)**.

In fact this species use of a large variety of organic compounds as a source of carbon and as electron donors for energy production. If oxygen, nitrate, and nitrite are not present, *P. aeruginosa* can also grow or survive by fermenting arginine or pyruvate, respectively. This capacity allows for growth to take place under anaerobic as well as microaerophilic conditions, where oxygen is present in lower concentrations than in the environment (which favors denitrifying conditions) **(Bédard 2016)**.

The majority of *Pseudomonas* grows at 42°C but not at 4°C.

Furthermore grow chemorganotrophically at neutral pH and at moderate temperatures around 28° C.

P. aeruginosa is naturally resistant to a wide range of antibiotics, making antibiotic treatment ineffective **(Chang 2018)**.

Structurally, *Pseudomonas aeruginosa*, is a rod-shaped bacterium, with length of 1,5±3 µL and width between 0,5 and 0,7 µm, mobile by one or more polar flagella; bacterium Gram-negative, aerobic, oxidases and catalases positive, with respiratory metabolism but able to use nitrate sas electron acceptors alternative to oxygen.

Species of the genus *Pseudomonas* are defined according to different physiological characteristics.

P. aeruginosa represents one of the typical microorganisms of the biofilm, in fact it is able to adhere to wet surfaces or in contact with liquids thanks to the production of lipopolysaccharides and extracellular glycoproteins.

Electron microscopy demonstrated that *P. aeruginosa* adheres to surfaces within 4 h; after 24 h at 37°C it begins to form adherent microcolonies and the first stages of a biofilm on different surface; in biofilms, *P. aeruginosa* is nearly 500 times more resistant to antibacterial agents than are bacteria in suspension **(Sagripanti 2000)**.

The ability to form biofilms is the crucial factor in fatal infections by *P. aeruginosa* **(Overhage 2005**).

The change of bacteria from planktonic to sessile state, in the form of biofilm, depends on the production of adhesins and components of the extracellular matrix which act as a scaffolding for the biofilm **(Ciofu 2019)**.

Thanks to the presence of a flagellum as a mediator of motility, *P. aeruginosa* is capable of forming cell monolayers on abiotic and biotic surfaces already after 4 hours of contact. This bacterium moves in liquids through the flagellum and its motility is due to the presence of pili IV. *P. aeruginosa* biofilms were reported as extremely resistant to sanitizing agents **(Caixeta 2012)**.

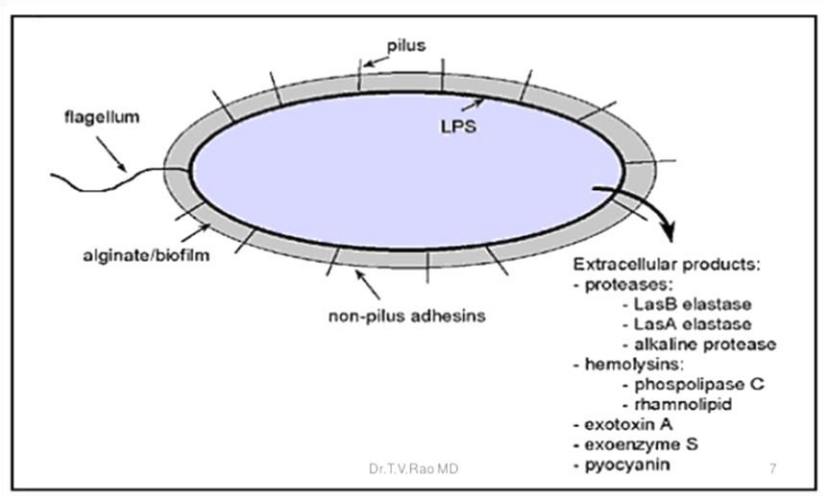


Fig 8: Molecular structure of *Pseudomonas aeruginosa*

In particular, the cell surface proteins, the pili and the flagellum are responsible for the initial attack on the surfaces **(Toyofuku 2016)** facilitating their colonization. The matrix proteins contribute to the structure and stability of the biofilm **(Fong 2015)**.

During chronic colonization, *P. aeruginosa* undergoes conversion from a nonmucoid to a mucoid phenotype. The most characteristic feature of the mucoid phenotype is the secretion of large amounts of highly viscous exopolysaccharides. The copolymer alginate, which is composed of mannuronic acid and guluronic acid, appears to be the major component of the secreted polysaccharide, and besides nucleic acids and proteins, is the key factor in the development of mucoid biofilms **(Overhage 2005)**.

Pseudomonas produces three different types of Extracellular Polymeric Substances (EPS) molecules as the main components of its biofilm matrix, they are extracellular polysaccharides: PSL, alginate and PEL.

PSL is a fundamental component of the biofilm matrix because it promotes both cell-cell interactions and adhesion to surfaces. Furthermore, the PSL also acts as an intercellular signaling molecule **(Irie 2017)**.

The Pel polysaccharide is instead a material rich in aglucose PEL is a cationic polymer that facilitates cell-cell interactions within the biofilm by electrostatic interactions with extracellular DNA **(Marmont 2017)**; Pel recently was described as an N-acetyl glucosamine (GlcNAc)- and Nacetyl galactosamine (GalNAc)-rich polysaccharide that is charged under slightly acidic pH and interacts with eDNA in the matrix6. Psl is

composed of a neutral pentasaccharide subunit that contains mannose, rhamnose, and glucose in a 3:1:1 ratio **(Passos da Silva 2019)**.

Pel and Psl can act as a scaffold of primary structure for biofilm development and are involved in the early stages of biofilm formation.

Pseudomonas produces also alginate.

Alginate is a negatively charged polymer containing guluronic acid and mannuronic acid. It is a very important molecule for *Pseudomonas* biofilm formation, in fact it provides structural stability. Alginate is a high molecular weight acetylated polymer with non-repetitive monomers of L-guluronic and D-mannuronic acids bonded with β-1,4 bond.

Alginate is a non-branched linear polymer, capsule-shaped **(Maunders 2017)**, composed of D-mannuronic acid and L-guluronic acid which contributes to the structural stability and protection of biofilms, as well as to water and nutrient retention **(Rasamiravaka 2015)**.

The production of polysaccharides by these biofilm-forming microorganisms facilitates their colonization through aggregation, surface adhesion and biofilm production.

In *Pseudomonas aeruginosa* biofilms, matrix consists mainly of polysaccharides, proteins, extracellular DNA and lipids, and its composition depends on the strain and depends the growing conditions and age of the biofilm. Together with the exopolysaccharides, proteins such as type IV pili, Cup type fimbriae, CdrA adhesins, LecAB lectins and Fap amyloid fibers can be present in the *P. aeruginosa* biofilm matrix. Furthermore, extracellular DNA (eDNA) functions as an important component of the matrix in *P. aeruginosa* biofilms **(Ciofu 2019)**.

The lectins, LecA and LecB (also named PAI-L and PAII-L, respectively) that interact with specific sugars. LecA binds to galactose and its derivatives, while LecB binds to fucose, mannose, and mannose-containing oligosaccharides (fig 8). Both lectins also are linked to biofilm formation on abiotic surfaces, although the underlying mechanism behind these observations are unknown **(Passos da Silva 2019)**.

Lectins are a specific class of carbohydrate-binding proteins different from enzymes or antibodies. They are in a wide range of organisms including viruses, bacteria, plants and animals, and play an important role in cell–cell interactions **(Funken 2012)**.

P. aeruginosa appears sporadically in drinking water distribution systems, for example as a consequence of contamination during construction or repair work **(Moritz 2011)**, it can be found in public drinking water systems and home installations, from which it comes from the source water or can be introduced, for example, during construction works.

It has been isolated from domestic plumbing systems, as shown in fig 9, from drinking water distribution systems, from technical water systems, from swimming pools and from numerous clinical settings **(Moritz 2011)**.

Several studies have been conducted to verify the direct influence of hydraulic material used and the ability of *P. aeruginosa* to adhere to it in the form of biofilm **(Bèdard 2016)**.

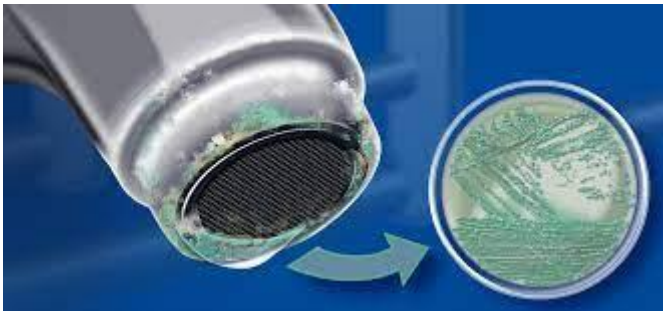


Fig 9: Ability of *P. aeruginosa* to form of biofilm

Non-tuberculous mycobacteria (NTM)

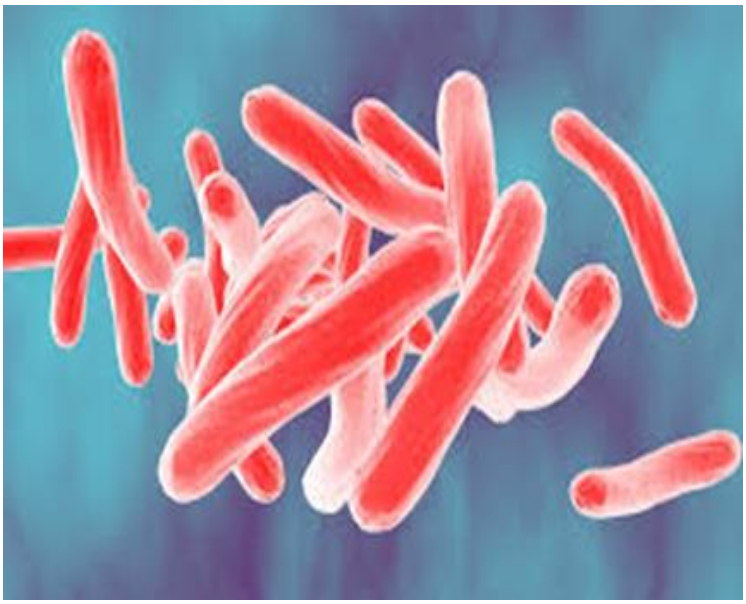


Fig 10: Non-tuberculous mycobacteria

The genus *Mycobacterium*, fig 10, includes bacilli, aerobic, straight or slightly curved rods between 0.2 and 0.6 *μm* wide and between 1.0 and 10 *μm* long, acid-fast alcohol stains. They are generally nonmotile bacteria, except for the species *Mycobacterium marinum* which has been shown to be motile within macrophages.

They do not readily stain with Gram stain, although they are weakly Gram positive. They do not form hyphae, are not motile, and do not form spores. They are aerobic bacteria, positive in catalase and aryl-sulfatase reactions.

They belong to the family *Mycobacteriaceae* (order *Actinomycetales*) with about 170 species, including pathogens and saprophytes, **(Pedley 2004)** and can be divided into two groups based on clinical relevance.

The first group includes obligate pathogens in humans and animals, namely the *Mycobacterium tuberculosis* complex (*M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, *M. pinnipedii* and *M. tuberculosis*), *M. leprae* and *M. lepraemurium*, which are generally not found in the environment. The second group includes mycobacteria potentially pathogenic to humans or animals.

Also, NTM can be divided into rapidly growing mycobacteria, which may form colonies within 7 days, of which the *M. abscessus* complex, *M. chelonae* and *M. fortuitum* are the most clinically relevant; and slow growing mycobacteria, which may take up to 12 weeks to grow, of which the *M. avium* complex (MAC), *M. xenopi*, *M. kansasii*, *M. simiae*, *M. malmoense* and *M. szulgai* are the most important **(Cowman 2019)**. Presumably the slow growth of many mycobacterial species is, at least in part, a consequence of the hydrophobic nature of their cell surface, which makes the cells rather impervious to nutrients (species with a lower lipid content grow, in fact, faster). Some mycobacteria are saprophytes (i.e., they live on decaying organic matter), and others are obligate parasites. Most of these species are found in soil and water in a free-living form or in diseased tissue of animals and can cause disease under certain circumstances, eg. skin lesions, lung or immune dysfunctions and chronic diseases. (**Vaerewijck)**

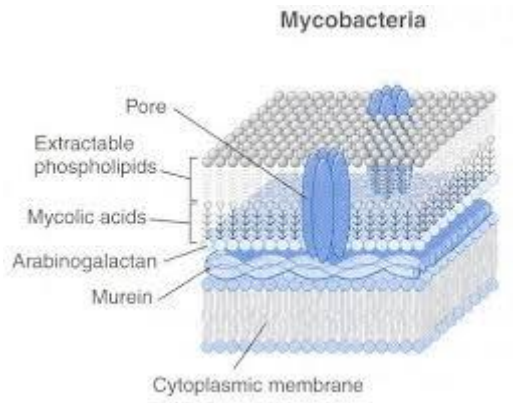


Fig 11: *Mycobacteria* cell wall

Mycobacteria have an outer membrane, fig 11. They possess capsules, and most do not form endospores. The distinguishing characteristic of all *Mycobacterium* species is that the cell wall is thicker than in many other bacteria, being hydrophobic, waxy, and rich in mycolic acids/mycolates. The cell wall consists of the hydrophobic mycolate layer and a peptidoglycan layer held together by a polysaccharide, arabinogalactan. The cell wall makes a substantial contribution to the hardiness of this genus.

Resistance to extremes of heat and pH, and to many disinfectants and antibiotics **(Cowman 2019)**.

The major determinant of NTM ecology and epidemiology is the presence of a lipid-rich outer membrane. The outer membrane's long chain mycolic acids contribute to the hydrophobicity, impermeability. Those features, in turn, lead to the preferential attachment to surfaces and resistance to disinfectants and antibiotics. NTM are oligotrophs and able to grow on a variety of organic compounds including some found in water and soil **(Falkinham 2009)**.

Also, *Mycobacteria* are able to multiply within low nutrient environment as in water pip systems and can survive in hospital hot water systems, and resist chlorination **(Khorosavi 2016)**.

Non-tuberculous *Mycobacteria* (MNT) have been recovered, in the environment, from fresh, brackish / sea water and wastewater, sometimes of high density.

NTM they were also recovered in drinking water systems before and after treatment, from distribution system and from raw spring waters. The water treatment itself risks being ineffective, in fact mycobacteria show a high resistance to disinfection; and these microorganisms are ozone resistant too **(Hilborn 2006)**.

The numbers of *Mycobacteria* appear to be higher in drinking water distribution systems (on average 25,000 times) than in those collected immediately afterwards treatment, suggesting that they are able to multiply along the distribution. It has also been shown that some environmental mycobacteria grow inside amoebas and ciliates and this one condition can provide a useful refuge when environmental conditions become adverse.

Mycobacterial infections related to contaminated hospital tap water have been recognized for many years and environmental mycobacteria have been isolated from systems hospital hot water **(Briancesco 2010)** and in domestic tap water **(Thomson 2013)**.

Those microorganisms can survive for months or years while some species can even proliferate in environment **(Klanicova 2013)** and they can cause life-threatening infections in humans, other mammals, and birds.

Moreover they appear in high numbers in waters and biofilms in drinking water distribution systems.

Habitats from which environmental opportunistic *Mycobacteria* have been isolated are listed in Table 3 (**Falkinham 2009)**.

Table 3. Habitats from which environmental opportunistic *Mycobacteria* have been isolated

Habitat	**Reference**
Natural waters	Falkinham et al. 1980; von Reyn et al. 1993
Drinking water distribution systems	Covert et al. 1999; Falkinham et al. 2001
Biofilms in drinking water distribution systems	Falkinham et al. 2001; Torvinen et al. 2004
Building, hospital, and household plumbing	Du Moulin et al. 1988; Wallace et al. 1998; Nishiuchi et al. 2007; Falkinham et al. 2008
Hot tubs and spas	Embil et al. 1997; Kahana et al. 1997; Mangione et al. 2001; Marras et al. 2005
Natural and household/building aerosols	Falkinham et al. 2008
Boreal forest soils and peats	Iivanainen et al. 1997, 1999
Acidic, brown-water swamps	Kirschner et al. 1992
Potting soils	De Groote et al. 2006
Metal removal fluid systems	Bernstein et al. 1995; Shelton et al. 1999; Moore et al. 2000

Infections occur in immunodeficient (e.g., people with HIV/AIDS) and immunosuppressed (e.g., people with cancer and transplant) patients. NTM, particularly *M. avium* and *M. intracellulare*, have been recovered from a variety of environmental niches with which humans come in contact, especially drinking water. NTM grow and persist in plumbing. For example, numbers of mycobacteria increase in pipes as the distance from the treatment plant increases. NTM cell surface hydrophobicity results in disinfectant resistance and a predilection to attach to surfaces where NTM grow and form biofilms that further increase disinfectant resistance. Because disinfectants inhibit the competing microflora, the slow-growing NTM can grow on the available nutrients in the absence of competition. *M. avium* can grow in

drinking water at concentrations of assimilable organic carbon of >50 μg/L. Thus, there is strong reason to hypothesize that NTM can colonize and persist in household plumbing **(Falkinham 2011)**.

The most common NTM isolated from humans are listed in Table 4 (**Falkinham 2009)**.

Table 4. The most common NTM isolated from humans, slowly and rapidly growing

Species Reference
Slowly growing mycobacteria (colony formation after 7 days)
Mycobacterium avium
Mycobacterium intracellulare
Mycobacterium kansasii
Mycobacterium xenopi
Mycobacterium marinum
Mycobacterium malmoense
Mycobacterium simiae
Rapidly growing mycobacteria (colony formation 3–7 days)
Mycobacterium abscessus
Mycobacterium chelonae
Mycobacterium fortuitum

Although the exact route of NTM infection is not established with certainty, based on NTM environmental distribution, it is very likely that the organism is ingested, inhaled, or implanted. Aerosolization of droplets small enough to enter the alveoli is the likely route of acquisition of pulmonary disease. Bathroom showers have been implicated as a primary source of exposure to aerosolized NTM **(Johnson 2014)**.

Among nontuberculous mycobacteria, *M. chimaera* was recognized as a cause of respiratory and disseminated infec-tions among immunocompromised patients **(Hasse 2020)**.

Hospital clusters of nontuberculous mycobacteria (NTM), are well recognized in the context of cardiac surgery. In 2014, 6 cases of severe infection due to *Mycobacterium chimaera*, a recently described slow-growing mycobacterium within the *Mycobacterium avium* complex (MAC; similar to Mycobacterium intracellular), were reported in cardiac surgery patients in Zurich. Investigators hypothesized that patients were infected by contaminated aerosols from the water tanks of heater-cooler units (HCUs) used during cardiopulmonary bypass **(Chand et al 2017)**.

- ***Legionella* spp.**

Fig 12: Legionella spp.

Species of the genus *Legionella*, in fig 12, are opportunistic waterborne pathogen, Gram-negative, non-spore-forming, rod-shaped, aerobic bacteria intracellular, monopolarly flagellated rod that measures 0.5 mm in width and 2 mm in length and with a diameter between 0,3 e 0,9 μm.

They generally appear as coccobacilli in tissues or secretions, but they can become filamentous in cultures **(UK NHS 2015)**.

They contain branched-chain fatty acids, have a non-fermentative metabolism, and require L-cysteine and iron salts for growth. Although they are Gram negative, legionellae stain poorly with the Gram procedure and other similar staining methods. This is attributed to the presence of branched-chain fatty acids, an important component of cell walls. Other staining methods have been described, such as Dieterle's silver impregnation method; while more effective methods include antibody-coupled fluorescent dyes and immunoperoxidase staining.

There cell wall consists of a cytoplasmic membrane on the inner surface, a thin layer of peptidoglycan and a outer membrane containing thermostable lipopolysaccharides (LPS) with species and serogroup specific O antigens. They do not have a capsule.

This bacterium does not form microcysts or endospores the known biochemical characteristics are those related to the *L. pneumophyla* species (catalase positive, produces β-lactamase, liquefies gelatin, has variable oxidase and reduction of nitrates and negative urease). It shows specific nutritional needs, in fact the main source of

carbon is constituted by aminocides, as it is not able to ferment or oxidize carbohydrates.

Legionella multiplies in a temperature range between 25 ° C and 42 ° C, fig 13, with optimal growth at 36 ° C and pH between 5.5 and 8.3. On the other hand, it does not grow at temperatures below 20 ° C and does not resist temperatures above 60 ° C.

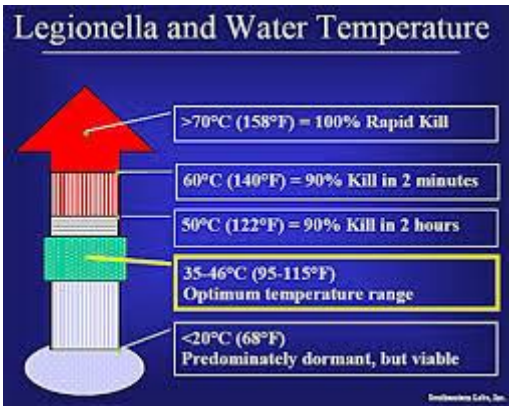


Fig 13: Temperature range favorable to *Legionella* multiplication

These characteristics allow it to adapt very well to aquatic environments of anthropogenic origin.

Currently, 60 species and 71distinct serogroups of *Legionella* have been identified. Although all species are potentially pathogenic, legionellosis is most often caused by *Legionella pneumophila* serogroup 1.

Other species of Legionella commonly identified as agents of disease in humans are *Legionella micdadei*, *Legionella bozemanae*, *Legionella dumoffii*, and *Legionella longbeachae* **(Burillo 2017)**.

Conditions of stagnation of water associated with weak disinfection treatments or not excessive heating, represent the ideal places for the proliferation of this microorganism. *Legionella pneumophila* is widespread in many water systems and poses a serious public health risk.

The diseases caused by *Legionella* are collectively termed legionellosis **(Borrella 2005)**. Legionnaires' disease is transmitted to humans via inhalation of contaminated water droplets **(Arwa 2018)**.

Legionella pneumophila, was recognized as being pathogenic to humans for the first time after an outbreak of acute *pneumonie* at a convention of the American Legion at Bellevue Stratford Hotel in Philadelphia, USA in July 1976. On that occasion, 221 people contracted this previously unknown form of pneumonia, and 34 died. The source of bacterial contamination was identified as the hotel's air conditioning system. Legionnaires' disease is acquired by inhalation or aspiration of *Legionellae* from a contaminated environmental source, and the hot water system is thought to be the most frequent source of cases or outbreaks found even within hospitals, where patients are most susceptible to serious infections **(Borella 2004)**.

The droplets of contaminated aerosol, once inhaled are able to reach the most distal branches of the respiratory system in a way that is more effective the smaller they are, being more dangerous the particles with diameter particles with a diameter of less than 5µm.

Legionellosis can be distinguished into two different clinical forms: severe infection legionnaires' disease, which includes pneumonia and the mortality rate estimated to be 5% to 30%, and Pontiac fever, with a flu-like symptomatology that usually resolves in 2-5 days **(Parr 2015)**.

From a clinical point of view, *Legionella* multiplies in the lungs, inside the alveolar macrophages, which are unable to destroy them or inhibit their growth: *Legionella* multiplies within these phagocytes until they cause lysis, with the consequent release of a large amount of bacteria that can infect other cells.

In the lungs, *L. pneumophila* replicates exponentially in alveolar macrophages causing a type of pneumonia called legionnaires' disease or legionellosis.

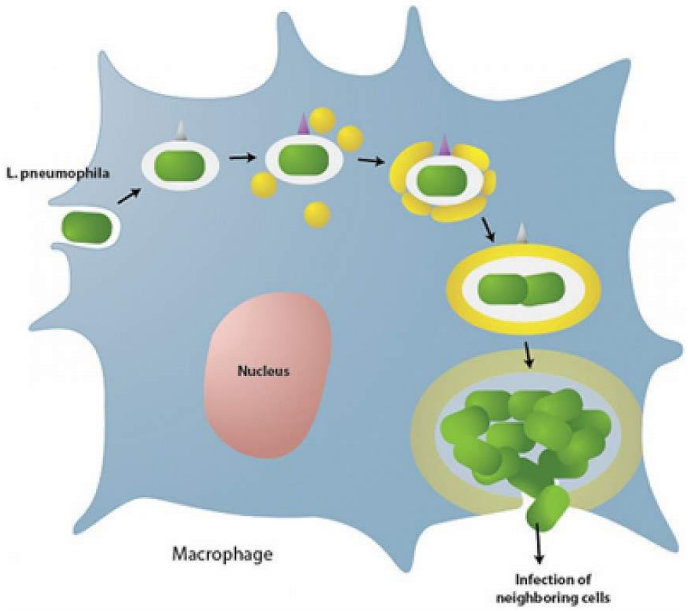


Fig 14: Infection cycle of L. pneumophyla

Some data suggest that biofilm-derived *Legionella pneumophila* evades the innate immune response in macrophages (fig 14).

Virulence factors of *Legionella* are:

- the *mip* (macrophage infectivity potentiator) gene, the first virulence-associated gene to be cloned **(Cianciotto 1992)**; the locus encodes for a 24-kDa surface protein with peptidyl-prolyl cis/trans isomerase activity capable of inhibiting calcineurin, a phosphatase present in numerous eukaryotic cells and implicated in several intracellular signaling mechanisms **(Wintermeyer 1995)**, which results in an alteration of the regulatory functions of the host cell;
- the lvh region (*Legionella* vir region), exclusive of the genus *Legionella*, located on a mobile element in Paris and Philadelphia strains of *Legionella pneumophila* and encoding for a particular secretion system involved in intracellular adhesion and survival as well as in replication **(Ridenour 2003)**;
- the *rtxA* region (ripeats in the toxin), belonging to the locus *enh1*, virulence factor, preserved among the strains of *Legionella pneumophila*, that encodes a large multifunctional protein of about 7000 amino acid residues; this region is in common with other pathogens as *Bordetella pertussis*, *Escherichia coli* o *Actinobacillus actinomycetemcomitans*.

The pathogenicity of *Legionella* in humans largely depends on the susceptibility of the host.

Children and young people are rarely affected, while immunocompromised individuals, especially transplant recipients, are at high risk of contracting the disease. Smoking and alcoholism are commonly recognized predisposing factors and infection is more common in males than in females and in people over 40 years of age. Individuals with end stage renal insufficiency or blood cancer, severely immunocompromised people (including AIDS patients) are significant risk of contracting Legionnaires' disease. Patients even with chronic lung disease, liver cirrhosis or diabetes are at risk, albeit slightly smaller extent. Pontiac fever, on the other hand, strikes healthy children and adults with the same frequency as immunocompromised individuals.

Only two factors of *Legionella* have been shown to be associated with 24-kDa virulence protein, enhancer of macrophage infectivity and the integral 113-kDa protein of cytoplasmic membrane, produced by the dotA gene.

It can be assumed that the infectious dose for humans is low.

Legionella is present both in the natural water environment (lakes, rivers, aquifers, deep wells, with highly variable concentrations ranging from $9.0x10^3$ to $3.3x10^7$ cfu / L, with higher concentrations in the summer months) and in the artificial one (water networks of buildings of various types such as hotels or hospitals, in tanks, cooling towers for water, in recirculation tanks, dental units).

The microorganism moves from its natural reservoirs to the water distribution systems. *Legionella pneumophila* is able to remain in the environment as free living planktonic bacteria or form bacterial biofilms that adhere to surfaces **(Khweek 2018)**.

It can not be excluded that *Legionellae* grow planctonically or in biofilms. However, a number of studies suggest that this pathogen only replicates within protozoa. Some species of protozoa are essential for the growth of *Legionella* in natural and anthropogenic environments: *Acanthamoeba*, *Hartmannella* and *Naegleria* they are

most commonly isolated from Legionella-contaminated plumbing systems. Other Legionella contaminated species are *Saccamoeba*, *Vexillifera* and *Platyamoeba* **(Steinert 2002)**.

L. pneumophila hides within protozoa, fig 15, as a survival strategy to overcome the low-nutrient environment and increases the resistance to disinfectant indeed protozoa not only provide nutrients for intracellular *Legionellae*, but also provide a refuge when environmental conditions become unfavorable. Particularly inside the *Acanthamoeba* cysts the bacteria are able to survive high temperatures, disinfection procedures and drought. Beyond protection and reactivation from dormancy *Legionella* can also use protozoa to colonize new habitats and to acquire greater resistance to stress **(Steinert 2002)**.

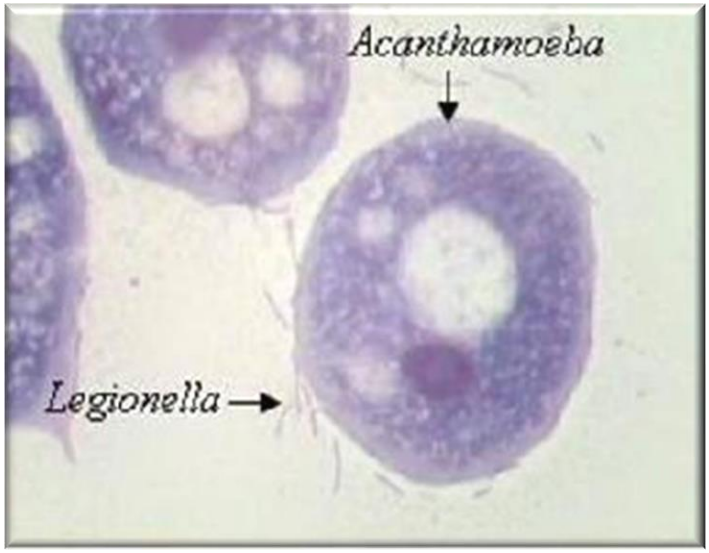


Fig 15: Protozoan colonized by *Legionella*

Legionella-protozoa relationship is central to the ecology of the organism in both aquatic and soil environments **(Newton 2010)**.

In addition, some species of moeba excrete biocide-resistant vesicles, containing a large quantity of *L. pneumophila*, which can be carried by air and act as vectors for the transmission of bacteria **(Newton 2010)**.

Recent studies have shown the existence of virulence genes of *Legionella*. These genes have been described as the main factors capable of influencing *Legionella* a grow and

survive within blood and alveolar monocytes macrophages or within free-living amoebas **(Zhan 2015)**.

This opportunistic pathogen most often thrives in biofilm. Biofilms have been recognized as one of the most important factors of survival and proliferation of *L. pneumophila* in warm, humid environments like showers, air conditioners, and spa baths **(Arwa 2013)**.

It is known that *L. pneumophila* can persist for long periods of time in water and biofilms commonly found in manmade water systems, such as plumbing systems, air conditioning equipments or whirlpool spas. It is widely accepted that biofilms play a critical role in the persistence of these bacteria within water systems, providing shelter and nutrients and preventing disinfectants from gaining access to the bacteria through the exopolysaccharide matrix **(Tesauro 2010)**.

Recent reports suggest that the growth of *Legionella* in biofilms may lead to enhanced virulence **(Abdel-Nour 2013)**.

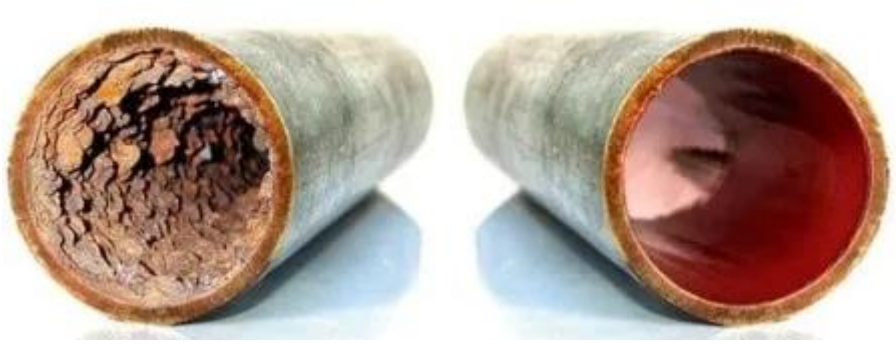


Fig 16: Adherence of *L. pneumophila* on surface material

In naturally occurring multispecies biofilms, the colonization with *L. pneumophila* can be influenced by several other species of microorganisms **(Abdel-Nour 2013)**.

Colonization of abiotic substrates by the *Legionella pneumophila* species in the form of bio-films is determined by a wide variety of parameters. A an important factor governing the adherence of *L. pneumophila* in anthropogenic water systems is the composition of the surface material, fig 16, to which the bacteria adhere in fact *L.*

pneumophila can adhere good for many plastics that are commonly used in plumbing, while copper inhibits them attachment.

For each bacterium there can be a preferential type of material that conditions a better adhesion; for example, *Legionella pneumophila* tends to adhere more to the following materials, in decreasing order: latex, ethylene-propylene, PVC, polypropylene, steel, stainless steel, polyethylene, glass. This is possible thanks to the different interactions that some nutrients have with the surfaces where they adhere, thus encouraging the development of biofilm by bacterial species that need those nutrients.

Cations are involved in the attachment of bacteria to different substrates and can contribute to the formation of biofouling.

Likewise, both calcium and magnesium have been shown to facilitate attachment of *L. pneumophila* to abiotic surfaces. High levels of zinc, magnesium and manganese are correlated with increased contamination by *L. pneumophila* in fact zinc increases the capacity of *L. pneumophila* to bind to host cells such as human lung epithelial cells; all this suggests that cations can increase the adhesion capacity of *L. pneumophila* to biotic surfaces in addition to abiotic substrates. The availability of carbon, in addition to the presence of cations, favors the colonization of biofilms with *L. pneumophila*, presumably because it provides nutrients for bacterial replication. In particular, the increase in biofilm production due to the presence of organic carbon was only reported at 20°C, suggesting that carbon can affect biofilm production only at certain temperatures.

Static and water flow conditions play an important role in biofilm formation and biofilm colonization with *L. pneumophila* in water systems. The stagnation of water in the distribution systems seems to favor colonization with *L. pneumophila*. In addition, cases of Legionnaires' disease have been linked to standing water in hospitals. Therefore, a constant flow of water can decrease the adhesion of bacteria to the internal surfaces of the water supply.

Little is known about the molecular factors of *L. pneumophila* that directly contribute to the surface adhesion process. *Legionella* collagen-like protein (Lcl) was initially

identified as a necessary adhesin in the infection of protozoa and macrophages. Furthermore, this protein facilitates biofilm production by promoting adhesion to abiotic substrates and cell-cell / cell-matrix interactions. In the *Legionella pneumophila* biofilm formation process, type IV pili are also involved, in fact these structures facilitate adhesion to protozoal cells **(Abdel-Nour 2013)**.

The control of *Legionella* spp. contamination is relevant in nosocomial environment, where patients, with compromised immune systems, are at increased risk of disease. For this reason, international and national guidelines advocate the adoption of preventive measures to control of *Legionella* water contamination. The *Legionella* prevenction contamination should start from a correct design and construction of water networks. During renovations or new construction the pipe runs should be short, insulated and long dead-legs avoided **(Borella 2016)**.

The WHO suggests that developing a water safety plans (WSP) is the preferred approach to the management of specific health risks of exposure to *Legionella* from water systems **(Borella 2016)**.

In the United States it is believed that, every year, cases of legionellosis are not less than 11,000.

In Italy, annually, the cases notified are about 150. However, there are compelling reasons to believe the cases actual are at least 10 times higher. One of the main reasons why the disease is underestimated is due to the fact that legionellosis has no clinical features in able to clearly distinguish it from other forms, atypical or bacterial, of pneumonia **(Idraulica 2002)**.

For its etiology, the utilities and systems most exposed to *Legionella* risk are:

- hospitals, clinics, nursing homes and the like;
- hotels, barracks, campsites and structures accommodation in general;
- facilities for sports and school activities;
- buildings with cooling towers;
- swimming pools;
- spas;

- decorative fountains and artificial waterfalls.

In plants, *Legionella* can be found isolated or host of protozoa such as amoebas.

Moreover, isolated or host of protozoa is present:

1. free in the water;

2. anchored to biofilm (aggregates)

And it is in these aggregates that the *Legionella* find the indispensable support, to live and develop. Some studies have highlighted the exchanges which normally occur between surfaces metals and biofilms **(Idraulica 2002)**.

Therefore, the formation of biofilms in water networks is fundamental in the fight against *Legionella* **(Idraulica 2002)**.

1.2.4 Biofilm

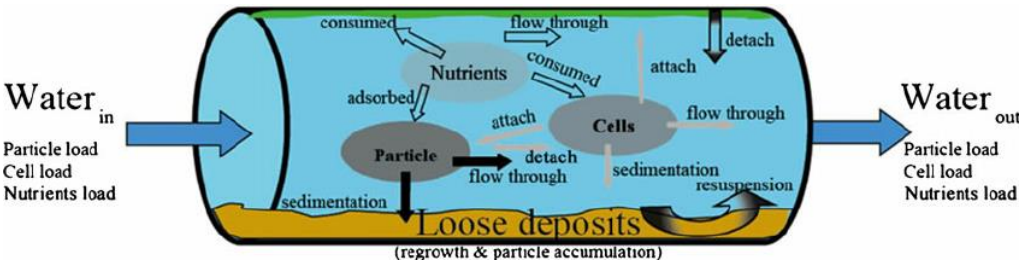


Fig 17: Biofilm deposition inside a water pipe

Biofilm is "a microbially derived sessile community" characterised by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of EPS (extracellular polymeric substances) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" **(Integration of hygienically relevant bacteria in drinking water biofilms grown on domestic plumbing materials 2011)**.

Biofilms are metaphorically called a "city of microbes" with EPS, which represents 85% of total biofilm biomass, as "house of the biofilm cells". EPS is composed mainly of biomolecules, exopolysaccharides, extracellular DNA (eDNA), and polypeptides that form a highly hydrated polar mixture that contributes to the overall structural scaffold and architecture of the biofilm **(Rasamiravaka 2015)**.

Bacteria form biofilms in response to environmental stresses such as UV radiation, desiccation, limited nutrients, extreme pH, extreme temperature, high salt concentrations, high pressure, and antimicrobial agents **(Muhammad 2020)**.

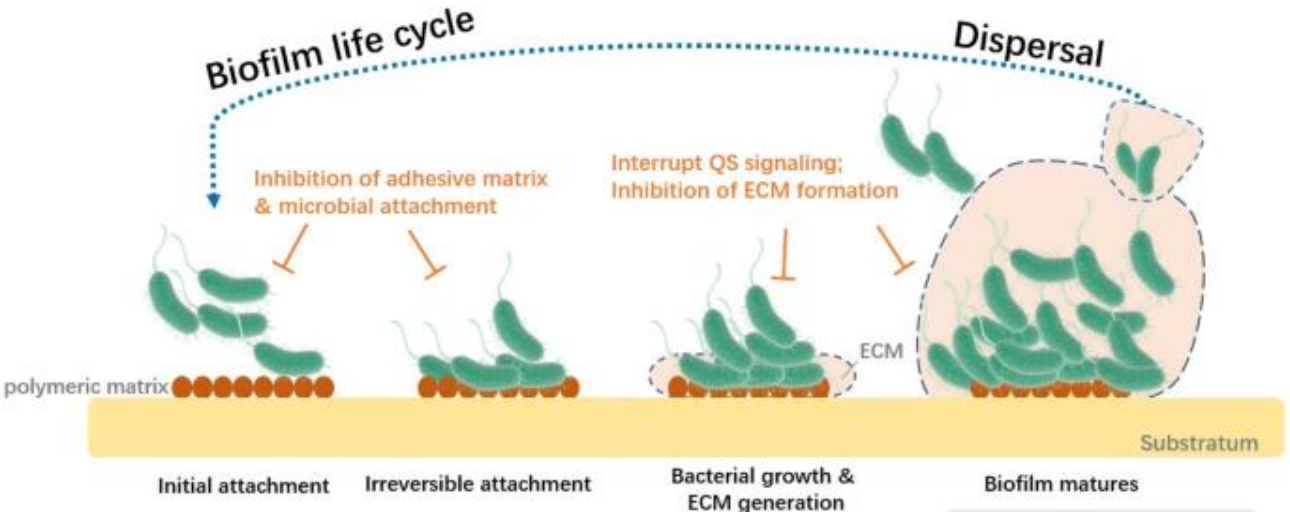


Fig 18: Biofilm formation

Important factors determining biofilm growth in pipes, by many species, are:

- presence and concentration of nutrient including carbon, nitrogen or phosphorous
- reduction in the concentration of disinfectants along the distribution system.

Other factors determining biofilm formation include the temperature, corrosion products (that are as microbial nutrient), and the pipe materials. The availability of nutrients is an important factor in the formation of biofilms; Carbon (C), nitrogen (N) and phosphorus (P) in the proportions 100:10:1 are required for heterotrophic microbial growth. To control biofilm formation in water distribution systems, the entry of biodegradable organic carbon (BOM) into the distribution system should be limited.

Most sources of carbon compounds in water supplies are natural in origin. The carbon is considered to be a major limiting nutrient of microbial growth.

Also temperature is an important environmental factor affecting biofilm formation in pipelines. Rogers et al (1994) noted that the overall trend of biofilm formation in a model system at 20, 40 and 50°C was related to both temperature and the piping materials. Other studies suggested that at temperature >15°C the risk of bacterial growth increased. Biofilm formation will be encourage if the pipe materials is able to provide the required nutrients for microbial growth. Roughness and porosity of

surface provide niches and protection for sessile bacteria from disinfectants **(Bimakr 2015)**.

The development of the biofilm is a multifactorial and dynamic process, fig 18 and 19, regulated by both environmental and genetic elements **(Lens 2003)**.

Biofilm formation is induced and regulated by numerous genes and environmental factors, among these, three are the most important.

1 The first is the quorum sensing (QS), which controls approximately 10% of the genes in *P. aeruginosa*, including many genes involved in the development and dispersion of the biofilm.

2 The second factor is the bis- (30-50) -cyclic diguanosine monophosphate (c-di-GMP), because its signaling network is the most complex secondary signaling system in bacteria and decides whether bacteria adopt both planktonic phenotype or biofilm.

3 The the third factor is represented by a small RNA (sRNA) whose role in the biofilm is not yet fully understood **(Yan 2019)**.

Among the environmental signals in the biofilm formation processes **(Marìc 2007)** and affect bacterial biofilm communities, such as the surface of the material, pipe diameter, spring water, the temperature and season, the availability of nutrients, the residue of disinfectants used (use of disinfectants can influence microbial communities), the hydraulic flows and time of stagnation of the water itself. These characteristics could explain the differences in the composition of biofilm communities found in several sites **(Luhrig 2017)**.

But also variations of pH of the external environment can influence the formation of the biofilm: bacteria respond to changes in internal and external pH regulating the activity and synthesis of proteins associated with many cellular processes. Indeed bacteria contain mechanisms that allow them to adapt to small environmental changes of pH **(Garrett 2008)**.

Bacteria can be transported to the surface in various ways, through sedimentation, through dynamic forces of fluids, through motility, pushed by attraction to chemotactic

factors or through passive forces such as Brownian motion and surface hydrophobicity of cells. Zobell in 1943 noted that adhesion to the surface by microorganisms was a distinct two-step process, a primary reversible surface attraction, and a secondary irreversible: indeed, the microorganisms must first of all reach the proximity of the surface in question and then adhere to that surface.

Adhesion to a substrate can be active or passive depending on the motility of the cell. Passive motility is governed by gravity, diffusion and fluid dynamics while active adhesion depends on the surface of the bacterial cell which facilitates the initial attack phase. The structures present on the cell surface, such as flagella, pili, adhesin, capsule and surface charge, influence adhesion.

The ability to move using flagella or pili is an indispensable prerequisite for the initial effective cell-surface adhesion.

The flagella allow the bacterium to move towards a specific site of attack, while the changes that occur in cell physiology affect adhesion, affecting the chemistry of some structures such as the surface membrane and surface proteins (adhesins and pili).

It seems that biofilm-associated proteins (BAPs) intervening in the early stages of surfaces adhesion **(Zubair 2014)**, bacteria begin development in response to specific environmental signals **(O'Toole 2000)**, initial reversible adhesion to a surface, irreversible adhesion, micro-colony formation, growth of the microcolony with formation of three-dimensional structures, biofilm maturation of the biofilm.

Microorganisms are generally believed to adhere more rapidly to non-polar hydrophobic surfaces than hydrophilic materials.

The hydrophobicity of the cell surface, the presence of fimbriae and flagella and the production of EPS facilitate the speed and degree of attack of microbial cells **(Donlan, 2002)**.

It has been observed that microorganisms attach more rapidly to hydrophobic surfaces such as plastic than glass or hydrophilic metals **(Ben-Yoav 2010)**.

The solid surface has important characteristics in the bacterial adhesion process.

The hydrophobicity of the bacterial cell surface, as well as that of the solid surface is important during adhesion as hydrophobic interactions tend to increase as the non-polar nature of one or both surfaces in question increases **(Donlan 2002; Simoes 2010)**.

In fact, most bacteria are negatively charged but contain a hydrophobic component, namely fimbriae. Most of the fimbriae that have been examined contain a high percentage of hydrophobic amino acid residues, and therefore play a fundamental role in overcoming the barrier of initial hydrostatic repulsion that exists between the cell and the substrate.

Bacterial adhesion which lasts about 3-5 hours, and can be divided into two phases.

Furthermore, adhesion stimulates the synthesis of polysaccharides and cell aggregation.

This aggregation occurs in two stages: reversible adhesion and irreversible adhesion. The reversible stage is an initial phase of weak interaction of the bacteria with the substrate and involves van der Waals forces, electrostatic forces and hydrophobic interactions. In this phase, the bacteria still exhibit a Brownian motion and can still be easily removed from the surface of simple rinsing operations.

The irreversible phase derives instead from the anchoring of appendages and / or from the production of extracellular polymers. In general, the repulsive forces prevent direct contact between the bacteria and the surface, since usually both are negatively charged. The anchoring between the bacterial appendages and the substrate involves short-range forces such as dipole-dipole interactions, hydrogen bonds, hydrophobic forces, and covalent bonds **(Chmielewski 2003)**.

"*When a bacterium is attached to a surface, it is the birth of a "biofilm" formation*"

Following irreversible adhesion to the supporting surfaces the bacteria involved begin the process of cell division, forming microcolonies and producing extracellular polymers (EPS) that act as an intercellular adhesive matrix of the biofilm **(Ben-Yoav 2010)**.

Subsequently threre are production and accumulation of an extracellular matrix composed by one or more polymeric substances such as proteins, polysaccharides, humic substances, extracellular DNA and sometimes other molecules such as those involved in cell-to-cell communication (Stage III); then, non colonized spaces are filled with bacteria, which finally cover theen tire surface (Stage IV) **(Rasamiravaka 2015, Azeredo 2017)**.

The maturation phase, follows the initial adhesion phase which pass to the formation of the first three-dimensional structures of the biofilm, microcolonies, until the completion of the mature biofilm, is completed in 12-48 hours, with the formation of the macrocolonies.

The microcolony is the basic structural unit of the biofilm. The microcolonies and macrocolonies that make up the biofilm are separated from each other by channels that allow the diffusion of nutrients, oxygen and antimicrobial agents and are therefore been likened to a kind of primitive circulatory system. In this way, within the biofilm, microorganisms develop in the community organized with a structural and functional heterogeneity similar to that of an organism multicellular.

The fully mature biofilm takes on a shape similar to that of a three-dimensional tower **(Roy 2018)**.

In most mature biofilms, EPS represents more than 90% of the dry mass. The extracellular matrix is made up of Extracellular Polymeric Substances (EPS) produced by the same bacteria and it is estimated that it represents 50-90% of the content total organic carbon of the biofilm.

The mature biofilm, aging to survive colonizes new niches, the detachment is in fact recognized as part of the life cycle of the biofilm.

From the mature biofilm, some bacterial cells break away in the form of planktonic cells. These scattered cells explore others niches to attach and a new surface. Therefore, dispersion is not only the final stage of the biofilm life cycle but also the beginning of another life cycle **(Toyofuku 2016)**.

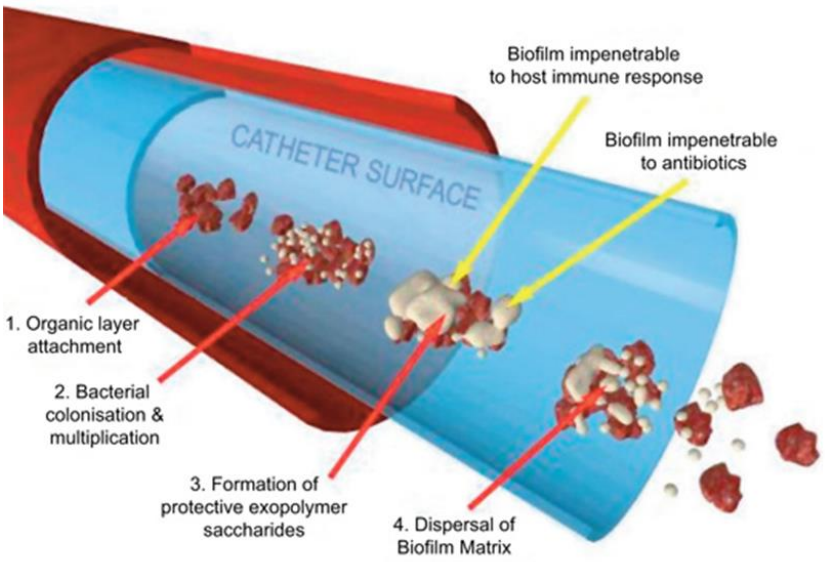


Fig 19: Steps of biofilm formation

Factors influencing dispersion can be both abiotic and biotic. Abiotic factors include shear forces and chemical factors, (nutrient concentration and their availability). Biotic factors, such as the metabolic activity of microorganisms and their gene expression, directly influence the behavior of biofilm shedding.

The dispersion was divided into 4 phases: erosion, abrasion, separation, colonization of new surfaces **(Garny 2008)**.

Microbial biofilms consist of a monolayer of cells or from multilayer with a thickness of 300-400 μm **(Lomander 2002)**.

Biofilms can be formed by a single species or by several species.

Multispecies biofilms are more resistant than the monospecies ones, because it is believed that EPS produced by different bacteria give greater stability.

Biofilms are widespread in the environment, it may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems.

Biofilms are more resistant to antibiotics than planktonic cells **(Spoering 2019)**: indeed it shows an increased survival and resistance to environmental and chemical stressors (e.g., antibiotics), conferred by the extracellular polysaccharide matrix, from 10 to 1,000 times less compared with their planktonic counterparts. This reduced susceptibility is caused by a combination of different factors, as: a poor antibiotic

penetration into the polysaccharide matrix; presence of cells showing a resistant phenotype (persisters); and the presence of either non-growing cells or cells that triggered stress responses under unfavorable chemical conditions within the biofilm matrix.

The exact compositions of biofilm matrixes differ greatly between different microorganisms and growth conditions under which biofilms are formed, but generally consist of exopolysaccharides, proteins, and nucleic acids. Proteinaceous components include cell surface adhesins, protein subunits of flagella and pili, secreted extracellular proteins, and proteins of outer membrane vesicles **(Fong 2015)**.

This matrix may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria **(Donland 2002)**, proteins, nucleic acids, lipids and phospholipids: proteins and polysaccharides form 75-89% of the composition of the matrix and this indicates that they are the major component **(Simoes 2010)**.

The biofilm matrix performs several functions for the benefit of the cells within it.

Matrix proteins, in EPS, contribute to biofilm structure and stability **(Fong 2015 and Fish 2017)**.

In drinking water distribution systems, it has been estimated that about 95% of all microbial cells present in drinking water distribution systems exist as biofilms on the surfaces of pipes and only 5% are found dispersed in the aqueous phase; the biofilms present in the networks dedicated to the transport of drinking water are mainly formed by the indigenous aquatic microflora without any relevance for human health. However, these biofilms have the potential to harbor opportunistic pathogens that can harm human health, especially if immunocompromised **(Moritz 2011)**.

Microorganisms can access the distribution system through treatment facilities, cross links or contamination inputs and exist as taxonomically diverse communities including bacteria, archaes, viruses and protozoa **(Deuterelo 2018)**.

Typically, bacteria grow at around 20-40 ° C, but some species develop between 4,5 and 70 ° C. The optimal pH for most microorganisms is around 7 **(Mota 2018)**.

The development of the biofilm, fig 20, depends on a series of variables such as the chemical and biological stability of the water, its temperature, the hydraulic conditions, any stagnation areas, as well as the type of material with which the net is made, from which they can be released organic compounds such as additives, monomers or degradation products of polymers derived from plastics. All these substances can be used by bacteria for their development **(Rozej 2015)**.

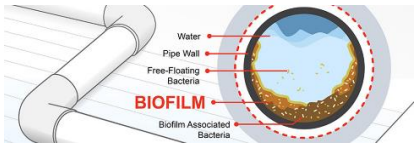


Fig 20: Biofilm development

1.2.5Biofilm and health risks

The 2004 Australian Drinking Water Guidelines, which incorporate the drinking water management framework Quality, has taken a risk-based approach from reservoir to tap to ensure high quality drinking water. The approach requires a thorough definition and understanding of water quality risks, thus enabling the implementation of adequate barriers and control procedures. The risk-based approach to drinking water quality management is now be adopted as best practice internationally, as well as in other sectors such as recycled water management and recreational waters.

The need for safe drinking water and the protection of water resources from contamination became evident when the relationship of microorganisms to disease and drinking water was revealed.

The link between disease and water contamination has led to the creation of protected source areas for drinking water and also to the decontamination of treated water to kill or remove microorganisms.

The requirements for the microbiological safety of drinking water specify that the microbial content should be very low without pathogenic microorganisms and the health risk for acquiring a waterborne infection should be below an accepted limit **(Buthelezi 2009)**.

To meet these requirements, effective protection of water resources, raw water treatment and quality control of the treatment process are required. However, due to the prevalence of biofilm in drinking water systems, it is also necessary to evaluate the factors that influence their formation, the control of the materials used and the appropriate disinfection treatments to be carried out.

Moreover, the presence of biofilm in water distribution systems also entails economic costs to cope with technical problems of biocorrosion structural networks and deterioration of water quality (taste, smell and color of water) **(Nya 2015)**.

1.2.7 Characteristics and differences of cells in the form of biofilm compared to cells in the planktonic form

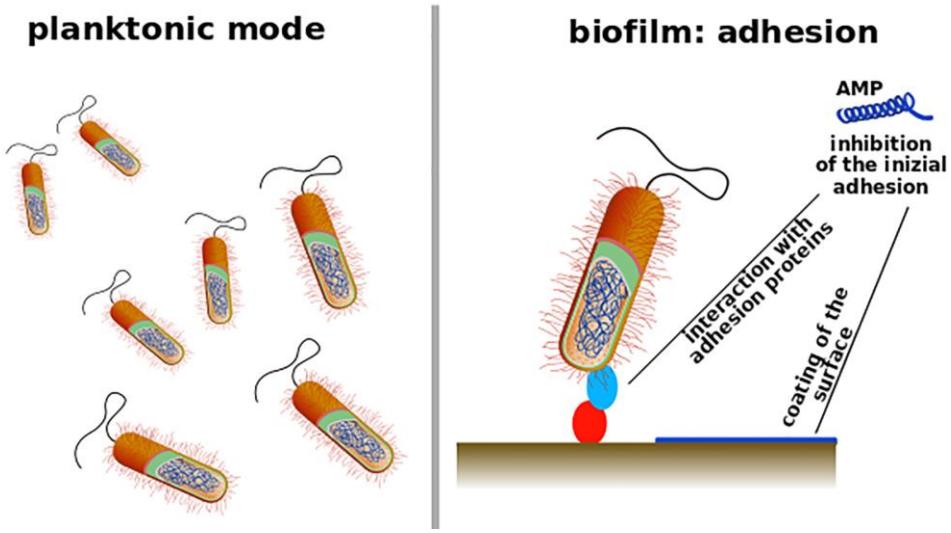


Fig 21: Planktonic and sessile cells

The ability to form biofilms represents a true survival strategy for microorganisms. The metabolic characteristics of bacterial cells within the community of biofilms exhibit distinct differences from the same cells that grow in shape planktonic. For example, with respect to planktonic cells i microbial biofilms, fig 21, generally show an induction of ribosomal genes, specific to the stationary phase or associated with stressful conditions and those responsible microbial adhesion to surfaces; on the contrary, the cells of the biofilm undergo a repression of the genes that code for flagella or other structures responsible for movement. Most of the cells associated with the biofilm have a slower growth rate than the same cells in planktonic form; this depends on the establishment within the biofilm of extremely heterogeneous environmental conditions due to the establishment of gradients of nutrients, oxygen or acid metabolites produced by the fermentation of substrates. Consequently, the physiological state that characterizes the passage from the planktonic form to that of biofilm is accompanied by a reduction of active cellular processes (the biosynthesis of DNA and wall proteins mobile phone). Biofilms exhibit resistance to antimicrobials from 10 to 1000 times higher than that shown by the same cells in planktonic form.

This is often a phenotypic resistance as it has been observed that bacteria resistant to antibiotic treatment in the form of biofilms become susceptible to the antimicrobial agent once the biofilm is broken down. This increase in resistance is attributable to several factors. First, the matrix extracellular physically hinders the spread of antimicrobial agents and prevents to them to reach their targets by seizing them or repelling them by interactions electrostatic. Due to the slowing of metabolism relative to the cells planktonic, the cells associated with the biofilm appear to be less susceptible antibiotics that inhibit metabolic activities such as peptidoglycan synthesis, proteins or nucleic acids.

Fig 22: Biofilm resistance to antimicrobial agents

Resistance of biofilms to antibiotics, as shown in fig 22, the activation of the general response to stress and bacterial resistance mechanisms encoded by mobile genetic elements, such as plasmids, whose exchange by conjugation is favored within the biofilm, given the close proximity between cells. Due to the formation of concentration gradients of nutrients, catabolites and signal molecules, within a biofilm an inhomogeneous cell population is created. For example the bacteria present in the deeper layers they have less oxygen than they do present in the outermost layers therefore they will be more resistant to antibiotics.

Furthermore, environmental heterogeneity can also create ecological niches in which some cells, called "persister", enter a state of quiescence, similar to that of bacterial spores, which makes them completely resistant to antimicrobials. Finally, the close proximity of the cells in the file biofilm can promote both the establishment of metabolic cooperation and transfer horizontal gene pool among community members, with the acquisition of new ones genetic characteristics.

1.3 Legislation

1.3.1 Normative regulation in Drinking Water Distribution Networks

The quality of water intended for human use," involves, in addition to potable use, the contact of water with the human body during various washing practices, taking into account both the average, adult, healthy population and sensitive groups such as children, the elderly, and especially the sick.

A drinking water distribution system aims to provide safe drinking water and protect public health. **(Fish 2017)**

From a normative point of view, the quality of drinking water is regulated by the Legislative Decree no. 31 of 2001, which implements the Directive 98/83/EC "Implementation of Directive 98/83/EC on the quality of water intended for human consumption" and applies to all waters intended for drinking, for the preparation of food and beverages both in the home and in the food industry, and establishes the requirements to be met in order for the water to be considered drinkable, identifying three families of parameters:

- chemical parameters
- microbiological parameters
- indicator parameters

The Decree establishes that water intended for human consumption must be wholesome and clean and for this purpose must not contain:

(a) microorganisms and parasites, or other substances, in quantities or concentrations that pose a potential danger to human health;

(b) meet the minimum requirements of Parts A and B of Annex I;

c) must comply with the provisions of the measures adopted pursuant to Article 14, paragraph 1.

The regulatory framework also includes:

Ministerial Decree 174 of April 6, 2004, relating to materials that may be used in water distribution systems and, most recently, the regulation governing equipment intended for changes in the organoleptic characteristics of drinking water.

Legislative Decree No. 28 of February 15, 2016, establishing requirements for the protection of the health of the population with regard to radioactive substances in water intended for human consumption.

1.3.2 New European Drinking Water Directive

On January 12, 2021, the New European Drinking Water Directive came into force, aiming to provide high quality tap water throughout the EU.
Member states now have two years to transpose the changes into their national rules.
The new document contains substantial changes to the nature and values of the parameters and a thorough review of the management and control methods that will significantly influence the future activities of integrated water service managers and water treatment operators.
Among the novelties in the text of the (EU) Directive are particularly relevant:

- Risk assessment through Water Safety Plans (WSPs);
- The identification of possible emerging pollutants present in supply sources;
- The assessment of risks related to distribution, including the domestic section that knows the meter from the tap;
- Effective and transparent communication to citizens about the quality of the water supplied, which is very important for the consumer in relation to mains water.

Through various tools and rules, the standard aims to encourage less consumption of bottled water, increasing and improving confidence in tap water.
In order to increase and improve confidence in tap water, the European legislator also intended to improve communication to citizens.
The new directive introduces a number of changes including the updating of water quality standards, fig 23.
With regard to microbiological parameters, the changes concern the parameters *Pseudomonas* and colony counts at 22 and 37°C, which arent' longer covered by the new directive.
The parametron Legionella is also included in the list of microorganisms to be investigated, for which the value of < 1 000 cfu/L**.**

Planned actions could be taken in cases of infections and outbreaks. In these cases, the source of infection should be confirmed and the species of Legionella identified.

The chemical parameters have undergone a major revision, with the values for some parameters being changed, but mostly with new substances being added to the list:

- **Bisphenol A**, used for example in resins that make up the coating of tanks for the storage of drinking water; it is considered an endocrine disruptor;
- **chlorate and chlorite**, by-products of chemical water disinfection using chlorine and its compounds; haematological effects and for chlorate also thyroid disorders are recognized;
- **haloacetic acids**, byproducts of the drinking water disinfection process, are considered potential carcinogens;
- **micro cystine-LR**, produced by algae commonly found in surface waters when environmental conditions favor their exponential reproduction, can cause gastrointestinal upset;
- **PFAS**, perfluoroalkyl substances widely used industrially for the production of numerous products, such as Gore Tex fabrics and the tefloning of non-stick cookware, are considered endocrine disruptors and the cause of widespread bioaccumulation and toxicity,
- **Uranium**, radioactive element, whose danger to health is due to its toxicity, causes kidney disorders and is considered a potential carcinogen **(Direttiva Europea 2020/2184).**

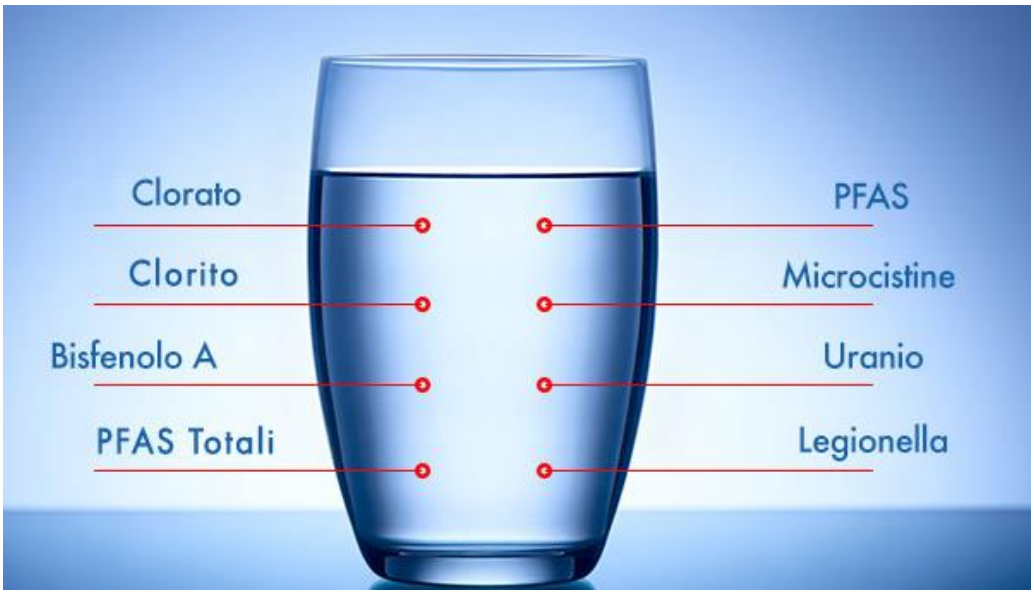


Fig 23: New parameters for water intended for human consumption

Main targets of the present research

The purpose of this study was to compare biocidal efficacy of two disinfectants, monochloramine and silver ion hydrogen peroxide, among the disinfectants currently most used in hospitals and hotels, against *Legionella pneumophila* species present in nosocomial water systems.

This comparison was made by evaluating:

- temporal chemical stability of each disinfectant;
- the Killing curves of each disinfectant against *Legionella pneumophila* under regulated conditions;
- the effect of each disinfectant on the microbiological composition of the biofilm stratified on materials used in the networks;
- the possible corrosive effect of the two disinfectants on materials in contact with water.

CHAPTER 2: MATERIALS & METHODS

Step I: Tests carried out at batch scale

2.1 Stability of monochloramine

2.1.1 Monochloramine stock solutions

A stock solution of monochloramine (30-60 mg/l) was prepared weekly by slowly adding 346 µl of 0.35 M sodium hypochlorite (as Cl_2) in 80 ml of 1.68 mM ammonium chloride maintained at 5-10°C under stirring. During the addition of NaClO, the pH of the resulting solution was constantly monitored with a glass electrode and maintained in the range 8.0-8.3 by adding few drops of 0.01 M HCl or 0.01 M NaOH. Finally, the solution was diluted to 100 ml and stored at 4°C until the subsequent use.

Immediately before the contact tests, the actual concentration of the stock solution was determined as described in 2.1.2.

2.1.2 Determination of monochloramine concentration

Monochloramine concentration in stock and test solutions was determined by applying an amendment to indophenol blue colorimetric method developed for the quantitative analysis of ammonia in aqueous solutions **(APHA 2016)**. To that end, an aliquot of sample not exceeding 2.5 ml was added with 100 µl of citrate buffer, 50 µl of phenate and 50 µl of nitroprussiate (reagent concentration as specified in before diluting to the final volume of 3 ml. After a reaction time of 1 h at least, the spectrophotometric absorption of the solution was measured at 640 nm.

In the case of test solutions, samples were filtered through a 0.45-µm membrane to remove suspended solids before the colorimetric determination of the disinfectant.

2.2 Stability of hygrogen peroxide

2.2.1 Hydrogen peroxide solutions

A standard solution of 30% H_2O_2 (Romil) and having a density of 1.10 g/mL was used for our study.

2.2.2. *PDV* reagent preparation

The reagent was prepared by dissolving in approximately 100 mL of demineralized water 0.575 g of V_2O_5 and 2.50 g of NaOH. The resulting solution was subjected to magnetic stirring and heating so as to promote solubilization of the solutes. Subsequently, 3 g of pyridin-2,6-dicarboxylic acid and 40 mL of concentrated H_2SO_4 were added. After cooling, the solution was diluted to the final volume of 250 mL with demineralized water. Excess pyridin-2,6-dicarboxylic acid, which precipitates at the bottom of the vessel over 24 h, was removed by filtration.

2.2.3 Determination of hydrogen peroxide

The determination of hydrogen peroxide was carried out spectrophotometrically by exploiting the absorption of light at a wavelength of 427 nm by the stable bone-peroxo-pyridin-2,6-dicarboxyvanadate (OPDV) complex in acidic environment formed by the reaction between hydrogen peroxide and the PDV reagent (vanadate ion + pyridin-2,6-dicarboxylic acid) in the reaction chamber of the above-described apparatus (Fig.24). The intensity of radiation absorption is directly proportional to the concentration of H_2O_2 in solution.

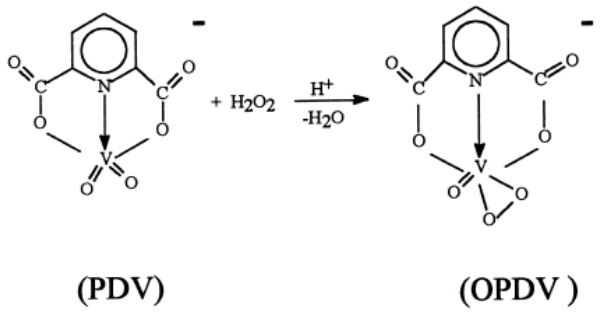


Fig 24: OPDV Formation

2.3 Killing curves of *Legionella pneumophila* in the presence of NH_2Cl

2.3.1 Hard water with Yeast Extract

According to the protocol CEN/TC216 No. 461, hard water for dilution of products, treatment of water and general purposes (HWGP) was used as aqueous media to test the monochloramine activity. During its preparation 10 ml of 0.5 g/l yeast extract was also added to the other saline components (final concentration: 0.0005 %) as interfering substance.

2.3.2 Bacteria test suspension

In-vitro determination of monochloramine biocidal activities was carried out according to an amendment to the protocol CEN/TC216 N°461 using a reference strain of *Legionella pneumophila* serogroup 1 subtype Philadelphia 33152. After thawing, the strain was grown in Buffered Charcoal Yeast Extract (BCYE) agar plates for 3 days at 37°C with 2.5% CO_2 and then sub-cultured for other additional 72 h in the same conditions. Bacteria were then collected from the plates and suspended in about 60 ml of Page's solution, prepared as described in the protocol, in order to obtain an optical density of 0.6 at 600 nm. The viable bacterial count at time zero was assessed as described in 2.4.4.

2.3.3 Test solutions for NH_2Cl and procedure

20-30 min before every contact test, 180 ml of the hard water with yeast extract and an aliquot of the monochloramine stock solution sealed in different vessels were thermostated in a first water bath at the test temperature (40, 45 or 50°C). At the same time, 20 ml of the bacteria suspension was kept at 40-45°C in a second water bath.

When the thermal equilibrium was reached, a suitable volume (2-4 ml) of the monochloramine stock solution was added to the hard water with yeast extract in the first water bath. After further 10 min during which the decomposition rate of the

disinfectant stabilized, the volume of bacteria suspension in the second water bath was entirely poured in the vessel containing the hard water with yeast extract and the disinfectant.

Aliquots of this final suspension were taken at fixed intervals of time (2-34 min) to determine the concentration of residual monochloramine and the number of surviving *Lp* bacteria.

2.3.4 Determination of the number of *Legionella pneumophila* bacteria

The viable bacterial count at time zero (N_0) was assessed by plating 10-fold serial dilutions of the suspension in BCYE agar plates. The latter were incubated at 37°C for 5-7 days before enumeration.

At the end of the selected contact time, the bactericidal activity of the residual concentration of monochloramine was neutralized by dilution. The viable bacterial count at the contact time (N_t) was assessed as described before for N_0.

2.3.5 Determination of residual NH_2Cl in aliquots of *in vitro* disinfected test solution

Monochloramine concentration in stock and test solutions was determined by applying an amendment to indophenol blue colorimetric method developed for the quantitative analysis of ammonia in aqueous solutions **(APHA 2016)**. To that end, an aliquot of sample not exceeding 2.5 ml was added with 100 µl of citrate buffer, 50 µl of phenate and 50 µl of nitroprussiate (reagent concentration as specified in before diluting to the final volume of 3 ml. After a reaction time of 1 h at least, the spectrophotometric absorption of the solution was measured at 640 nm.

In the case of test solutions, samples were filtered through a 0.45-µm membrane to remove suspended solids before the colorimetric determination of the disinfectant.

2.3.6 Data analysis

Acquired data were processed using first-order kinetic models already developed by ISS research group for the study of other disinfectants.

For each examined temperature, the decimal logarithm of the survival ratio N_t/N_0 was linearly correlated to the product of the contact time and the average concentration of the disinfectant detected during the *Lp* exposure to the biocide. The latter parameter was calculated by integrating the temporal trend of the disinfectant concentration between the time zero and the contact time *t*.

2.4 Killing curves of *Legionella pneumophila* in the presence of H_2O_2-Ag^+

2.4.1 Hard water with Yeast Extract

According to the protocol CEN/TC216 No. 461, hard water for dilution of products, treatment of water and general purposes (HWGP) was used as aqueous media to test the monochloramine activity. During its preparation 10 ml of 0.5 g/l yeast extract was also added to the other saline components (final concentration: 0.0005 %) as interfering substance.

2.4.2 Bacteria test suspension

In-vitro determination of monochloramine biocidal activities was carried out according to an amendment to the protocol CEN/TC216 No. 461 (11) using a reference strain of *Legionella pneumophila* serogroup 1 subtype Philadelphia 33152. After thawing, the strain was grown in Buffered Charcoal Yeast Extract (BCYE) agar plates for 3 days at 37°C with 2.5% CO_2 and then sub-cultured for other additional 72 h in the same conditions. Bacteria were then collected from the plates and suspended in about 60 ml of Page's solution, prepared as described in the protocol, in order to obtain an optical density of 0.6 at 600 nm. The viable bacterial count at time zero was assessed as described in 2.4.4.

2.4.3 Test solutions for H_2O_2/Ag^+ and procedure

20-30 min before every contact test, 180 ml of the hard water with yeast extract and an aliquot of the hydrogen peroxide/Ag^+ solution sealed in different vessels were thermostated in a first water bath at the test temperature (40, 45 or 50°C). At the same time, 20 ml of the bacteria suspension was kept at 40-45°C in a second water bath.

When the thermal equilibrium was reached, a suitable volume (2-4 ml) of the H_2O_2/Ag^+ solution was added to the hard water with yeast extract in the first water bath. After further 10 min during which the decomposition rate of the disinfectant stabilized, the volume of bacteria suspension in the second water bath was entirely poured in the vessel containing the hard water with yeast extract and the disinfectant.

Aliquots of this final suspension were taken at fixed intervals of time (2-34 min) to determine the concentration of residual monochloramine and the number of surviving *Lp* bacteria.

2.4.4 Determination of the number of *Legionella pneumophila* bacteria

The viable bacterial count at time zero (N_0) was assessed by plating 10-fold serial dilutions of the suspension in BCYE agar plates. The latter were incubated at 37°C for 5-7 days before enumeration.

At the end of the selected contact time, the bactericidal activity of the residual concentration of monochloramine was neutralized by dilution. The viable bacterial count at the contact time (N_t) was assessed as described before for N_0.

2.4.5 Determination of residual H_2O_2 and Ag^+ in aliquots of *in vitro* disinfected test solution

An aliquot (0.3-2.5 mL) of test solution disinfected with H_2O_2-Ag^+ was introduced into a 5-mL volumetric flask containing 200 µL of the PDV reagent. After dilution to volume with distilled water, the resulting solution was analyzed

spectrophotometrically at a wavelength of 427 nm using a Lange Xion Σ500 colorimeter equipped with a cuvette with a 1-cm optical path.

Determination of silver in water samples disinfected with H_2O_2/Ag^+, taken along the water supply and acidified with HNO_3 at pH ≤ 2, was performed by atomic emission spectrometry with an inductive plasma source at wavelengths of 328.07 nm and 338.28 nm, using a Perkin-Elmer Optima 4300DV spectrometer and a CETAK ultrasonic nebulizer **(Istisan 07/31, APHA 1998)**.

2.4.6 *In vitro* activity of H_2O_2-Ag^+

For the *in vitro* determination of the activity of the solution containing hydrogen peroxide and silver ions some tests have been carried out applying, after modification, a new protocol still in draft elaborated by the European Commettee for Standardisation. According to this protocol, the activity of the disinfectant is assayed in a culture medium suitable for the growth of *Legionella*, even in the presence of interfering substances that simulate any agents that interact with the activity of the disinfectant. The tests in question were performed using a reference strain of *Legionella pneumophila* serogroup 1 subtype Philadelphia ATCC 33152, grown in agar BCYE (Buffered Charcoal Yeast Extract). At the end of the contact time the action of the disinfectant was blocked with the enzyme catalase which selectively decomposes hydrogen peroxide. At the same time an aliquot of the test solution was subjected to chemical analysis to evaluate the residual concentration of the two components of the biocide.

After some preliminary experiments to fine-tune the method, four different concentrations of the disinfectant solution H_2O_2-Ag^+ were tested at contact times between 1.5 and 72.0 hours, according to the scheme shown in Tab. 5, and in the presence of quantities of *Legionella pneumophila* included in a range of $3{,}7 \cdot 10^7$-$3{,}5 \cdot 10^9$ CFU/mL.

Subsequently, in order to integrate the data already in possession with further experimental values capable of defining more precisely the correlation between the

activity of the biocide and the conditions of use, further tests were scheduled at closer contact times according to the scheme in Tab. 6.

Table. 5. In vitro activity of H_2O_2–Ag^+

Contact Time (h)	H_2O_2 Concentration (mg/L)	Ag^+ Concentration (µg/L)
1,5	150	100
1,5	150	100
1,5	50	50
1,5	50	50
1,5	30	30
1,5	30	30
1,5	15	10
1,5	15	10
3	150	100
3	150	100
3	50	50
3	50	50
3	30	30
3	30	30
3	15	10
3	15	10
6	150	100
6	150	100
6	50	50
6	50	50
6	30	30
6	30	30
6	15	10
6	15	10
24	150	100
24	150	100
24	50	50
24	50	50
24	30	30
24	30	30
24	15	10
24	15	10

48	150	100
48	150	100
48	50	50
48	50	50
48	30	30
48	30	30
48	15	10
48	15	10
72	150	100
72	150	100
72	50	50
72	50	50
72	30	30
72	30	30
72	15	10
72	15	10

Table 6. Subsequent in vitro testing of H_2O_2-Ag^+ disinfectant activity

Contact Time (h)	H_2O_2 Concentration (mg/L)	Ag^+ Concentration (μg/L)
2	150	150
2,5	100	100
3	100	100
3,5	100	100
4	100	100
6	50	50
8,5	30	30
10,5	30	30
13	30	30
26	15	15
31	15	15

2.4.7 Data analysis

Acquired data were processed using first-order kinetic models already developed by ISS research group for the study of other disinfectants.

For each examined temperature, the decimal logarithm of the survival ratio N_t/N_0 was

linearly correlated to the product of the contact time and the average concentration of the disinfectant detected during the *Lp* exposure to the biocide. The latter parameter was calculated by integrating the temporal trend of the disinfectant concentration between the time zero and the contact time *t*.

2.5 Biofilm growth on different materials

2.5.1 Selected microbial species

The bacteria used in this study were those belonging to the species *Pseudomonas aeruginosa* ATCC 27853 (fig 25).

Bacterial cells stocked at -80°C, were passaged to Tryptic Glucose Yeast Agar (Plate Count Agar Standard Methods Agar) plates.

After being incubated for 24 h at 37°C, a bacterial solution was prepared and diluted with a concentration of 10^6 ufc/mL measured with the Miles-Misra technique (Miles and Misra).

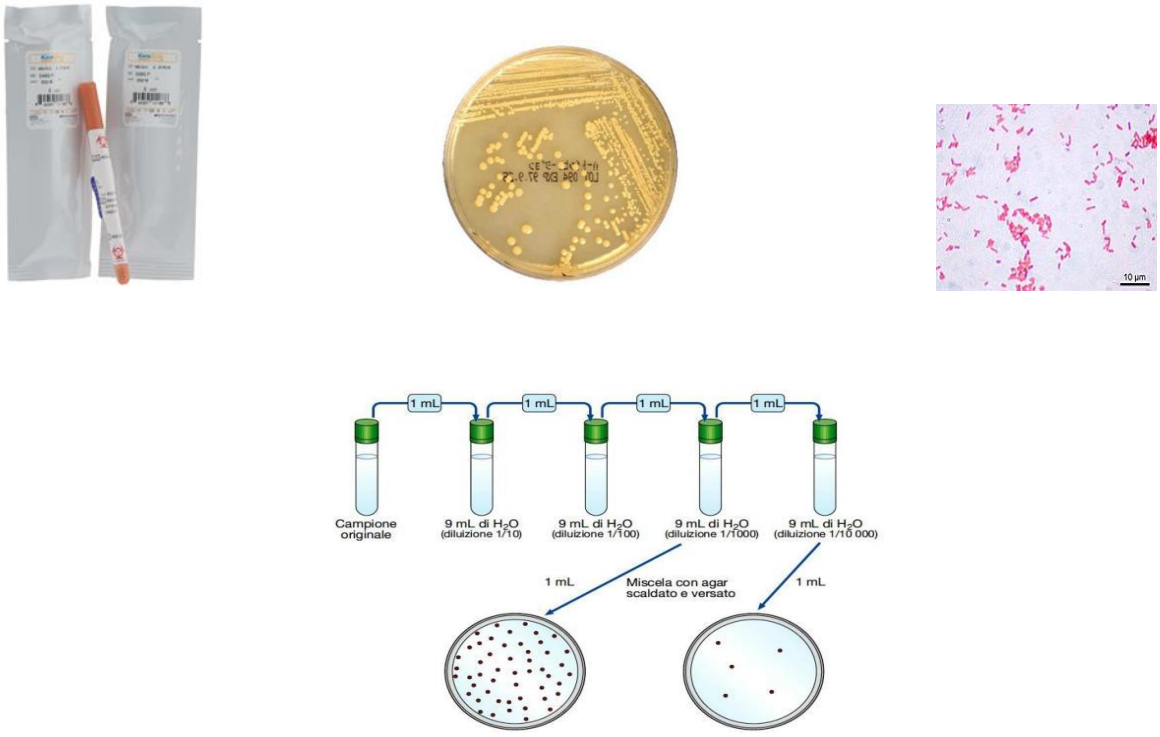


Fig 25: *Pseudomonas aeruginosa* ATCC 27853

2.5.2 Preparation of materials to be tested

Four types of materials, fig 26, were used in this study: polyvinylchloride (PVC), high density polyethylene (HDPE), rubber ethylene propylene diene monomer (EPDM) and polytetrafluoroethylene, to investigate the formation of biofilms produced by the species alone *Pseudomonas aeruginosa,* under laboratory conditions (in the dark and at 25°C).

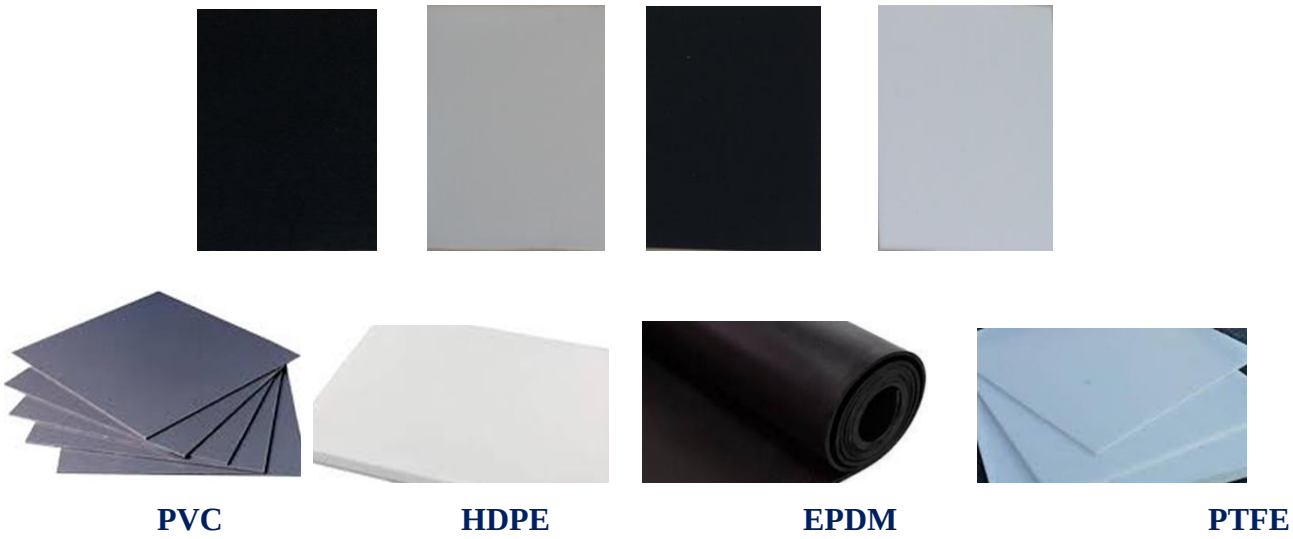


Fig 26: Tested materials

All the materials, PVC, HDPE, EPDM and PTFE, were cut so to obtain specimens of 2x10 cm area and 1mm thick.

Specimens were drilled using a Black and Decker 18.0 V screwdriver drill with a 0.5 mm bit (EPDM and PTFE) and using a cutter (EPDM and PVC) and then individually washed and sterilized at 121°C for 15 minutes.

2.5.3 Preparation of materials used as positive control (C+)

AISI 304 non-magnetic austenitic stainless-steel specimens were used for positive control. Specimens with an area of 2x10 cm and a thickness of 1mm were obtained. The specimens were washed and sterilized at 121°C for 15 minutes before being placed in the positive control tank.

All operations were carried out under aseptic conditions. The tanks were then incubated at 25°C in the dark.

2.5.4 Preparation of materials used as negative control (C-)

Glass specimens (Specimen slides) measuring 76x26 mm in area and 1.2 mm thick were used for the negative control. The specimens were washed and sterilized at 121°C for 15 minutes before being placed inside the negative control tank. All operations were carried out under aseptic conditions.

The tanks were then incubated at 25°C in the dark.

2.5.5 Tank preparation

Glass tanks, rectangular in shape, (length 26 cm, width 18 cm and height 8 cm) were set up under aseptic conditions, one for each type of material to be tested (PVC, HDPE, EPDM e PTFE).

Tanks, previously washed and sterilized at 121°C for 15 minutes, were set up with specimens of the materials to be tested, fill with 2 litres of tap water sterilized, to eliminate the environmental bacterial flora present, and inoculated with a concentration 10^6 of *Pseudomonas aeruginosa* ATCC 27853.

Three glass rods, previously washed and sterilized (having these dimensions: length 25.8 cm, width 1 cm, thickness 2 mm) were placed in each glass tank, on which specimens were placed (10 specimens for each rod), drilled with a Black and Decker 18.0 V screwdriver drill with a 0.5 mm tip (EPDM and PTFE) and with a cutter (EPDM and PVC) and then washed and sterilized at 121°C for 15 minutes.

All operations were carried out under aseptic conditions.

The tanks were then incubated at 25°C in the dark.

Fig 27: Glass tanks

Biofilm formation produced by *Pseudomonas aeruginosa* on specimens of the materials analysed has been evaluated by two different techniques: the quantitative cultivation method and the bioluminometric method for the measurement of ATP (adenosin triphosphate).

2.5.6 Test analysis T_0

On the same day that the material and control tanks were set up, analyses were performed by cultural (Tab 7) and bioluminometric methods (Tab 8) at T_0 to check for any contamination in the materials tested.

Tab 7. Cultural Analysis at T_0

Analysis T_0	**Read at 24 h**	**Read at 48 h**
EPDM	0	0
PTFE	0	0
PVC	0	0
PEAD	0	0
C+	0	0
C-	0	0

Tab 8. Bioluminometric analysis T_0

Analysis T_0	**Read at 24 h**	**Read at 48 h**
EPDM	0	0
PTFE	0	0
PVC	0	0
PEAD	0	0
C+	0	0
C-	0	0

2.5.7 Test pieces analysis

2.5.7.1 Cultural method

Cultural method allows to determine the concentration of bacteria present on the analysed surfaces that have formed colonies on agarized medium Tryptic Glucose Yeast Agar (as indicated in the **ISO 4833:2003**-Microbology of food and animal feeding stuff- Horizontal method for the enumeration of microorganisms-Colony count technique at 30°C).

The biofilm was removed from the surface of each specimen with by swab; the swabs were eluted in 5 mL of diluent solution (buffer physiological solution) and vortexed.

Known volumes (100 μL) of the prepared solutions by swabs were sown on to agarized plates of Tryptic Glucose Yeast Agar (Plate Count Agar Standard Methods Agar), decimal dilutions of 10^{-1} and 10^{-2}, respectively, were then prepared from the buffered solution, and 100 µL of the two dilutions were seeded in duplicate always on agarized plates of Tryptic Glucose Yeast Agar.

all plates have been incubated at 30°C for 24h.

After incubation the number of colonies was counted as cfu/mL.

Cultural media

- **Tryptic Glucose Yeast Agar (TSA)**

Tryptone 5,0 g

Yeast extract 2,5 g

Glucose 1,0 g

Agar 1,5 g

Distilled water 1000 mL

pH 7,0±0,2

The medium is also commercially available in dehydrated form and is prepared and stored following the manufacturer's instructions.

Rehydrate the medium in distilled water, heat until boiling.

Sterilize at 121 ± 3 °C for 15 minutes. Leave to cool down to 50°C, distribute in Petri dishes and allow to solidify. Store at (5 ± 3) °C for no more than two weeks in optimal condition.

- **Physiological buffered solution**

Na_2HPO_4 1,2 g

NaH_2PO_4 0,22 g

NaCl 8,5 g

Distilled water 1000 mL

pH = 7,2

The medium is also commercially available in dehydrated form and is prepared and stored following the manufacturer's instructions.

Rehydrate the medium in distilled water, heat until boiling.

After dissolving the powders distribute in 100 mL flasks and sterilize at 121 ± 3 °C for 15 minutes.

Store at (5 ± 3) °C for no more than two weeks in optimal condition.

2.5.7.2 Bioluminometric method for ATP measurement

At the same time as the cultivation method, the measurement of the ATP was determined.

The method allows to evaluate the level of organic material and the concentration of microorganisms present in the biofilm formed on the internal walls of the tubes (fig 28).

The specimens were analysed using bioluminometer the results expressed as Relative Light Units (RLU).

The ATP is a molecule used by all living organisms as a usable energy reserve. In every living organism ATP represent the molecular form to store the energy required in cellular metabolic processes in DNA replication synthesis and in other processes.

The ATP molecule is an excellent index of the quality of the living material present in a sample and, the higher the biomass, the higher the ATP present: the quantification of the organic ATP represents an excellent index of the presence of viable cells.

Bioluminescence methods with the use of the firefly luciferase enzyme are most commonly used for the determination of adenine nucleotides. The advantages of these methods are their high sensitivity, selectivity, and relative ease of application **(Khlyntseva 2009)**.

In the bioluminometric method, the biofilm was removed from the surface of each specimen with by a specific swab, present inside a test tube containing a diluent solution (containing luciferase, luciferin, $MgSO_4$, DTT, EDTA, BSA, and tricine buffer salt). After spatulation, each swab was placed back into its tube and gently shaken for a few moments, avoiding the formation of bubbles. Then the tubes containing the swabs were placed inside the bioluminometer for the measurement of the corresponding Relative Light Units (RLU).

For each swab three measurements were taken and then the arithmetic mean of the values was calculated for each specimen tested.

ATP collected on the buffer determines a light signal using the luciferin-luciferase system: the amount of ATP present on the tested surface is directly proportional to the amount of light emitted.

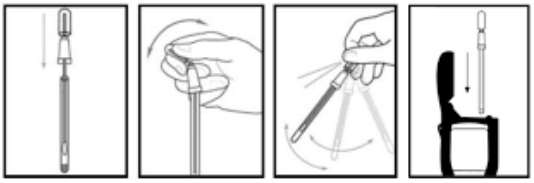


Fig 28: Bioluminometric method

Adenosine triphosphate

The ATP test is a process of rapid measurement of actively growing microorganisms through the detection of adenosine triphosphate or ATP (fig 29).

ATP is quantified by measuring the light produced through its reaction with the natural firefly enzyme luciferase using a luminometer. The amount of light produced is directly proportional to the amount of ATP present in the sample.

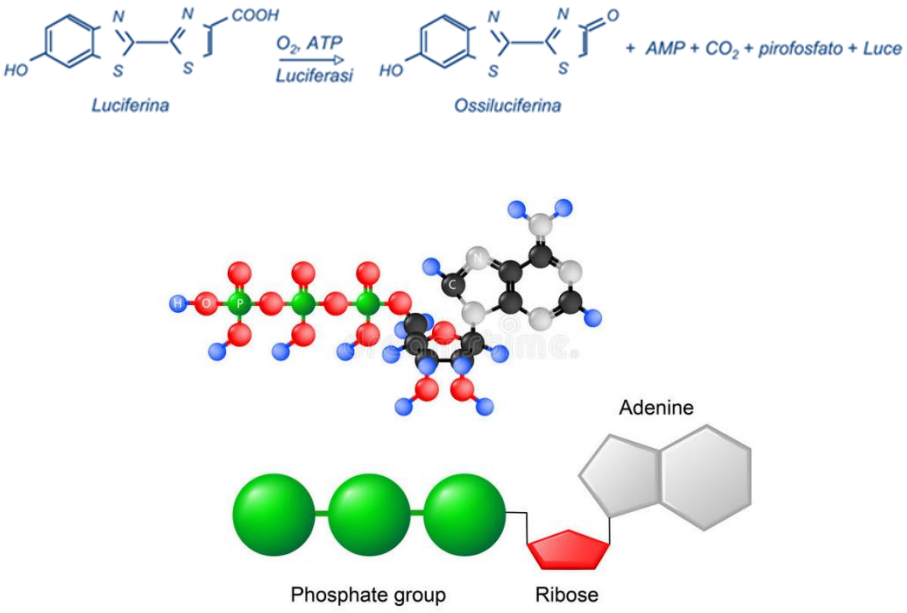


Fig 29: adenosine triphosphate or ATP

Adenosine triphosphate (ATP) is formed in the oxidation reactions and in the course of the glycolytic cleavage of carbohydrates, it is a starting substance in the synthesis of nucleic acids; participates in the regulation of many biochemical processes and is a mediator in synapses. ATP also participates in metabolic processes. It interacts with actomyosin to break down into adenosine diphosphate (ADP) and inorganic phosphate. In this case, energy is released and most of this energy is used by the muscles to perform mechanical work and to synthesize proteins, urea and metabolic intermediates. Therefore, the main function of ATP in the body is to provide energy for many biochemical reactions.

The luciferin-luciferase reaction was discovered as early as 1884. However, detailed studies on firefly luciferase began in 1947 when McElroy was the first to apply this reaction for the determination of ATP.

The mechanism of this reaction has been studied in sufficient detail. It is based on the oxidation of D-luciferin in the presence of ATP and oxygen catalysed by firefly luciferase:

$$\textbf{ATP + D-luciferin + } O_2 \rightarrow \textbf{AMP+ oxyluciferin + PP + } CO_2 \textbf{ + h}\nu$$

where PP is pyrophosphate

The emission spectrum in the region of 470–700 nm is asymmetric with a maximum at 562 nm.

Step II: Tests carried out at distribution-network scale

2.6 Study area

Subject of the present study were two healthcare facilities, indicated as H1 and H2, where sampling and subsequent chemical and microbiological investigations were carried out in order to search for the parameter Legionella pneumophila and to verify the reduction of the latter in the presence of two disinfectants, NH_2Cl and H_2O_2-Ag^+.

2.6.1 Facility H1: general features

Fig 30: H1 facility

The H1 facility, fig 30, has 85 beds and is spread over four floors.

- Ground floor with emergency room, specialist clinics, medical clinic for blood collection.

-First floor with analysis laboratories, blood sampling point, radiology, departments of obstetrics, gynecology, pediatrics, ticket office, offices.

-Second floor with chapel, surgery and orthopaedics departments, day surgery clinic, endoscopy service, minor surgery clinic, operating block.

-Third floor with medical department, echocardiography outpatient clinic, oncology outpatient clinic.

-Fourth floor with medical direction, administration service direction, public relations office.

The H1 facility is a dated structure, although it has undergone recent renovation works both in the HWS production sub-station and in some sections of the water network.
The pipelines used in the HWS circuit are made of galvanized steel, multilayer and C-PVC. Another characteristic of this hospital is represented by the heterogeneity of the materials that make up the network, which is the result of various interventions over the years, including PVC-C.
The HWS hydraulic circuit underwent sanitizing treatment with a hydrogen peroxide - silver salts mixture (H_2O_2 - Ag^+). Checks carried out prior to the start of the experiment covered by this report had revealed a sporadic presence of *Legionella pneumophila* serum group 3 at attention levels ($\leq 10^3$ CFU/l).

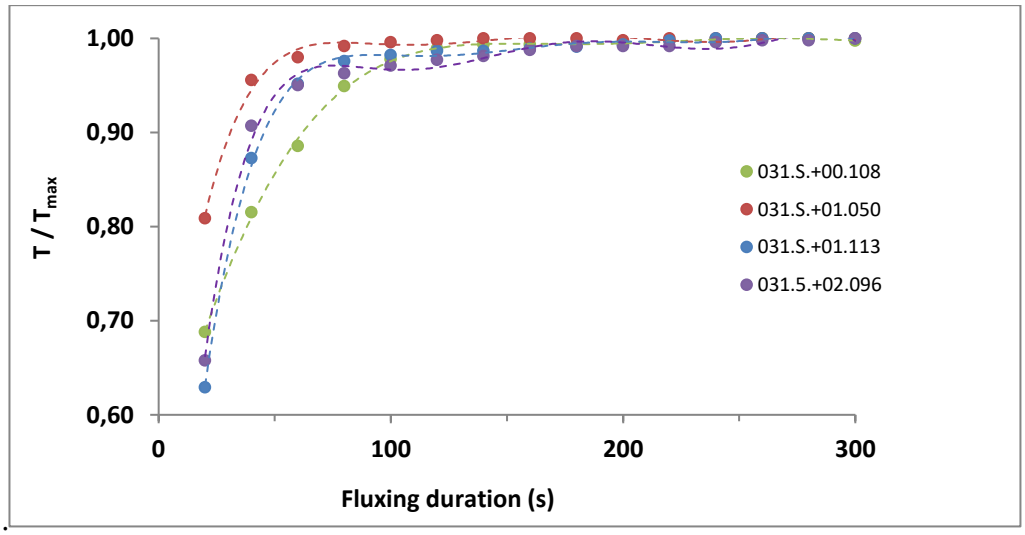
System *status*

In the DHW production plant there is only one boiler group composed of two elements.
The cold-water make-up line (CWS, sanitary cold water) is made up of a single pipe and has a DN (normal diameter) 80 detachment near the single boiler.
There is also a dosing station for the H_2O_2 - Ag^+ mixture, a static mixer and a flow meter to regulate the concentration of the sanitizer in the make-up water.
The cold-water supply to feed the mixing valve is positioned after the injection of the sanitizer.
The distribution of HWS, sanitary hot water, takes place through a manifold connected to a single supply line of the points of use (equipped with a mixing valve) and a second line intercepted by a valve, reserved for possible future expansions. A second sanitizer dosing point has been inserted along the delivery line, downstream of the three-way mixing valve, operated by a timed logic capable of carrying out additional injections of sanitizer in the absence of cold-water replenishment (during the night).
A pair of corrosion-coupon holders were installed along the supply line and the corresponding HWS recirculation line.

HWS temperature trend at consumers as a function of flushing time

Trend of the instantaneous temperature T of the HWS with respect to the maximum value Tmax as a function of the time elapsed from the opening of the tap to the utilities of the structure H1 is shown in the figure 31.

Fig 31. Trend of the instantaneous temperature T of the HWS with respect to the maximum value Tmax as a function of the time elapsed from the opening of the tap to the utilities of the structure H1.



In all cases tested, the HWS temperature reached its maximum value within 4 min of opening the tap.

As a precautionary measure, in subsequent sampling operations under flowing conditions, it was agreed to wait 5 min before sampling HWS.

2.6.2 Facility H2: general features

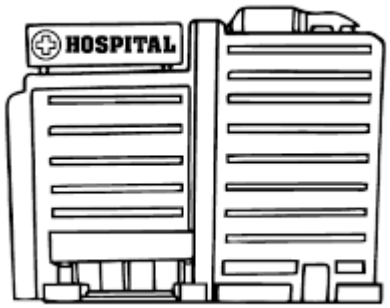


Fig 32: H2 facility

The H2 facility, fig 32, has 60 beds and is spread over three floors.

-Ground floor with entrance-acceptance, physical therapy, hydrotherapy pools, aids center, occupational therapy and chapel.
-First floor with inpatient area I, with two-bed rooms with bath, rehabilitation therapy gyms, neurophysiology area, urodynamic clinic, physiatric clinics and movement analysis laboratory.
-Second floor with inpatient area II, with two-bed rooms with bathroom, gyms for inpatient therapy, logo-therapy area, psychological clinic, offices and management.

This H2 facility is a newly constructed facility.
In this structure, the hydraulic circuit of HWS is made of galvanized steel and multilayer and has been subjected to sanitizing treatment with a mixture of hydrogen peroxide - silver salts following the detection of the presence of *Legionella pneumophila* serum group 1.

System *status*
In the substation intended for HWS production there are three groups of boilers, each of which is composed of two boilers in parallel, activated one group at a time, in rotation, during normal operation.
The cold-water make-up line (CWS) of the three groups of boilers is formed by a single

manifold and has a DN 80 connection near each boiler.

There is a H_2O_2 - Ag^+ mixture dosing station downstream of the flow meter. The derivation of cold water to feed the mixing valve occurs after the injection of the sanitizer.

After inspection, a static mixer was inserted at a distance of about 1 m from the injection point of the sanitizer and a new withdrawal point was defined downstream of the mixer.

The three groups of boilers are connected to the HWS delivery manifold with which two circuits are fed:

1. a high-flow line, consisting of a DN 100 galvanized steel pipe to supply lots 1, 2 south and 3;
2. a low flow line, consisting of a DN 65 galvanized steel pipe for the supply of lot 2 north.

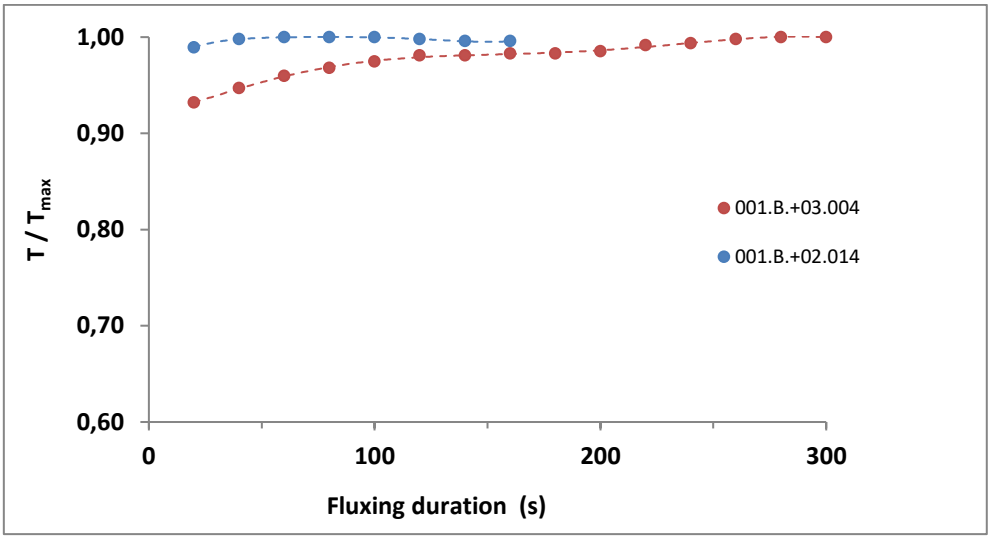
Along each line, a high temperature flow meter was installed to operate the sanitizer dosing pumps with negative feedback, given the imbalance between the flows of the two lines. In addition, a static mixer was installed on each line downstream of the corresponding sanitizer dosing point.

A pair of corrosion-coupon holders were installed along each of the two delivery lines and the corresponding HWS recirculation lines.

<u>HWS temperature trend at consumers as a function of flushing time</u>

Trend of the instantaneous temperature T of the HWS with respect to the maximum value Tmax as a function of the time elapsed from the opening of the tap to the utilities of the structure H2 is shown in the figure 33.

Fig 33. Trend of the instantaneous temperature T of the HWS with respect to the maximum value Tmax as a function of the time elapsed from the opening of the tap to the utilities of the structure H2.



In all cases tested, the HWS temperature reached its maximum value within 4 min of opening the tap.

As a precautionary measure, in subsequent sampling operations under flowing conditions, it was agreed to wait 5 min before sampling HWS.

2.7 Disinfectant residues in use at the investigated networks

2.7.1 Determination of NH_2Cl residues in HWS samples

Monochloramine was assayed spectrophotometrically. For this purpose, 10 ml of sample, filtered through 0.45 µm nitrocellulose membranes, was spiked with 400 µl of citrate buffer, 200 µl of phenate, and 200 µl of nitroprusside (concentration of reagents as provided in APHA et al., 2016). After a minimum reaction time of 1 h, the absorbance of the solution at 640 nm was measured using a Lange Xion Σ500 colorimeter and a cuvette with a 1-cm optical path.

2.7.2 Determination of H_2O_2 residues in HWS samples

Residual hydrogen peroxide contained in HWS samples taken along the water mains of the two facilities was assayed spectrophotometrically at a wavelength of 427 nm

following the formation of the stable oxoperoxypyridin-2,6-dicarboxyvanadate (OPDV) complex in an acidic environment by reaction between hydrogen peroxide and the PDV reagent (vanadate ion + pyridin-2,6-dicarboxylic acid).

The intensity of radiation absorption is directly proportional to the concentration of H_2O_2 in solution.

For this purpose, 10 ml of the sample, filtered through 0.45 µm nitrocellulose membranes, was spiked with 400 µl of the 25 mM PDV reagent. The resulting solution was analyzed spectrophotometrically at a wavelength of 427 nm using a Lange Xion Σ500 colorimeter equipped with a cuvette with an optical path of 1 cm.

The reagent was prepared by dissolving 0.575 g of V_2O_5 and 2.50 g of NaOH in approximately 100 ml of demineralized water. The resulting solution was subjected to magnetic stirring and heating so as to promote solubilization of the solutes. Subsequently, 3 g of pyridin-2,6-dicarboxylic acid and 40 ml of concentrated H_2SO_4 were added. After cooling, the solution was diluted to a final volume of 250 ml with demineralized water. Excess pyridin-2,6-dicarboxylic acid, which precipitates at the bottom of the vessel over 24 h, was removed by filtration.

2.6 Presence of *Legionella* in the investigated networks

Significant sampling point were identified at two facilities studied H1 and H2.

2.8.1 Water Sampling

At each sampling point, water samples were collected in special sterile bottles (2000 ml of HWS). To neutralize the residual-free chlorine, sodium thiosulfate was added to sterile bottles for *Legionella* spp. determination. The water sampling was carried out from the established sampling points along the delivery and recirculation pipes inside the heating plants and from the utilities (sinks or showers) of the hospital facilities.

Once collected, samples protected from sunlight were processed within 24 hours of collection.

2.8.2 Analysis of *Legionella* in HWS samples by culture methods

An aliquot (1000 ml) of homogenized sample was analyzed according to the standard **ISO 11731:1998** *"Water quality, detection and enumeration of Legionella"*, fig 34.

2.8.2.1 Membrane Filtration

Filter the samples and place the untreated membrane filter directly on the plate of *Legionella* Buffered Charcoal Yeast Extract Agar Base (BCYE).

Fig 34: Cultural method

2.8.2.2 Heat Treatment

After filtration, bacteria collected on the membranes were resuspended in 10 ml of the original water sample, and place in a water bath at (50°C±1) °C for (30±2) min.

0,1 ml of the suspension was spread on a Petri dish containing BCYE agar and MWY agar.

2.8.2.3 Incubation

The inoculated plates were then incubated for 7–10 days at 37 ± 1°C, in humidified atmosphere (air with 2,5% CO_2 can be beneficial for the growth of some *Legionella* but it is not essential).

2.8.2.4 Examination of the plates

Legionella colonies appear as convex, as in fig 35, circular white colonies having a centre that resembles ground glass. Colony edges are entire and tend to have speckled

green or pinkish purple iridescent edges. The colour of the colonies may be a variety of shades of purple or green or a range of colours depending on the thickness of the agar plate and the age of the culture (colonies become grey with age). Under an ultraviolet lamp, colonies of several species (*L. bozemanii, L. dumoffii, L. gormanii, L. parisiensis* and *L. tucsonensis*) autoflorence brilliant white. Colonies of *L. pneumophyla* arrear dull green often tinged with yellow.

The colour of the fluorescence can help to differentiate colonies in samples containing different species of *Legionella*.

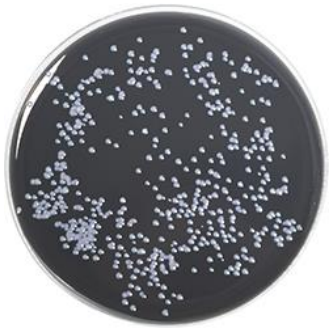


Fig 35. *Legionella* colonies on BCYE

2.8.2.5 Confirmation of presumptive *Legionella* colonies

If more morphological different types of presumptive colonies of *Legionella* are growing on the plate, take at least one colony from each type and place on BCYE agar and on Blood agar, more selective medium, and incubate at (36±2) °C for 2 d to 5 d.

Isolated colonies were identified using an agglutination test (*Legionella* latex test; Oxoid).

- **Catalase Test**

If the tested bacterial strain is capable of producing catalase, a small inoculum of it (3-4 colonies) is mixed in a 3% hydrogen peroxide solution and bubbles are produced.

The lack of catalase is evident by the lack or weak production of bubbles. Positive catalase bacteria include obligate aerobes and facultative anaerobes, which have the ability to use oxygen as a final electron acceptor.

Negative catalase bacteria can be obligate anaerobes or facultative anaerobes that ferment by not using oxygen as a final electron acceptor (e.g., *Streptococci*). All *Legionella* species are positive.

- **Agglutination Test**

Presumptive identification at the species level is done using agglutination techniques with specific antisera.

The latex agglutination test, "*Legionella* Latex Test", uses blue latex particles sensitized with rabbit antibodies that agglutinate in the presence of latex antigens.

Sensitized with rabbit antibodies that agglutinate in the presence of specific antigens of the cell wall antigens, forming a precipitate visible to the naked eye. This screening method allows for the rapid recognition of serotypes of the pathogenic species of *Legionella* isolated: at present there are commercially available antisera related to 14 different *Legionella* serotypes (fig 36). Serogroup 1 is considered the most pathogenic and is responsible for the greatest number of cases of cases of disease.

For the agglutination test, 4 to 10 colonies with the same morphology are picked up with a loop and diluted in a tube containing 0.4 ml of saline (0.85% NaCl). The cell suspension is vortexed for 5 seconds and taken with an automated pipette calibrated at 250 µl. This aliquot is distributed within each of the four circles provided on the reaction card and mixed with the drop of each latex reagent (3 test reagents and the control reagent).

Gently rotate the reaction card for about one minute and observe for any agglutination: the result is positive if agglutination of the blue latex particles occurs within one minute and no agglutination occurs in the control area. A positive reaction indicates that the agents of a certain serogroup of *Legionella* species have been detected in the sample.

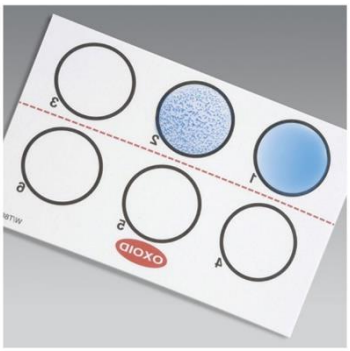


Fig 36: Agglutination test

2.8.2.6 Gram stain

Legionella stains poorly with gram stain and stains positive with silver or Giemsa stain. Gram stain should be prepared from culture on charcoal yeast extract agar with iron and cysteine.

2.8.3 Analysis of *Legionella* in HWS samples by molecular methods

An aliquot (1000 ml) of homogenized sample was analyzed by Real Time PCR according to the standard ISO/TS 12869:2012 *"Water quality -- Detection and quantification of Legionella spp. and/or Legionella pneumophila by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)".* Dopo aver filtrato il campione (fig 37) attraverso filtri in policarbonato da 0,4 µm, il filtro è stato utilizzato per l'estrazione diretta del DNA mediante Aquadien Kit (BioRad), in accordo con le procedure fornite con il kit.

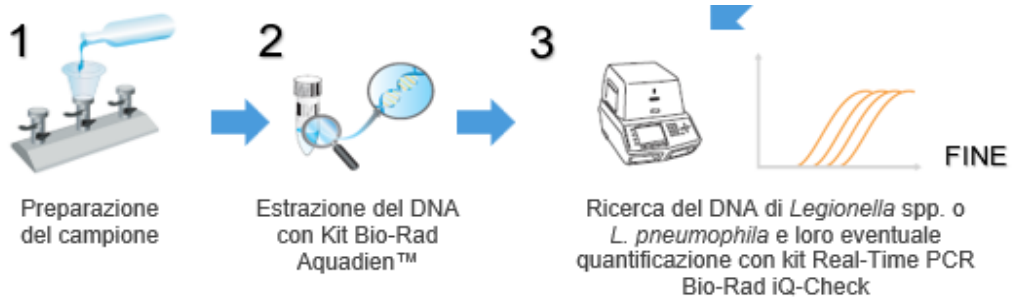


Fig 37: Molecular method

2.9 Microbial characterization of biofilm grown on test specimens

2.9.1 Specimens submitted to analysis

Trials were conducted using specimens of the following materials shown in Fig 38-39:

- certified coupons of AISI 316L stainless steel and galvanized steel (Fig 38)

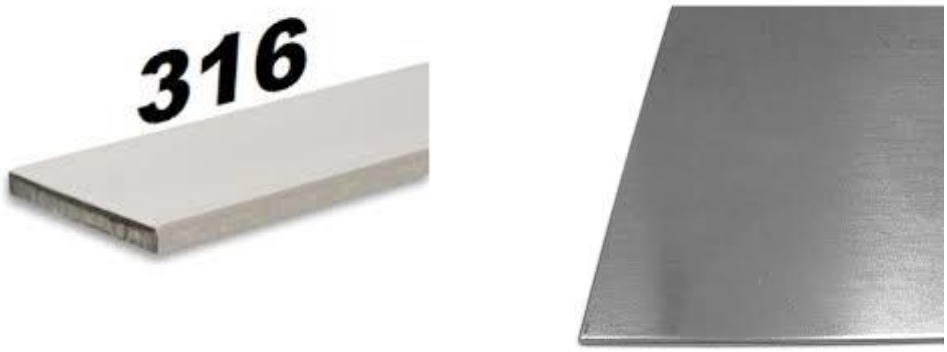


Fig 38: AISI 316L stainless steel and galvanized steel

- handcrafted specimens of C-PVC and multilayer, obtained by longitudinally cutting pieces of pipe. For this last material, the Teflon inner layer has been selected for the direct contact with the HWS flow (Fig 39).

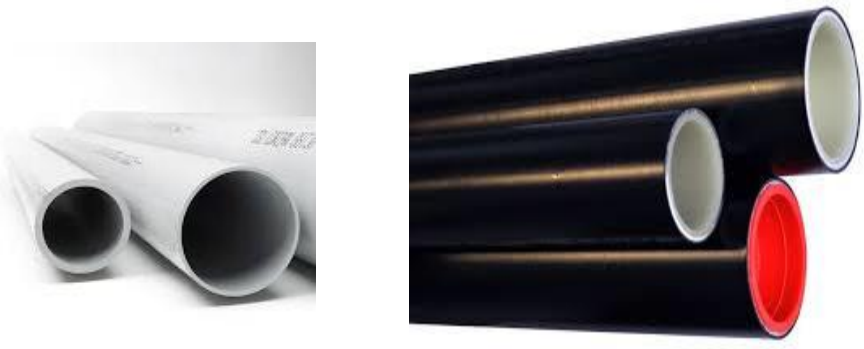


Fig 39: C-PVC and multilayer

2.9.2 Preliminary specimens processing

Each specimen was stored in a silica gel desiccator for at least 24 hours and then weighed using an analytical balance with a sensitivity of no less than 0.1 mg.

All specimens were preliminarily washed with distilled water, dried with paper, immersed in 70% ethyl alcohol for 10 min, rinsed with sterile distilled water, and stored in a clean sterile container until further use.

2.9.3 Installation of specimens in the HWS networks

Before the start of the contact tests, each specimen was hooked to the appropriate support bar, as shown in Fig. 40, and immersed in different solutions: 40 mL 70% ethanol for 10 minutes

- 40 mL sterile water
- 40 mL sterile water

Fig 40: Connection of support bar - specimen

In a second step, the "support bar - specimen" system was inserted into the HWS recirculation hydraulic circuit as shown in Fig. 41, noting the specimen identification code.

Fig 41: Contact chambers between ACS and specimens

2.9.4 Recovery of test specimens

After the contact time inside the HWS water system, each "support bar - specimen" system was taken out of the hydraulic circuit, carefully avoiding both any kind of mechanical shaking and any contamination deriving from direct contact with other surfaces.

Each specimen, using the support bar, was washed, by immersion, in 40 mL of demineralized water contained in 50 mL Falcon tubes. It was then recovered from the holder by slowly sliding it into a 50 mL Falcon and stored dry until the next analysis. Tubes containing the collected samples were kept at 4°C.

2.9.5 Detection of *Legionella* in biofilm samples

The biofilm samples, present in the tube were subjected to sonication treatment at 51 W for 4 min inside the same Falcon tube containing the biofilm immersed in water, keeping the level of the latter below the level of the ultrasonic bath;

- Subsequently, they were vortexed for 5 s of the biofilm suspension;
- - biofilm suspensions were filtered onto 0.22 µm polycarbonate membranes. The remaining biofilm, which may still be present on the specimen surface, was removed using a sterile scraper and suspended in a small volume of sterile water. The newly obtained biofilm suspension was vortexed for 5 s and then filtered

onto 0.22 µm polycarbonate membranes;

- The membranes on which the biofilm was filtered were placed in a sterile 50 mL Falcon tube and stored at -20°C or -80°C.

2.9.6 DNA extraction from biofilm and microbiome analysis

The total DNA extracted from the coupons was subjected to determination of the microbiome present on these devices by 16S rRNA analysis. For this purpose, the Miseq2 instrument (ILLUMINA) was used to obtain the sequences. Bioinformatic analysis of the microbiomes was performed using the software "Amplicon Analysis Pipeline analyse 16S rRNA data from Illumina Miseq paired-end reads" present in the Galaxy-ARIES platform. The content of operational taxonomic units (OTUs) in the samples and subsequent analyses were performed using MEGAN 6 software.

2.10Analysis of the corrosion degree of the specimens

The degree of corrosion of the specimens and the extent of sediment accumulated on their surface during exposure to HWS flow was evaluated by applying the procedure described in both the American ASTM G1 standard and ISO/FDIS 8407, adapted as appropriate.

To this end, each specimen was first immersed in 50 ml of demineralized water directly inside the Falcon tube in which it was stored. weakly adhered to the surface of the coupon. Subsequently, the coupon was transferred to a new Falcon tube and treated successively with 50 ml of demineralized water and 50 + 50 + 50 ml of diammonium citrate 200 g/l, always at a temperature of 70°C for 20 min. The second treatment in water allowed the elimination of the stratifications strongly adhered to the coupon surface, while the following treatments with diammonium citrate allowed the evaluation of the coupon corrosion degree by removing the corrosion products and part of the underlying substrate. Correct estimation of the individual contributions was performed by graphing the weight changes of the specimen at the end of each treatment (Fig. 42), as described in both the American ASTM G1 standard and ISO/FDIS 8407,

and applying the following equations:

- Weight of weakly adhered strata = A-B
- Weight of strongly adhered layers = B-C
- Weight loss due to corrosion = C

The results obtained were expressed as specimen corrosion rate and stratification formation rate using the following equation:

Rate = 10^4 *ΔWeight/ Specimen surface * Exposure time

where specimen area is expressed in cm^2, exposure time in hours, weight change in g, and rate in g/ (m^{2}·h).

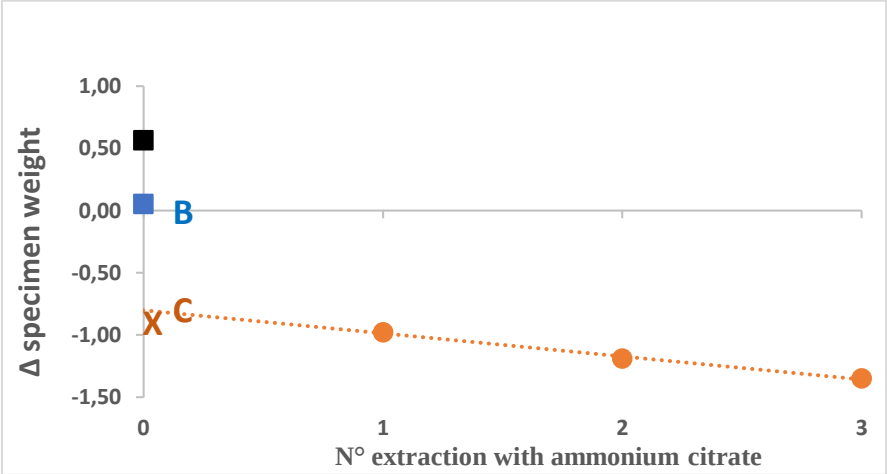


Fig 42: Analysis of the corrosion degree of the specimens

CHAPTER 3: RESULTS

3.1 Stability of monochloramine (NH_2Cl) and hydrogen peroxide/silver ions (H_2O_2-Ag^+)

3.1.1 Decomposition kinetics of monochloramine

Fig.43 shows the trend of monochloramine decomposition, measured at 50°C. No significant decomposition was observed for monochloramine in demineralized water at least with regard to the time interval under investigation (0-60 min).

In addition, no significant effects were detected as the temperature of the test solution changed over the range 40-50°C.

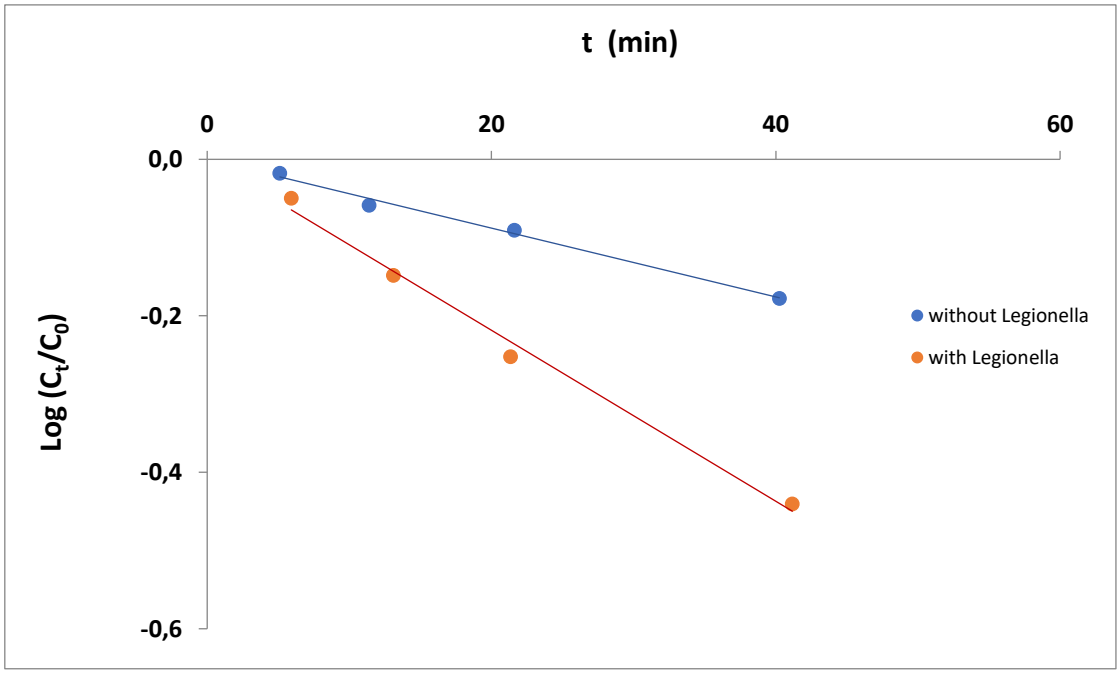


Fig 43. Decomposition kinetics of monochloramine before (●) and after (●) inoculation of *Legionella* species at a temperature of 50°C.

3.1.2 Preliminary tests to identify the starting conditions to be adopted in subsequent kinetic tests

Preliminary tests were conducted by introducing different amounts of monochloramine (final concentration of 0.2-3.5 mg/l) into the test solution inoculated with *Legionella pneumophila* at approximately 10^8 CFU/ml and thermostated at 45°C for 10-120 min. Most of these tests did not provide useful data due to total bacterial kill and/or complete decomposition of monochloramine. However, the following information could be obtained:

- monochloramine is rapidly decomposed by the test solution matrix, especially in the presence of *Legionella pneumophila*;
- in order to avoid total killing of *Legionella pneumophila*, the concentration of monochloramine should not exceed 1.2 mg/l by significantly limiting the test contact time (e.g., ≤ 10 min at 1.2 mg/l, ≤ 25 min at 0.8 mg/l);
- Initial disinfectant concentrations of less than 0.7 mg/l should not be tested directly due to decomposition by the matrix in the test solution;
- In order to increase the reproducibility of the acquired data, the single components of the test solution must be thermostated separately, and then mixed, taking care to proceed with the inoculation as soon as the rate of decomposition of the disinfectant is stable.

3.1.3 Killing curves in the temperature range 40-50°C

During the study, tests were performed at different temperatures 40°C, 45°C, and 50°C, from which quantitative data were obtained.

First-order kinetic models applied to survival relationships recorded as a function of contact time and residual disinfectant concentration are shown in Fig. 44.

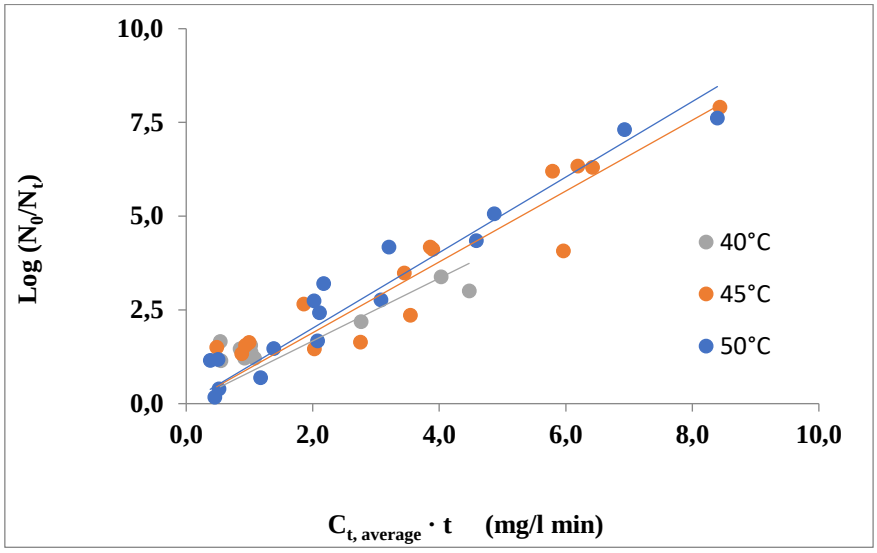


Fig 44. Killing curves at different temperatures of 40, 45 e 50°C

The data collected confirmed that the hypothesized kinetic model allows for an R_T abatement rate estimate from the value of the slope of the lines.

A slight increase in the R_T value was observed as the temperature increased from 40 to 50°C, although this increase appears to be of the same order of magnitude as the experimental uncertainty.

As expected, the abatement rates follow the Arrhenius equation (Fig. 45), which describes the dependence of R_T on the absolute temperature value T (in Kelvin).

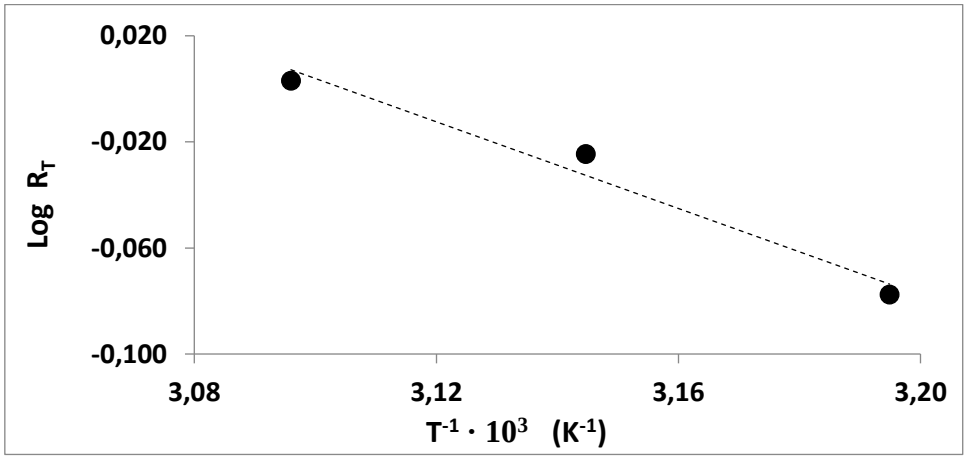


Fig 45. Application of the Arrhenius equation to the knockdown rate of *L. pneumophila*

It follows that the survival ratio of *Legionella pneumophila* in the presence of monochloramine is related to the operating conditions according to the following equation:

$$log\frac{N_0}{N_t} = 10^{2.5-\frac{800}{T}} \cdot Ct$$

3.2 Stability of hydrogen peroxide/silver

3.2.1 Decomposition kinetics of hydrogen peroxide-silver

Fig.46 shows the trend of hydrogen peroxide-silver decomposition, measured at 50°C. The addition of *Legionella pneumophila* to the test solution accelerated the decomposition of the disinfectant. Significant effects were detected as the temperature of the test solution changed over the range 40-50°C.

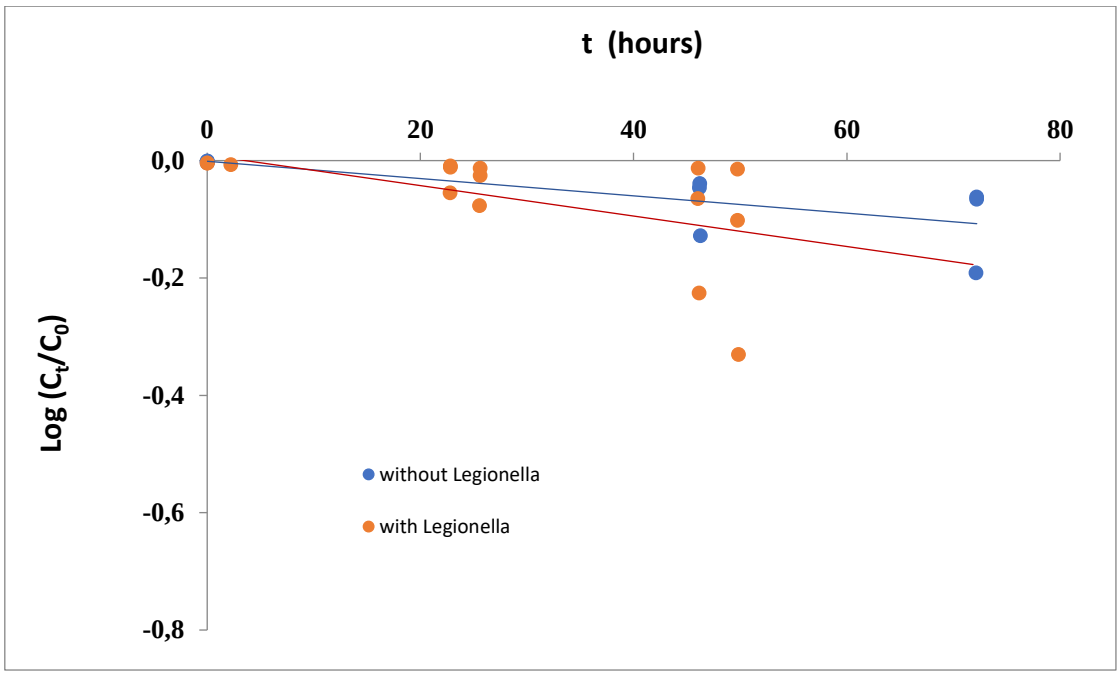


Fig 46. Decomposition kinetics of hydrogen peroxide before (●) and after (●) inoculation of *Legionella* species at a temperature of 50°C.

3.2.2 Preliminary tests to identify the starting conditions to be adopted in subsequent kinetic tests

Tab. 9. Microbiological killing tests with H_2O_2−Ag^+: series 1. $N_0 = 3{,}5 \cdot 10^9$ CFU/mL.

Time h	H_2O_2 mg/L	Ag^+ µg/L	N_t UFC/mL	log N_t/N_0
1,5	150	100	$2{,}2 \cdot 10^5$	-4,2
1,5	50	50	NA	
1,5	30	30	NA	
1,5	15	10	NA	
3,0	150	100	$1{,}3 \cdot 10^2$	-7,4
3,0	50	50	NA	
3,0	30	30	NA	
3,0	15	10	NA	
6,0	150	100	$0{,}0 \cdot 10^0$	
6,0	50	50	$5{,}7 \cdot 10^4$	-4,8
6,0	30	30	NA	
6,0	15	10	NA	
24,0	150	100	$0{,}0 \cdot 10^0$	
24,0	50	50	$0{,}0 \cdot 10^0$	
24,0	30	30	$1{,}0 \cdot 10^0$	
24,0	15	10	$3{,}8 \cdot 10^4$	-5,0
48,0	150	100	$0{,}0 \cdot 10^0$	
48,0	50	50	$0{,}0 \cdot 10^0$	
48,0	30	30	$0{,}0 \cdot 10^0$	
48,0	15	10	$0{,}0 \cdot 10^0$	
72,0	150	100	$0{,}0 \cdot 10^0$	
72,0	50	50	$0{,}0 \cdot 10^0$	
72,0	30	30	$0{,}0 \cdot 10^0$	
72,0	15	10	$0{,}0 \cdot 10^0$	

NC: non-accounting.

Tab. 10 Microbiological killing tests with H_2O_2–Ag^+: series 2. $N_0 = 1{,}1 \cdot 10^9$ C/mL.

Time h	H_2O_2 mg/L	Ag^+ µg/L	N_t UFC/mL	log N_t/N_0
1,5	150	100	$1{,}6 \cdot 10^5$	-3,8
1,5	50	50	NA	
1,5	30	30	NA	
1,5	15	10	NA	
3,0	150	100	$0{,}0 \cdot 10^0$	
3,0	50	50	$9{,}1 \cdot 10^5$	-3,1
3,0	30	30	NA	
3,0	15	10	NA	
6,0	150	100	$0{,}0 \cdot 10^0$	
6,0	50	50	$2{,}3 \cdot 10^4$	-4,7
6,0	30	30	$1{,}5 \cdot 10^6$	-2,9
6,0	15	10	NA	
24,0	150	100	$0{,}0 \cdot 10^0$	
24,0	50	50	$0{,}0 \cdot 10^0$	
24,0	30	30	$1{,}0 \cdot 10^0$	
24,0	15	10	$2{,}6 \cdot 10^3$	-5,6
48,0	150	100	$0{,}0 \cdot 10^0$	
48,0	50	50	$0{,}0 \cdot 10^0$	
48,0	30	30	$0{,}0 \cdot 10^0$	
48,0	15	10	$0{,}0 \cdot 10^0$	
72,0	150	100	$0{,}0 \cdot 10^0$	
72,0	50	50	$0{,}0 \cdot 10^0$	
72,0	30	30	$0{,}0 \cdot 10^0$	
72,0	15	10	$0{,}0 \cdot 10^0$	

NC: non accounting

3.2.3 Killing curves in the temperature range 40-50°C

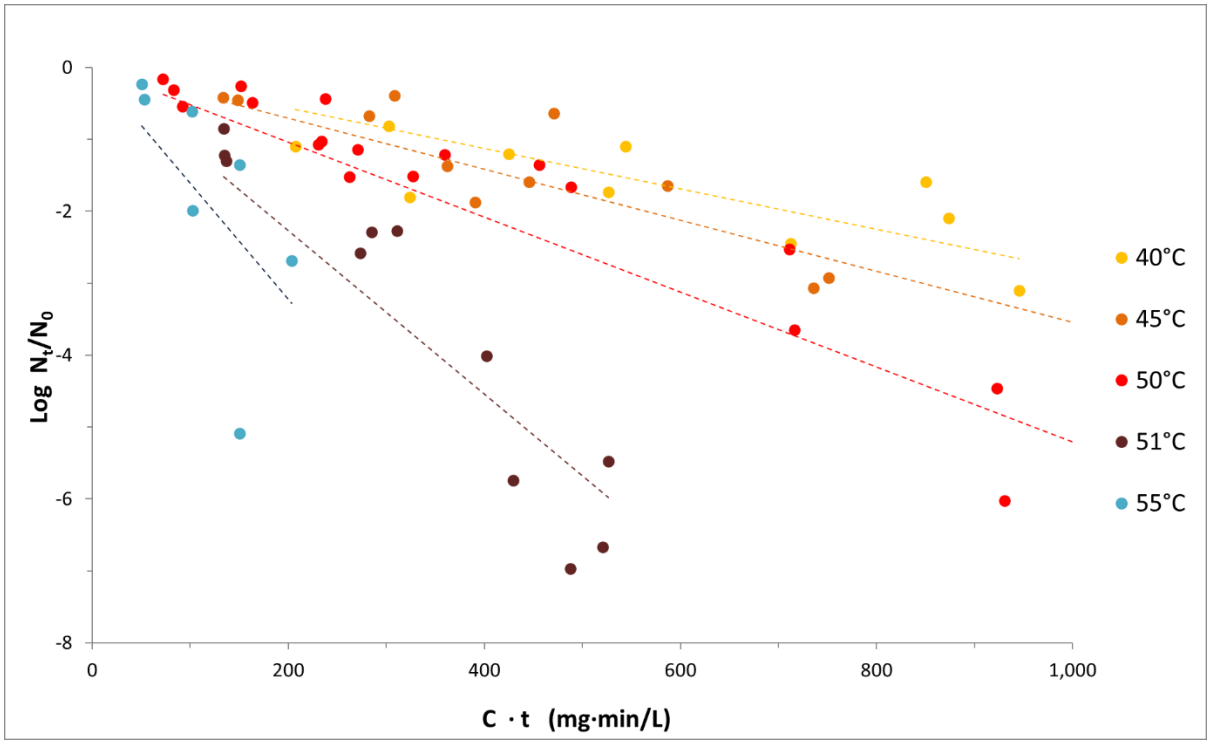


Fig 47. Killing curves in the range 40-50°C with hydrogen peroxide-silver

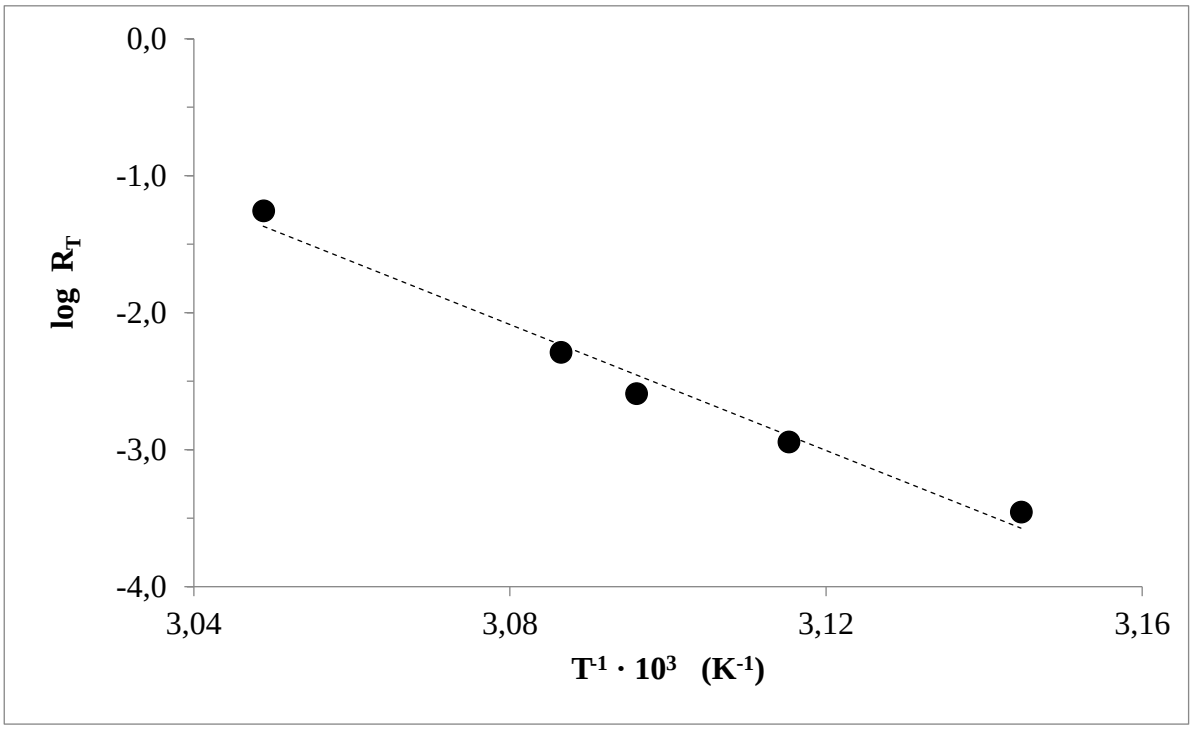


Fig. 48

$$log\frac{N_0}{N_t} = 10^{69-\frac{23000}{T}} \cdot Ct$$

3.3 Killing curves of *Legionella pneumophila* in presence of NH_2Cl

3.3.1 Minimum concentration of disinfectant required to reduce *L. pneumophyla* contamination in a defined time interval

The preceding equation provides an estimate of the minimum dose of NH_2Cl required to achieve a significant reduction in *Legionella pneumophila* contamination in a defined time interval.

A reduction of 3 decimal logarithmic units can be expected after 5 min of contact time when the concentration of monochloramine in water is not less than 100 mg/l at 50°C, as shown in Tab.11.

Tab 11. Monochloramine concentration required to reduce *Legionella* species by 3Log in 5 minutes

T (°C)	Ct (mg*min/L)	C (mg/L)
40	91100	18200
45	6370	1270
50	484	97
55	40	7,9

3.4 Killing curves of *Legionella pneumophila* in presence of H_2O_2-Ag^+

3.4.1 Minimum concentration of disinfectant required to reduce *L. pneumophyla* contamination in a defined time interval

The preceding equation provides an estimate of the minimum dose of H_2O_2-Ag^+ required to achieve a significant reduction in *Legionella pneumophila* contamination in a defined time interval.

A reduction of 3 decimal logarithmic units can be expected after 5 min of contact time when the concentration of hydrogen peroxide/silver ion in water is not less than 0,57 mg/l at 50°C, as shown in Tab.12.

Tab12. Hydrogen peroxide concentration required to reduce *Legionella* species by 3Log in 5 minutes

T (°C)	Ct (mg*min/L)	C (mg/L)
40	3,41	0,68
45	3,11	0,62
50	2,84	0,57
55	2,67	0,52

3.3 Biofilm formation on tested materials

Fig. 49-50 show the trends of microbial biofilm growth on the different types of materials tested, analyzed by the cultural method (Fig. 49) and with the bioluminometric method (Fig 50).

Both methods showed increased biofilm growth on EPDM synthetic rubber while the material least contaminated by biofilm formation was found to be Teflon (PTFE).

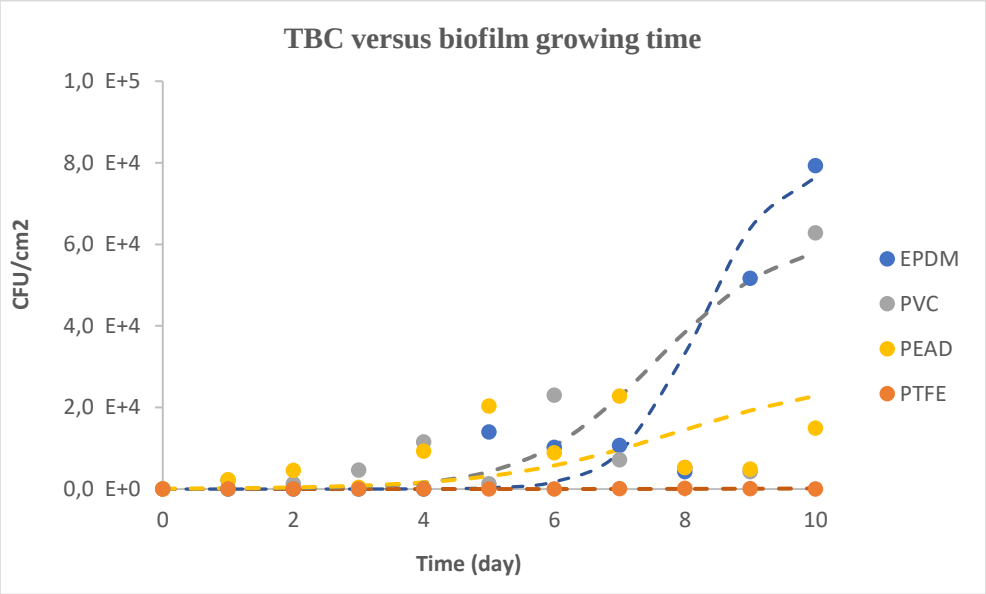


Fig. 49. Total results of cultural method

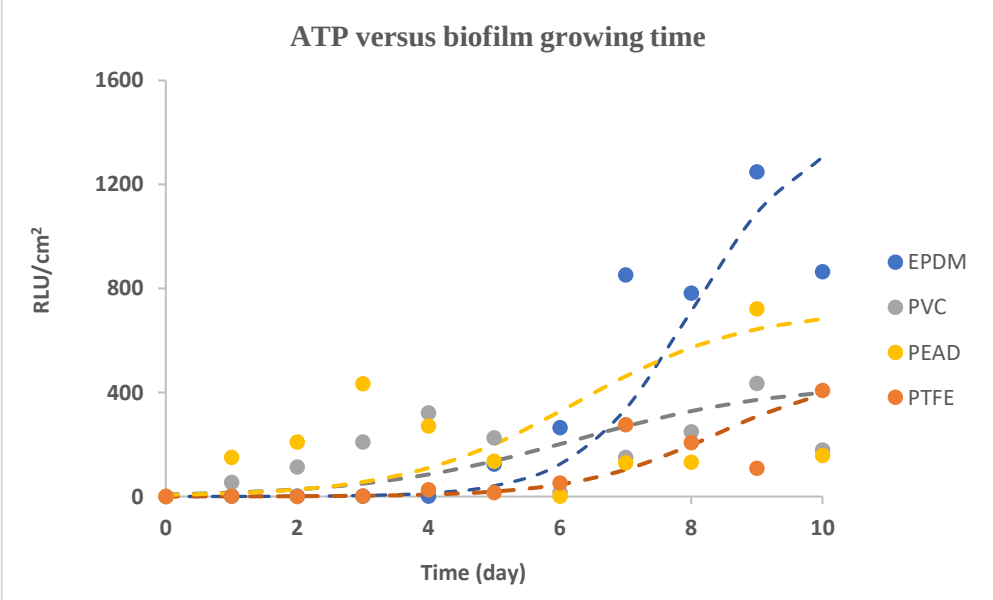


Fig. 50. Total results of bioluminometric method

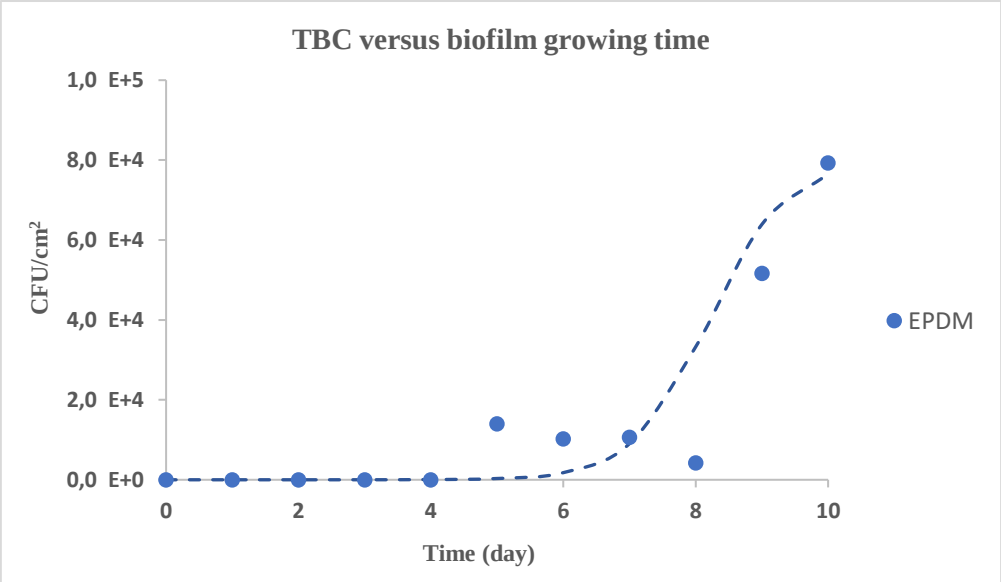


Fig 51. Microbial growth trend measured over time interval on EPDM with cultural method

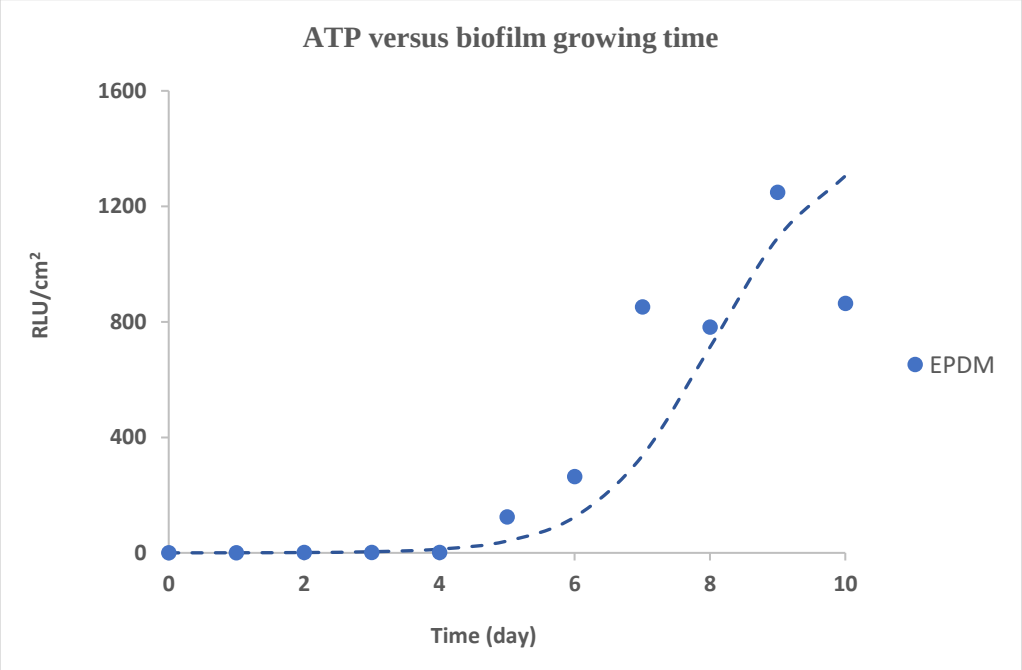


Fig 52. Microbial growth trend measured over time interval on EPDM with bioluminometric method

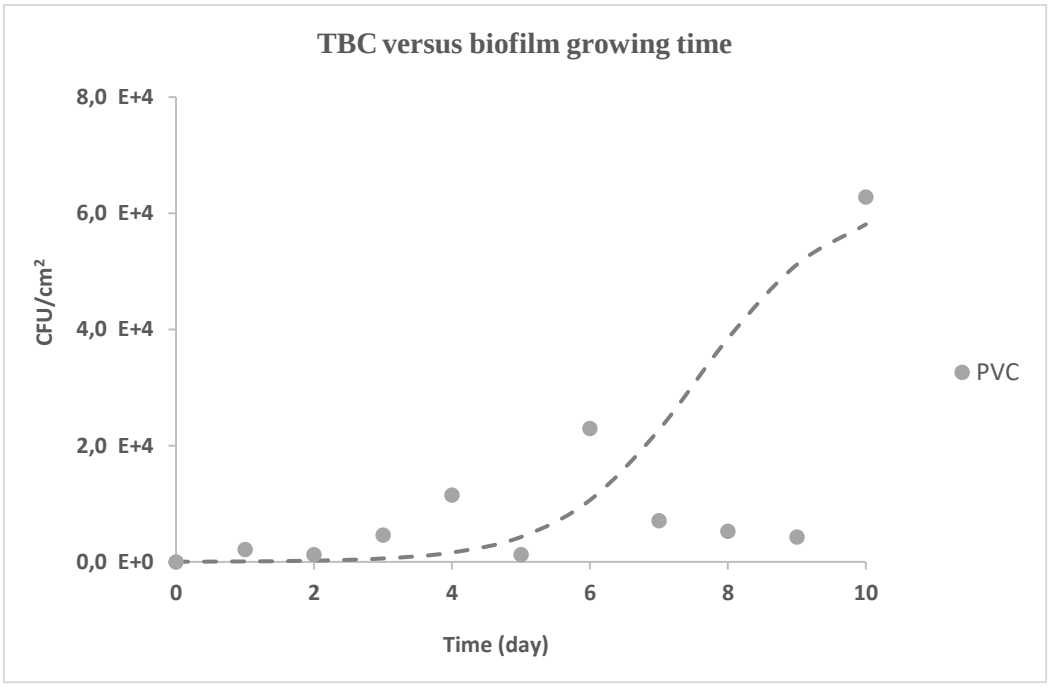


Fig 53. Microbial growth trend measured in the time interval on PVC with cultural method

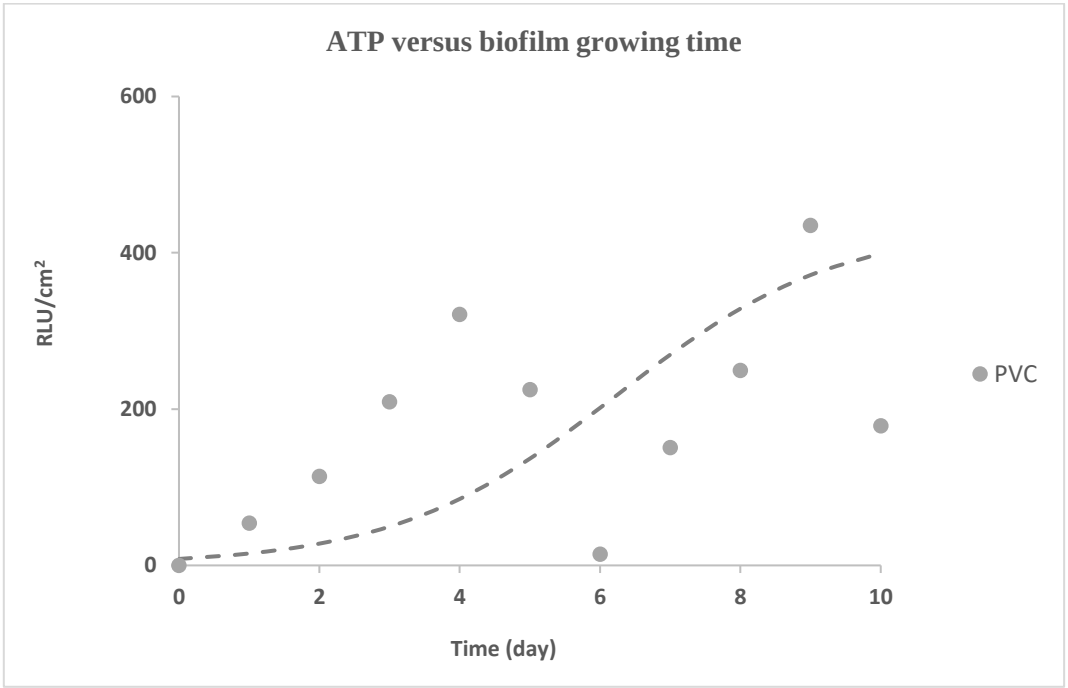


Fig 54. Microbial growth trend measured over time interval on PVC with bioluminometric method

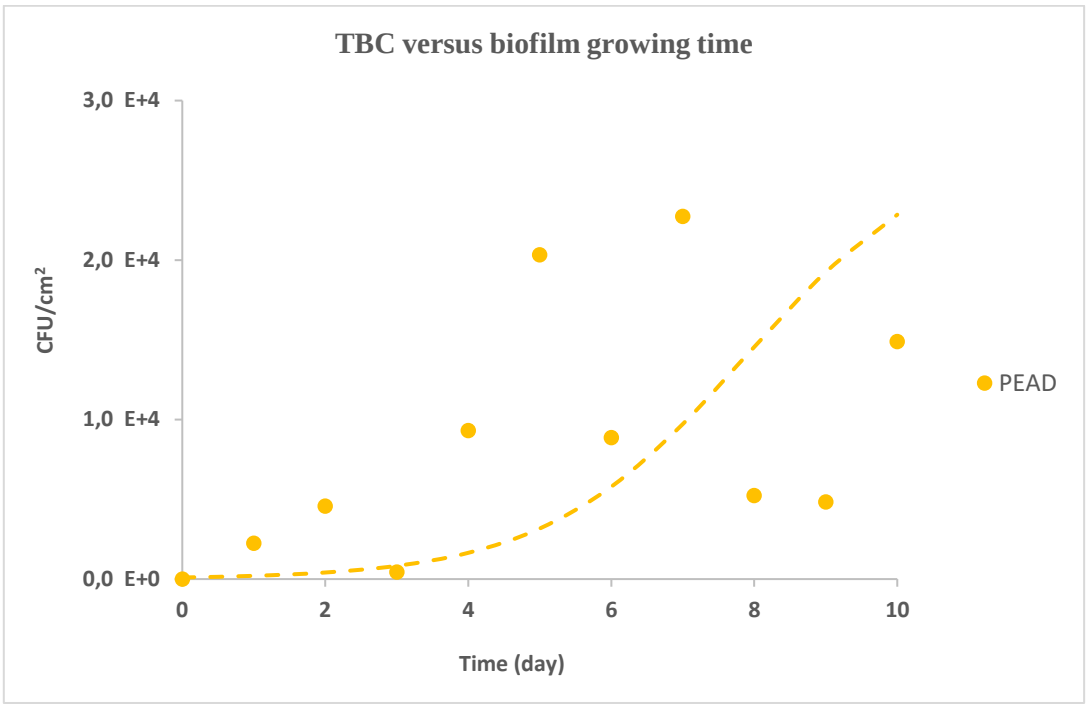


Fig 55. Microbial growth trend measured in the time interval on PEAD with cultural method

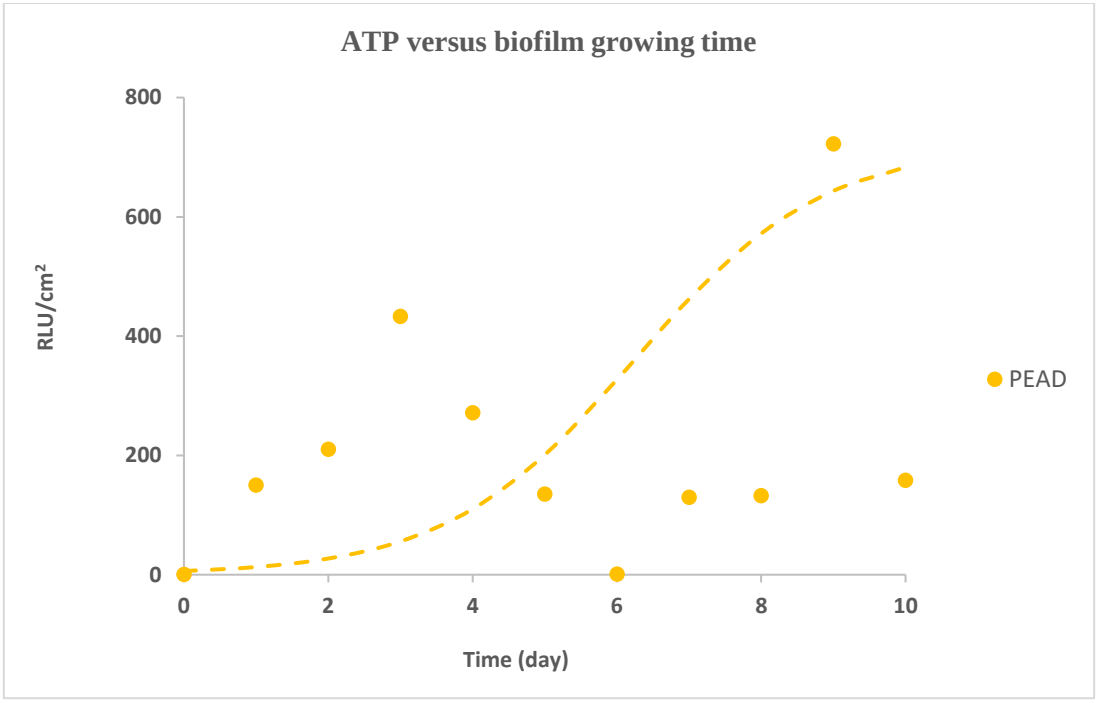


Fig 56. Microbial growth trend measured over the time interval on PEAD with bioluminometric method

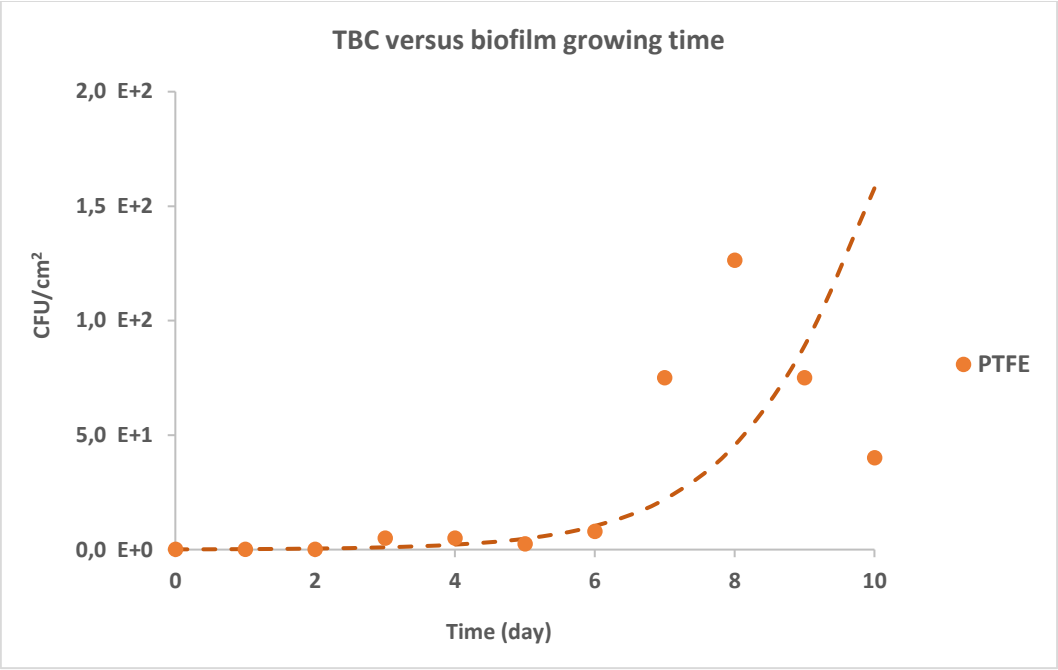


Fig 57. Microbial growth trend measured over time interval on PTFE with cultural method

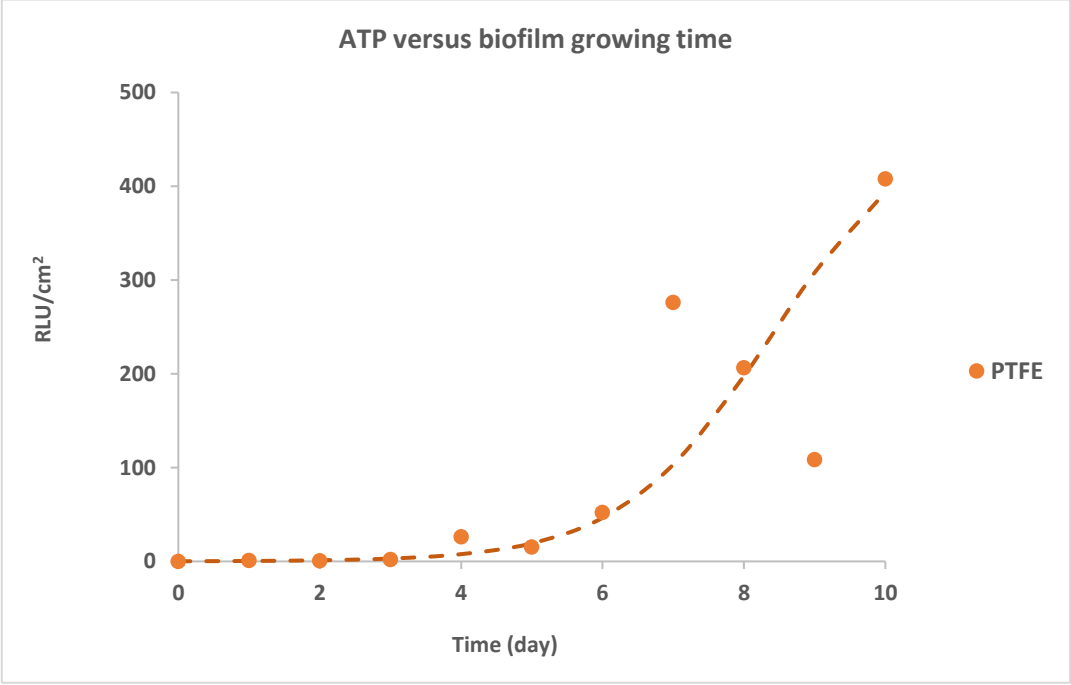


Fig 58. Microbial growth trend measured over time interval on PTFE with bioluminometric method

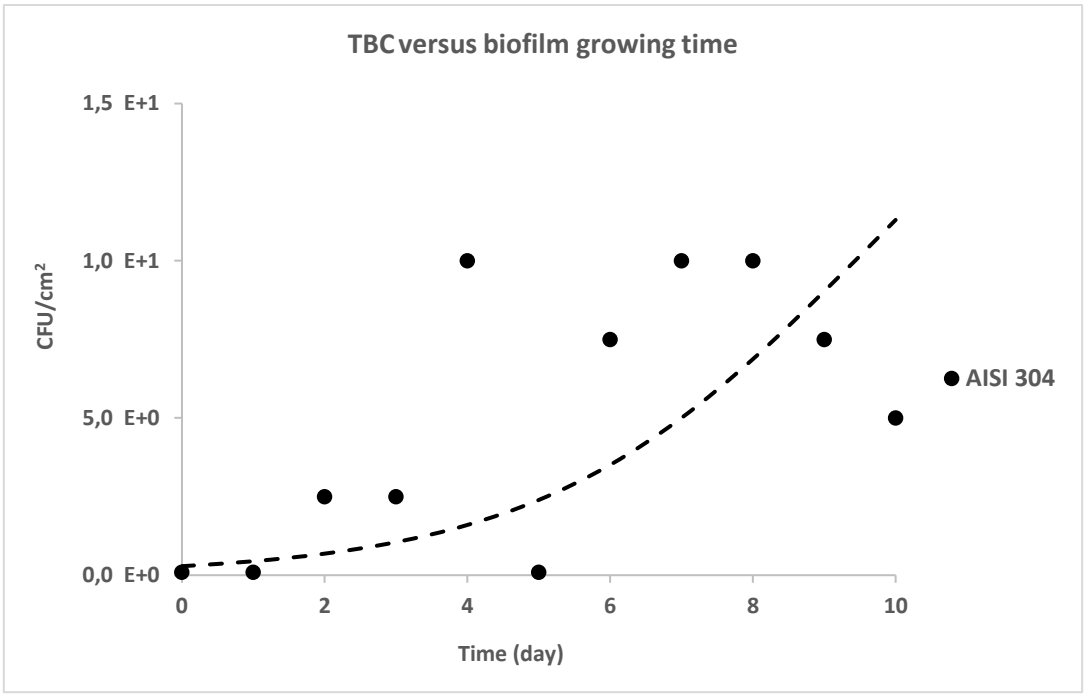


Fig 59. Microbial growth trend measured in the time interval on AISI 304 with cultural method

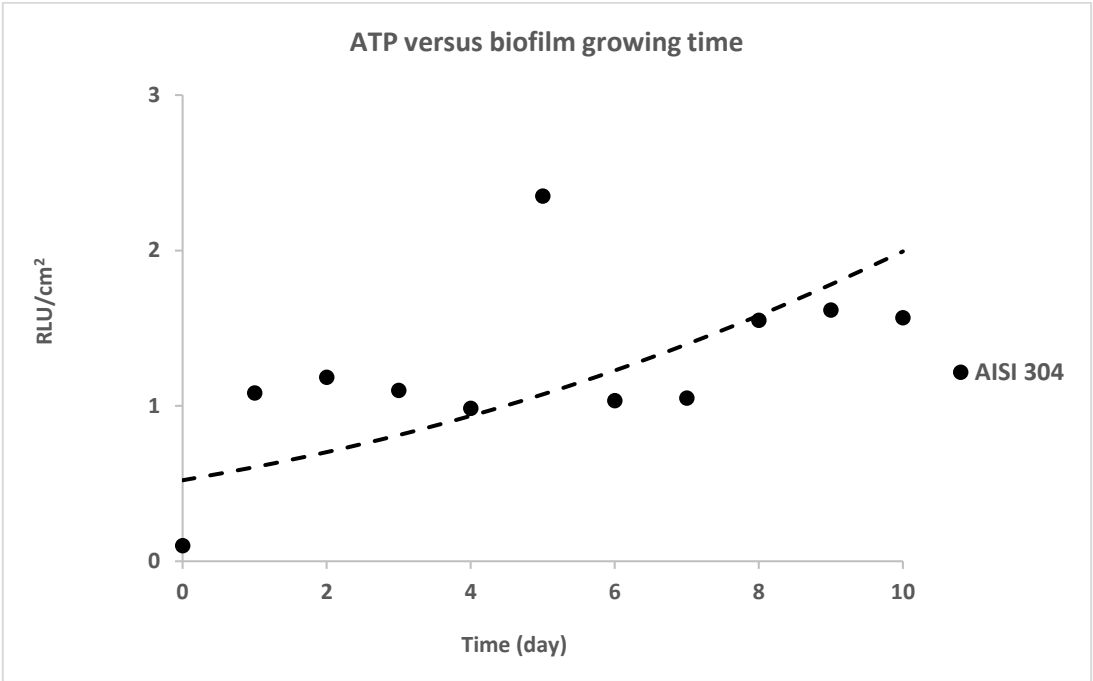


Fig 60. Trend of microbial growth measured in the time interval on AISI 304 with bioluminometric method

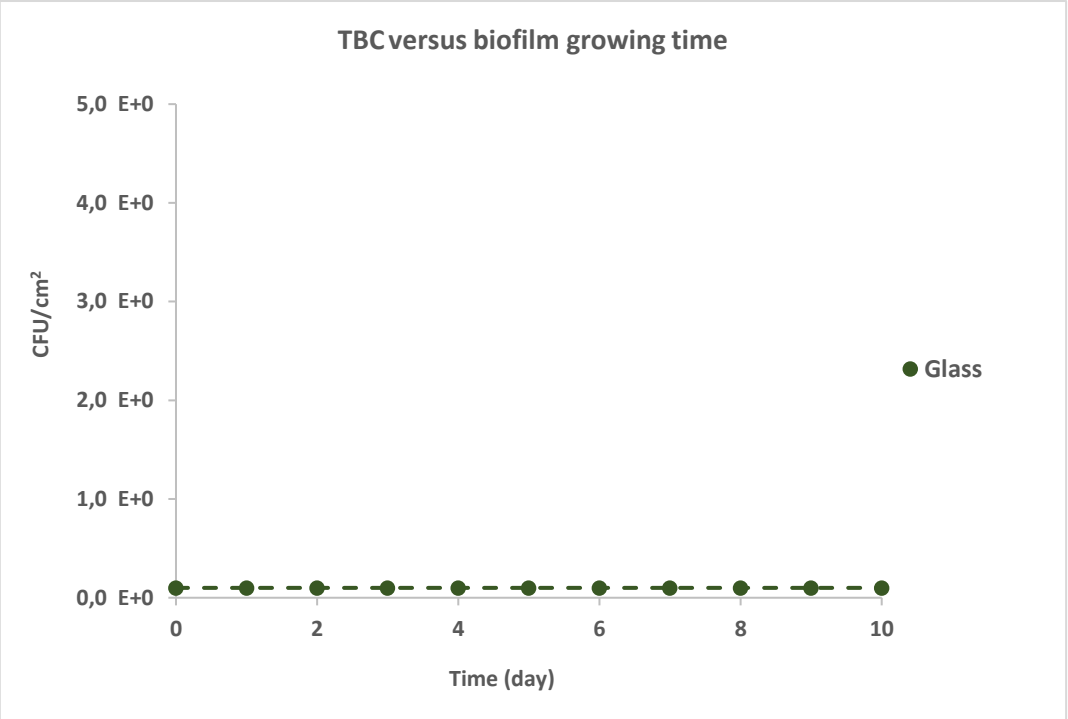


Fig 61. Trend of microbial growth measured over the time interval on GLASS using a cultural method

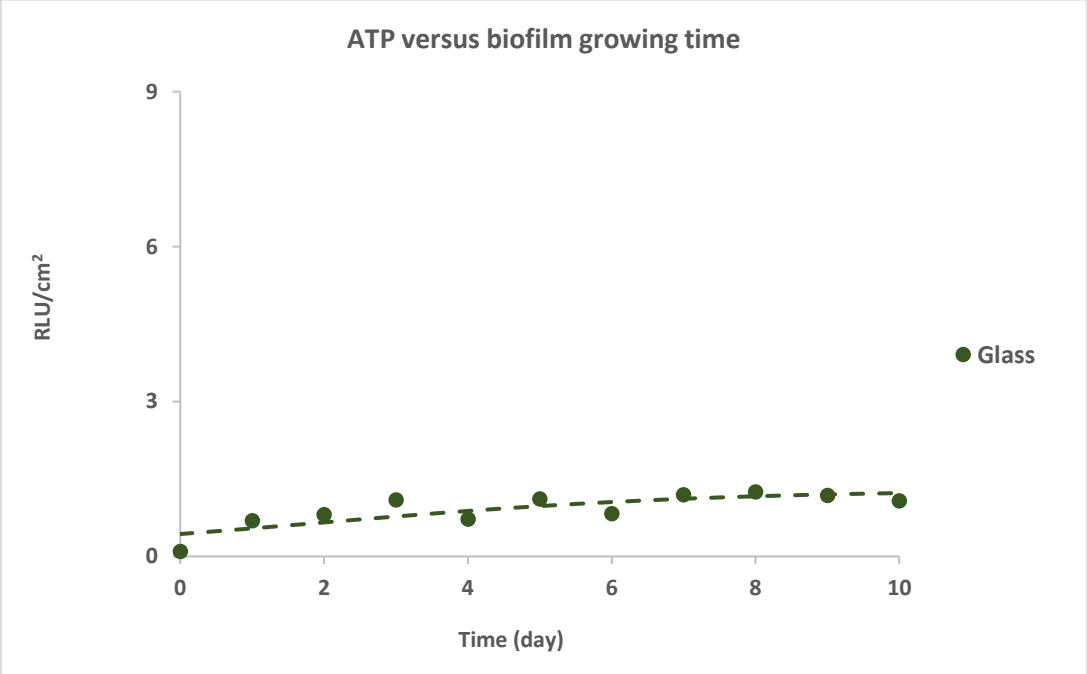


Fig 62. Microbial growth trend measured in the time interval on GLASS with bioluminometric method

3.6 Disinfectant residues in the investigated network

Fig 63 shows the results of the analysis of HWS samples disinfected with H_2O_2-Ag^+ taken along the water mains of the two hospitals under investigation.

Comparison of the concentrations of hydrogen peroxide present at the discharge with those found along the HWS water mains shows an average loss of approximately 5-9% biocide (9% for hospital H1; 5% for both batches of hospital H2).

The median disinfectant concentration in the recirculations of the two principals was 20 mg/L (with an interquartile range of 1 mg/L) throughout the first phase of the trial.

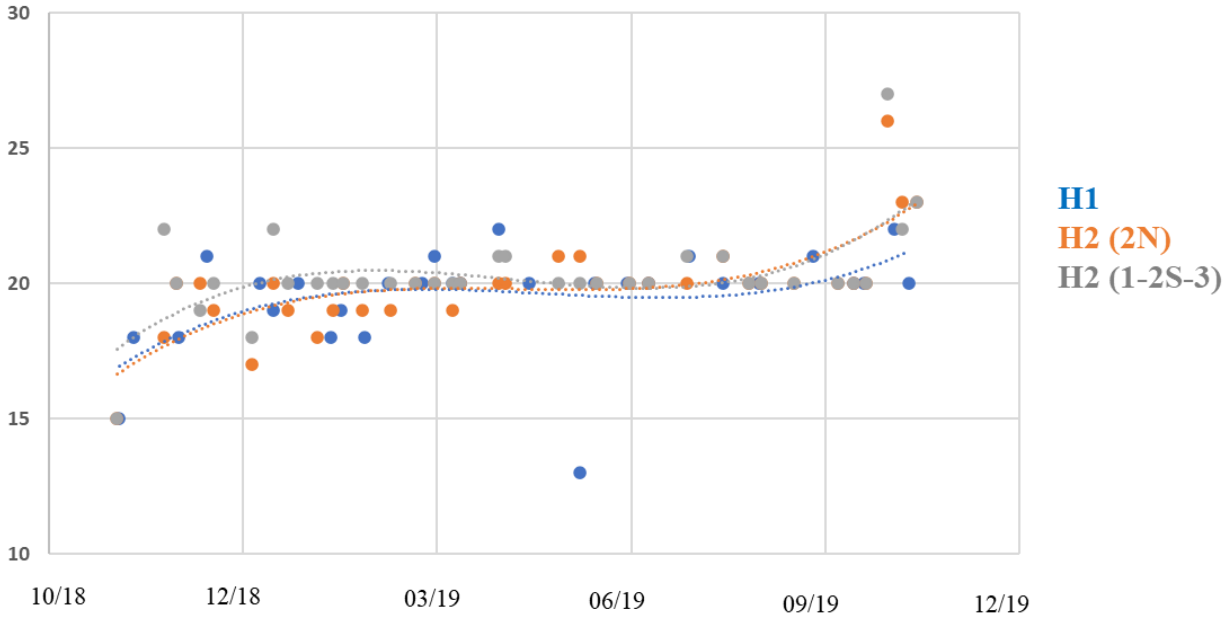


Fig 63. Trend of H_2O_2 concentration in the two structures under study

Fig 64, on the other hand, shows the results of the analysis of HWS samples disinfected with NH_2Cl taken along the water networks of the two structures under investigation H1 and H2.

Comparison of monochloramine concentrations present at the discharge and those found along HWS water mains during the second and third sampling campaigns shows an average loss of approximately 3-17% biocide (3% for structure H1; 12% for structure H2 lots 1,2S,3; 17% for structure H2 lot 2N).

The median concentration of the disinfectant in the recirculations of the two principals was 1.1-1.5 mg/L (with an interquartile range of 0.5-0.7 mg/L) throughout the second phase of the trial, i.e., above the minimum thresholds.

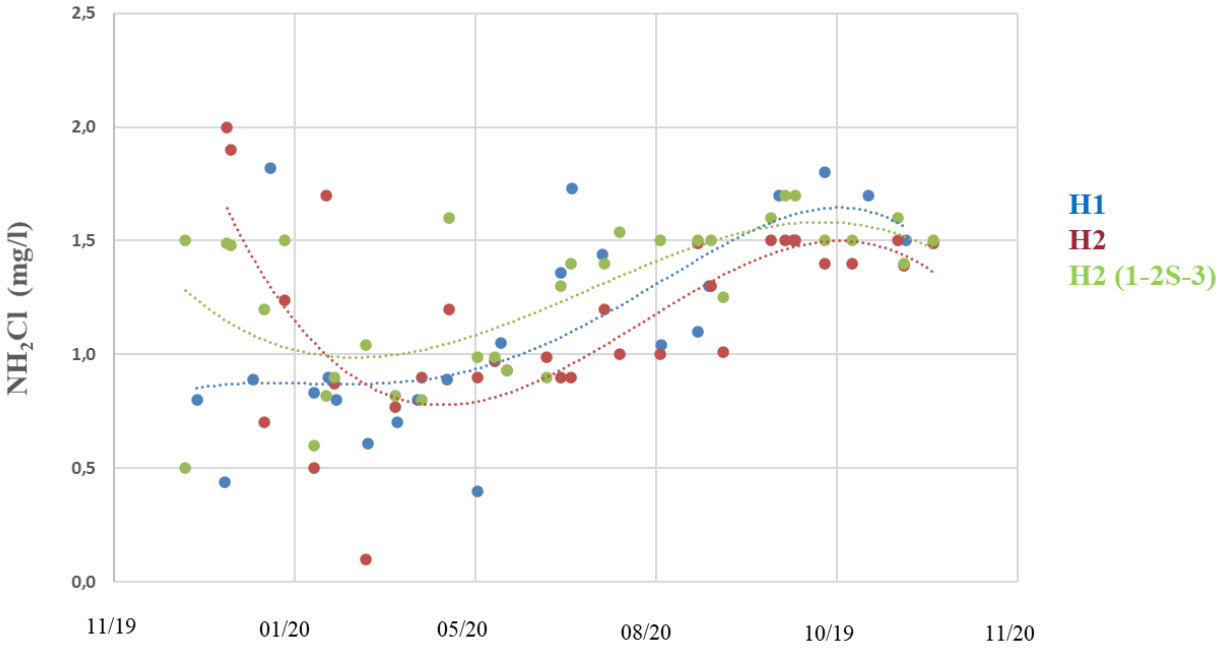


Fig 64. Trend of NH_2Cl concentration in the two structures examined

3.7 *Legionella* presence in the investigated networks

Figs. 65-66 show the average values found in the two structures H1 and H2 over time. The results of culture testing of HWS samples taken from both health care facilities examined showed low *Legionella* contamination in building H2 and no contamination in building H1.

The results of the analysis by Real Time PCR of the same samples analyzed by the culture method showed on the contrary a positivity in both structures that tends to reduce with the passage of time.

Positive values found by Real Time PCR analysis could be associated with the presence of the microorganism in a non-viable form or in a viable but non-culturable form and/or its occult presence within amoebae or biofilms.

In fact, as known from the literature, disinfection treatments could induce *Legionella* to choose such survival strategies and thus remain hidden until conditions favourable to multiplication reappear.

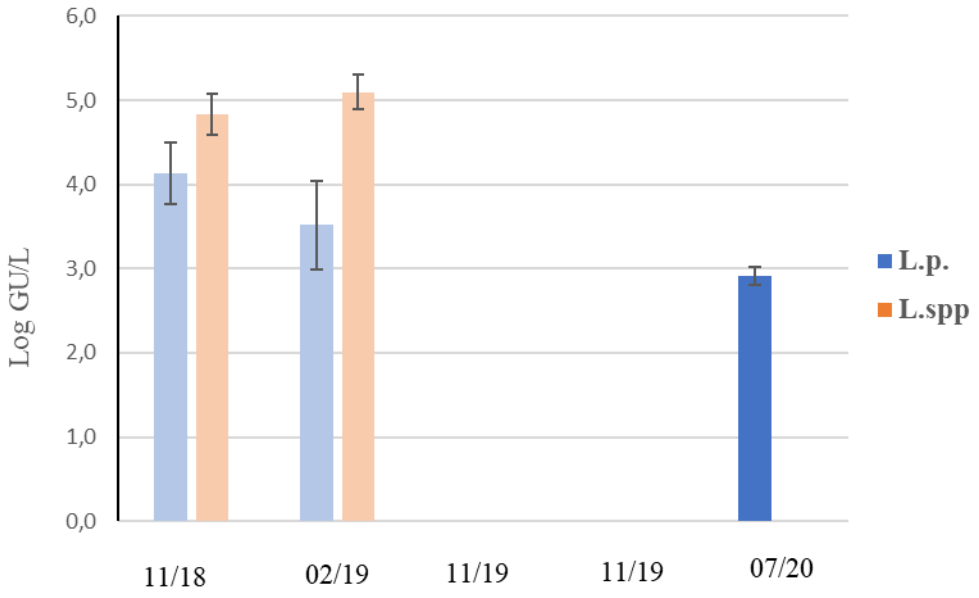


Fig 65. Trend of the average concentration of *Legionella pneumophila* and *Legionella* species determined by Real time PCR in the HWS network of Hospital H1. Lighter and darker colored bars indicate values acquired during the use of H_2O_2/Ag^+ and NH_2Cl, respectively.

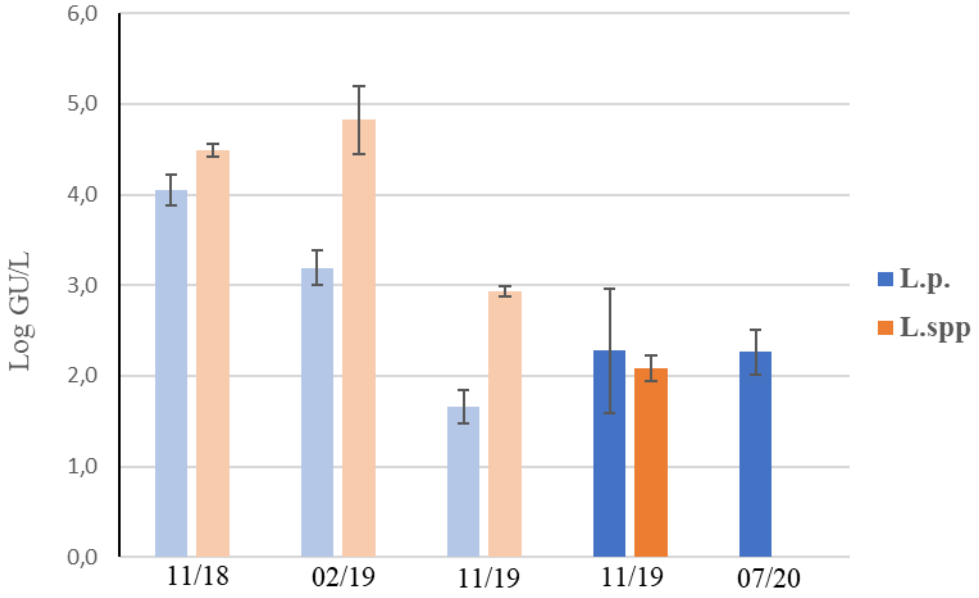


Fig 66. Trend of the average concentration of *Legionella pneumophila* and *Legionella* species determined by Real time PCR in the HWS network of Hospital H2. Lighter and darker colored bars indicate values acquired during the use of H_2O_2/Ag^+ and NH_2Cl, respectively.

3.8 Microbial characterization of specimens placed in the HWS networks of structures H1 and H2

3.8.1 *Legionella* research results on biofilm grown on specimens

Figs. 67-68-69-70 below show the results obtained from the analysis of *Legionella* spp. and *Legionella pnemophila* by Real Time PCR of DNA extracted from biofilm recovered from the surface of coupons exposed to the flow of HWS of the two structures H1 and H2.

Fig. 67 shows the average values obtained during the experiment for each material and each disinfectant tested.

Strong variability was observed in the genomic units of both *L.pneumophila* and *Legionella* spp. poorly influenced by the nature of the disinfectant.

On average, the least contaminated material was found to be galvanized steel, however, for which there were consistent fluctuations in the analytical data.

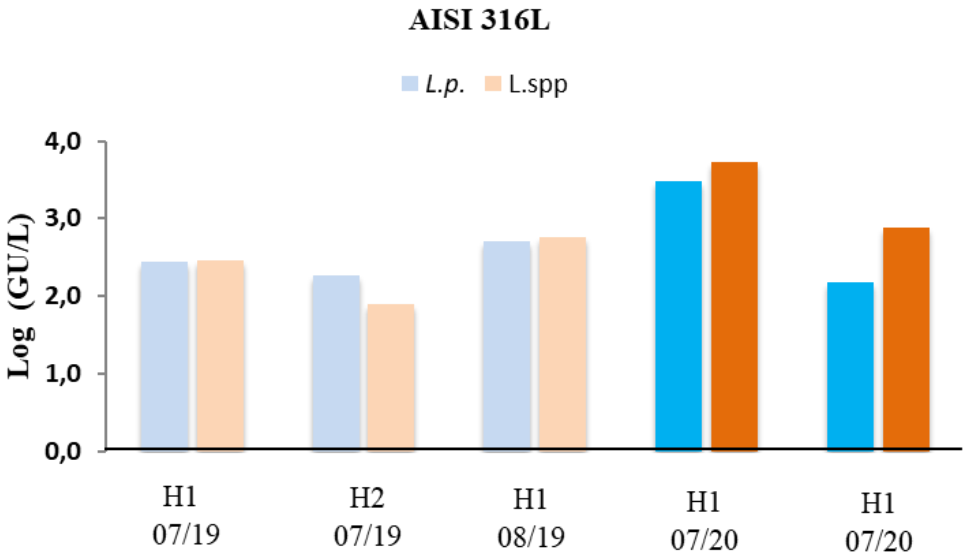


Fig 67. *Legionella pnemophila* and *Legionella* spp. in biofilm recovered from the surface of AISI 316 L steel coupons exposed to the HWS flow of the H1 and H2 structures.

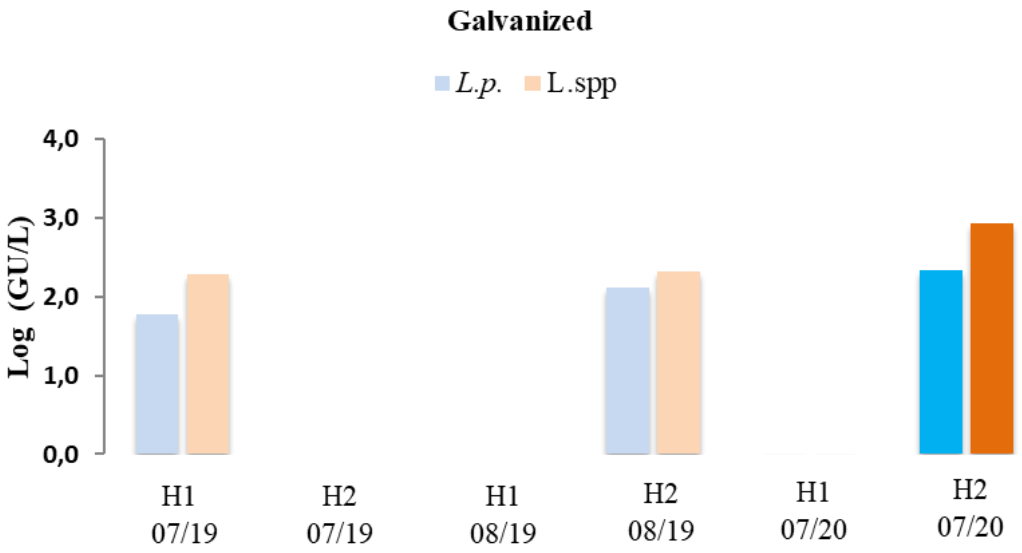


Fig 68. *Legionella pnemophila* e *Legionella spp.* in the biofilm recovered from the surface of galvanized steel coupons exposed to the flow of HWS from the two hospital wards.

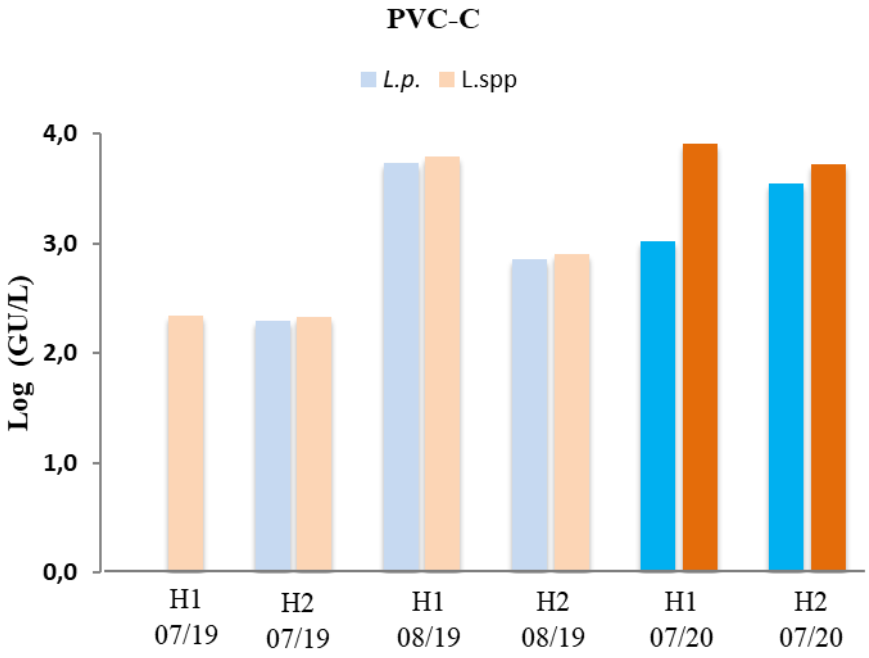


Fig 69. *Legionella pnemophila* e *Legionella spp.* in the biofilm recovered from the surface of the C-PVC coupons exposed to the HWS flow of the two hospital buildings.

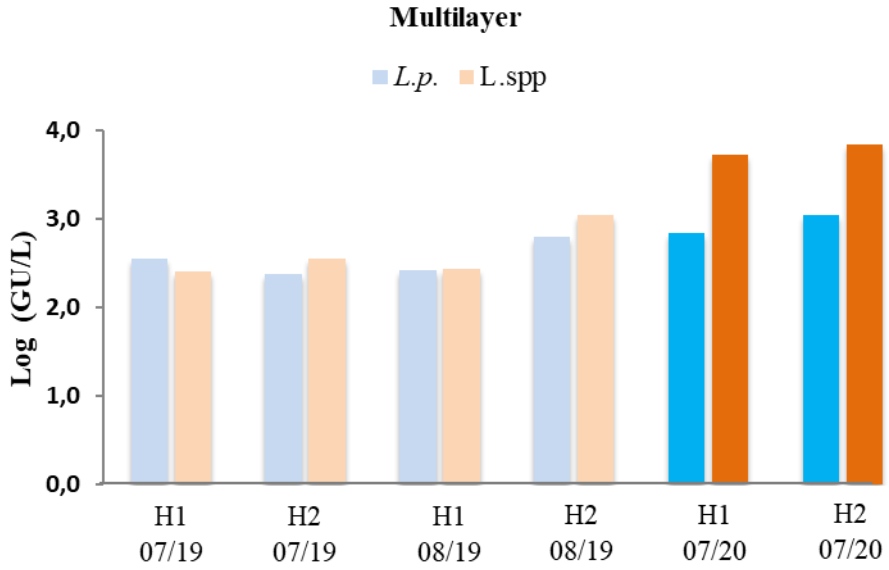


Fig 70. *Legionella pnemophila* e *Legionella spp.* in the biofilm recovered from the surface of the multilayer coupons exposed to the flow of the HWS of the two hospital wards.

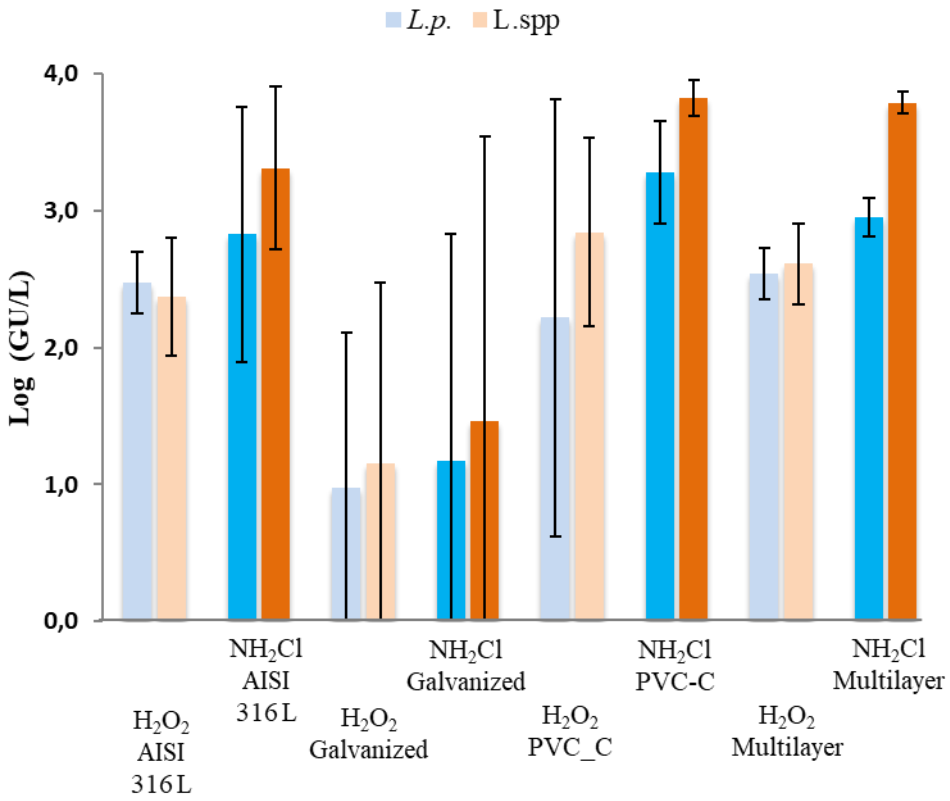


Fig 71. Average values *of Legionella pnemophila* and *Legionella* spp. in the biofilm recovered from the surface of the coupons exposed to the flow of the HWS of the two hospitals disinfected with hydrogen peroxide/silver and with monochloramine.

3.8.2 Characterization of the microbiome extracted from the biofilm deposited on the specimen's surface

The 16S rRNA analysis of the DNAs, extracted from the biofilm attached to the coupons under study, allowed the determination of the microbiome characterizing the biofilm extracted from the surface of the materials examined.

Below are histograms depicting the relative abundances of the microorganisms most represented in the microbiomes of the HWS networks of the two hospitals (Fig. 72).

An enrichment of bacterial flora was observed in building H1 between 2019 and 2020. At the species level, high concentrations of *Legionella pneumophila* were observed in 2020.

Bacterial population diversifications were also observed in building H2 between 2019 and 2020. The genus *Legionella* was only found to be present on the galvanized steel specimen taken in the summer of 2020. In the PVC specimen taken in the summer of 2020, the species *L. micdadei* was detected.

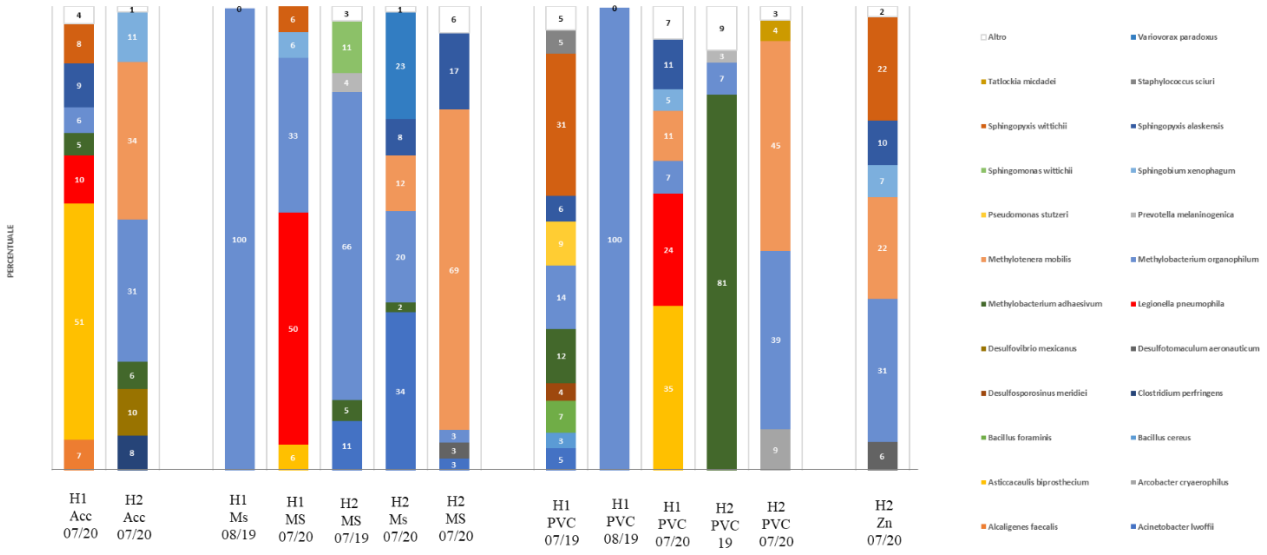


Fig 72. Relative abundances of the most represented microorganisms in the microbiomes of the HWS networks of the two facilities

3.9 Degree of corrosion of the specimens and formation of inorganic and organic stratifications on their surface

The results obtained from the analysis performed on the specimens exposed to the HWS flow of the two principals H1 and H2 are shown below.

Fig. 73 shows the average degree of corrosion found, for each material and for each type of disinfectant tested, at the end of an exposure time of 32-42 days (average of 37 days): a significant decrease in the degree of corrosion of the materials tested can be observed following the introduction of monochloramine.

The most corrosion-prone material was galvanized steel and, to a significantly lesser extent, C-PVC.

Fig. 44 shows the average rates of formation of weakly and strongly adhered inorganic and organic stratifications on the surfaces of the tested materials in the presence of the two disinfectants: a higher deposition of weakly adhered stratifications is observed on the surface of C-PVC and galvanized steel; strongly adhered stratifications were found almost exclusively on the surface of galvanized steel.

In all cases, the level of stratification was moderately reduced with the introduction of monochloramine.

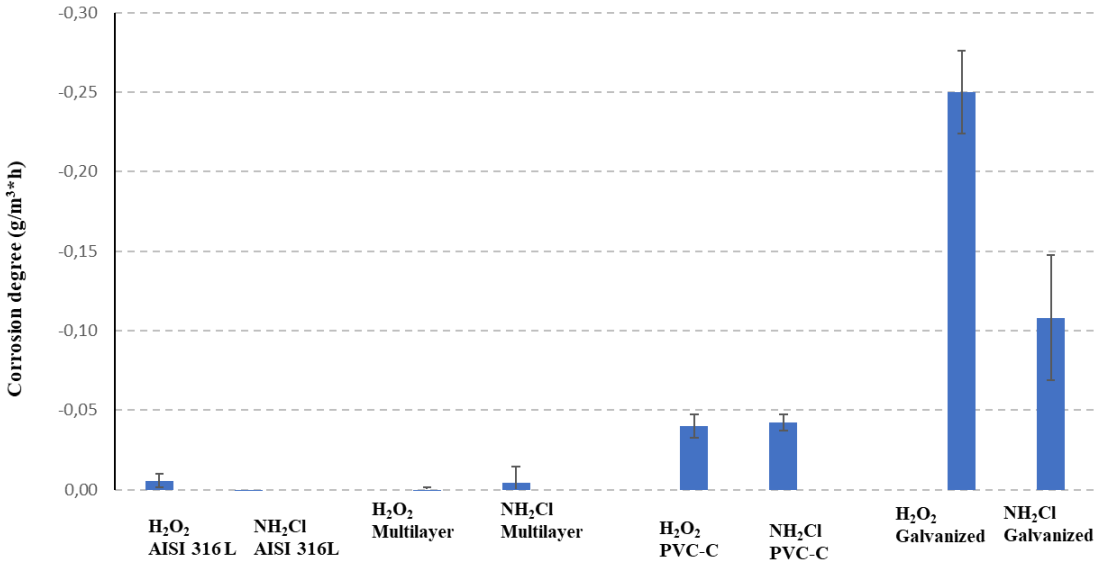


Fig 73. Corrosion of the specimens after 37 days (SE: 1 day) of exposure to HWS of the two structures H1 and H2.

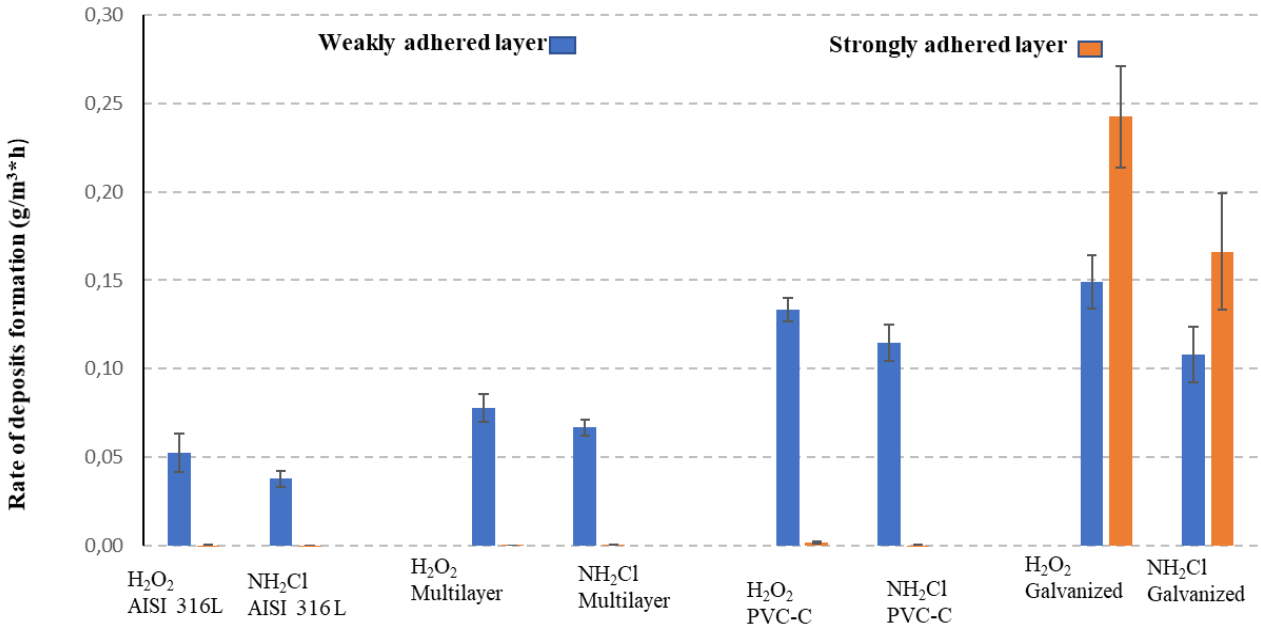


Fig 74. Rate of deposit formation on the coupon surface after 37 days (SE: 1 day) of exposure to HWS of the two structures H1 and H2.

CHAPTER 4: CONCLUSIONS

The experimental activities carried out as part of this study resulted in the acquisition of the following information:

1. The rate of decomposition of monochloramine was higher than that of hydrogen peroxide;
2. Under regulated conditions to obtain a reduction of 3 decimal logarithmic units of *L. pneumophyla* species after 5 minutes of contact, a 50°C, was required a lower concentration of hydrogen peroxide than that of monochloramine, due to the higher disinfecting capacity of hydrogen peroxide at these temperatures.
3. Among the tested materials (EPDM, PVC, PEAD, PTFE), the material that showed a higher degree of microbial adhesion was found to be EPDM, with both the cultural and bioluminometric methods, while the material with the lowest degree of microbial adhesion was found to be PTFE, with both methods tested;
4. The disinfectants present in the network showed different decomposition rates: in fact, the decomposition of monochloramine was greater than that of hydrogen peroxide at the most distal points of use.
5. Culture examination of HWS samples from both health care facilities examined showed low *Legionella* contamination in facility H2 and no contamination in facility H1;
6. The analysis carried out by Real Time PCR of the same samples analysed with the culture method has been found, however, a positivity for both structures, expressed as genomic units (UG), probably due to the presence of the microorganism in a viable or non-viable form not cultivable and / or its occult presence within amoebae or biofilm;
7. Strong variability was observed in the genomic units of both *L.pneumophila* and *L.*spp in the biofilm deposited on the surface of the HWS-exposed coupons of the two hospitals, scarcely influenced by the nature of the disinfectant.

Unexpectedly, the least contaminated material was galvanized steel, for which, however, consistent fluctuations in analytical data were recorded;

8. Analysis of the microbiome extracted from the biofilm deposited on the surface of HWS-exposed coupons from the two hospitals revealed a complex diversification of the bacterial population with the presence of *Legionella pneumophila*;
9. All materials examined (AISI 316L stainless steel, galvanized steel, C-PVC, and multilayer) experienced less corrosion and weakly adhering stratification of inorganic and organic deposits when hydrogen peroxide, at a median concentration of 20 mg/L, was replaced with monochloramine, at a median concentration of 1.1-1.5 mg/L. As expected, the material most prone to corrosion and weakly adhered layers was galvanized steel followed, to a significantly lesser extent, by C-PVC. Galvanized steel was also found to be prone to accumulate strongly adhered stratifications on its surface.

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