Microbial Dynamics in Drinking Water Biofilms

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Memory presented by **Francesc Codony Iglesias** to obtain the degree of

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Supervisor	Supervisor
Dr Jordi Mas Gordi	Dr Jordi Morató Farreras

Per tots els meus, per la Irene
, en record de l’Anna Cabané
i per totes les *Victories Morals*

"Many of the truths that we cling to depend on our point of view."

Yoda

(Jedi Master)

Acknowledgment

A work like this is the result of the sum of multiple positive actions. I have spent the best years of my life doing research and living the life. My priority was not to write a thesis, though I have to recognize that cycles like this must have a end. Now is the moment to do it, and it is the moment to remember good people found along way.

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Introduction

The water supply released into the distribution system is altered during its passage through the system due to a large extent to microbial activity. These quality changes most often result in complaints about taste, odour, and colour. In other cases, the sanitary quality of water is altered due to bacterial regrowth, which may be associated with non-compliance to national regulations.

Most of the network components may become excellent microbial environments, though their persistence and proliferation will be influenced by a variety of factors, including distribution system age, soil stability and corrosivity, pipe material used, water flow reversal, static water conditions, water chemistry, seasonal warm water temperatures, disinfection, and operational practices.

The water body is the major habitat of drinking water systems, though the most significant changes in drinking water properties are at contact surfaces.

Most exposed surfaces in water distribution systems are colonized by microorganisms in biofilms [1], diverse microbial communities adapted to low nutrient and high chlorine levels. The characteristics of biofilms vary widely, from sparsely colonized surfaces to thick complex layers several micrometres in depth, and are formed by an interwoven structure of extracellular polymers, microbial cells, and ion channels [2,3].

Considering the many kilometres of water mains and pipes from the treatment plant to the tap, biofilms represent a far more significant reservoir of microorganisms than the water phase [4]. The pipe network is the most extensive surface of the distribution system. Several factors modulate microorganism colonization, such as the pipe material, age, corrosion, sediment accumulation, and water flow.

Various materials have been used for water supply lines, including ductile iron, cast iron lined with cement, steel, reinforced concrete, asbestos combined with Portland cement, and three types of plastic: polyvinyl chloride (PCV), polyethylene, and polybutylene. In Catalonia, domestic lines and building pipe networks are most often copper, galvanized iron, or more recently plastic.

In each case, several factors related to the characteristics of the material contribute considerably to the level of surface colonization by microorganisms. Firstly, each material has a specific surface area. Two pipes identical in length and diameter but made of different materials do not expose the same surface area for bacterial colonization. The presence of microscopic pores and the superficial roughness offers microorganisms more surface area than the theoretical surface area.

Secondly, internal corrosion caused by galvanic actions in metal pipe may protect microbial colonies from disinfection, increase the hydraulic mixing and transport of nutrients to the surface, and increase the specific surface area of the material [5]. This phenomenon is often aided by the microbial activity of both sulphate-reducing bacteria and other aerobic heterotrophic bacteria [6,7]. In these corrosion tubercles, a variety of microbial groups can be detected, such as sulphate reducers, nitrate reducers, nitrite oxidizers, ammonia oxidizers, sulphur oxidizers, and various heterotrophic microorganisms. Exposed iron is decisive because the amount of corrosion products formed on the pipe surface is directly related to the solubility of iron from the pipe [8]. Corrosion products are formed when iron molecules are oxidized to various iron oxides by dissolved oxygen and oxidizing disinfectants present in the bulk fluid. The common corrosion tubercle is a layered formation of iron oxides in various oxidation states. The deepest layer in direct contact with the pipe surface is formed by siderite ($FeCO_3$) and is coated with a layer of goethite (α-FeOOH). On the external surface is a thin layer of a higher oxidized iron oxide or magnetite (Fe_3O_4).

Moreover, most materials used as coatings, sealants, joints, etc. support microbial growth and, in some cases, have a growth-promoting effect [9].

An early but detailed study of pipe scrapings, tubercles, biofilms, flocs, and sediments from a water distribution system in New Jersey[2] reported 1.0×10^7 bacteria/m^2 in the pipe scrapings, finding mainly *Flavobacterium* sp. and *Pseudomonas vesicularis*. The same species were the predominant planktonic species found in the bulk water. The number of cells accumulated on the pipe wall surface was not determined. Nevertheless, the number of heterotrophic plate count (HPC) bacteria in tubercle material was 1.3×10^7 cells/g, and it was the only particulate sample containing significant levels of coliform (>160 cfu/g).

Over the last three decades, the advent of molecular techniques, as qPCR or sequencing techniques, all of them improved our current understanding of microbial diversity in these environments, expanding on the initial knowledge based on culture techniques [10]. Whole-genome amplification has become an important tool for evaluating the genetic information in environmental samples, but the level of bias can affect our understanding, as happens with groundwater and drinking water [11].

Yet, microbial structure and activity need to be linked to differentiate between active (or at least live) microorganisms and the surrounding genetic background. At this stage, viability PCR procedures can help [12], even though each procedure needs to be optimized [13]. Approaches are now available for estimating the percentage of live microorganisms [14].

The water supply in the community varies throughout the day as a consequence of the activities of the general public and local industries. The industrial use of potable water is more constant and predictable, but all other uses often present with sudden changes influenced by multiple factors; thus, it is not possible to ensure water treatment operations are constant with appropriate production and supply. Therefore, storage structures are essential components of the distribution network, which acts as a buffer for water demand in different scenarios, equilibrating water production and demand.

Drinking water systems often have a very complex structure because they have continuously grown and adapted to the urban structure, topology, and geology. Very different pipe with different diameter and materials can be found in the same distribution system, and along the system are different water reservoirs of different volumes, construction materials, and water flow.

System integrity is essential for the distribution of safe water, the prevention of contamination from soil, non-treated water with faecal matter, and to control bacterial regrowth, especially for pathogens.

The microbial load of the water is key to the role of bacterial communities attached to surfaces in biofilms. These communities can become very complex and diverse with different types of microorganisms. Thus, water quality is influenced by the complex interactions that occur in these microbial communities.

The presence of biofilms in water distribution networks constitutes one of the hazards currently recognized as affecting the microbiological quality of drinking water [15]. Microbial growth within the biofilm and release of offspring into the overlaying water contributes to increased microbial counts in the water phase. On the other hand, the structure of the biofilm itself contributes to sheltering occasional contaminants from the effects of chlorine, preventing adequate disinfection and potentially allowing regrowth of contaminants in the water distribution network [16,17]. In addition, the different relationships between diverse microorganisms present in biofilms, such as amoebas, can enhance the survival and spread of pathogens.

In most cases, the main strategy for microbiological quality control in the water industry is based on the use of bacteriological indicators tested by culturing. The common approach is a combination of process indicators, such as total bacterial and total coliform counts, with faecal pollution indicators, such as *Escherichia coli* in Council Directive 98/83/EC [18]. As a rule of thumb, microbiologically safe water can be delivered to customers when these parameters are under control.

This approach allows us to manage complex structures, such as a water distribution network, realistically [19]. Nevertheless, the microbial ecology of these systems is quite complex. Despite good potabilization and disinfection practices, microbial proliferation occurs not only throughout all water columns, but also on surfaces and biofilms [20]. The biofilms and amoebas [21] each play a key role in pathogen survival and in promoting their regrowth and proliferation throughout the entire water distribution network, even in the absence of clear external pollution episodes.

Although only a few indicators are required to manage the complexity of a drinking water system, the continuous improvement in our knowledge of the microbial dynamics of these systems as result of DNA-based technologies should not be neglected. These tools provide some clarity in our understanding of pathogen survival, proliferation, and spread. Furthermore, molecular analysis allows us to detect and study new threats and to detect uncultivable pathogens [22].

During the last few decades, we have surpassed the first data suggesting a strong relationship between pathogenic bacteria and amoebas to evidence that this interaction is responsible for many examples of pathogen proliferation.

The presence of amoebas in drinking water systems is not sporadic; they can colonize virtually any kind of water system and support harsh physical and/chemical conditions, including elevated temperature and biocides [23]. Interestingly, for all bacteria included in the CCL3, there is published laboratory-based evidence of intra-amoebal growth, implying the potential for amoebas to act as environmental vectors and/or transport hosts for the CCL3-listed bacterial pathogens (*Campylobacter jejuni, E. coli* O157:H7*, Helicobacter pylori, Legionella pneumophila, Mycobacterium avium, Salmonella enterica,* and *Shigella sonnei*).

Although further research regarding bacteria–amoeba interactions is necessary, current knowledge of all bacteria present in the CCL [24,25,26,27,28,29,30] indicates that amoebas could play a key role in bacterial resistance to disinfection, survival, and spread in drinking water systems. Bacteria–amoeba interactions also suggest that, after a contamination episode, the presence of faecal-related pathogens, such as *C. jejuni*, *E. coli* O157:H7, *H. pylori*, *S. enterica,* and *S.*

sonnei, would not be detected using only conventional faecal indicators because some enteric bacterial pathogens would survive within the amoebas and still be present at lower concentrations. Once again, it is the old problem of detecting all health risk-associated indicator micro-organisms or finding a new more appropriate one.

Biofilm development seems to be governed by the interplay between several factors: the microbial load in the circulating water, the amount of nutrients available, the concentration of disinfectant, and the hydraulic regime of the system [31]. Currently, suppliers involved in the management of water distribution networks who are aware of the problem posed by biofilms are attempting to control their growth by a combination of several factors [32]. First, an adequate level of disinfectant is maintained throughout the distribution network via regular chlorination at stations placed at select points along the network. Second, the presence of organic carbon in the water is minimized by several procedures (e.g., activated carbon filtration, ozonization, UV treatment) in order to minimize the availability of substrates for growth. Third, low microbial levels at the input of the water distribution system are minimized through adequate disinfection along the potabilization sequence. Implementation of biofilm control measures can require a substantial investment in both infrastructure and subsequent operating costs.

The role of chlorine is very clear, but the speed of biofilm formation, microorganism diversity, and density may also be modulated by surface shear stress [33], pipe material [34], temperature [35], and Biodegradable Dissolved Organic Carbon (BDOC)[36]. Among these parameters, the levels of organic matter have been postulated to not be the most influential [37]. However, reduction of BDOC has to be considered along with other perspectives. All bacterial regrowth in drinking water systems will not be stopped by limiting nutrient levels, though the occurrence of coliform bacteria is dependent on a complex interaction between several factors, including nutrient levels [5,38]. For this reason, although BDOC has little impact on biofilms, it may be a key point for preventing undesirable growth. Moreover, a reduced level of organic matter reduces chlorine demand and increases disinfectant stability, allowing optimization of the chlorine dosage and minimizing its disappearance during distribution. Consequently, this low level, improves the ability of chlorine to act against free or attached microorganisms despite the limited capacity of chlorine on biofilms. Although not universal, some European countries (e.g., the Netherlands) have had a tendency to reduce or eliminate the use of chlorine, which does not have a dramatic impact on public health. Nevertheless, a drinking water distribution system is a complex structure with kilometres of pipes, and it is very difficult to ensure correct chlorine levels at all points. If problems exist with chlorine levels, biofilm formation may be allowed or accelerated. Without chlorine addition in the presence of a biofilm, the capacity to control microbial quality is reduced. Therefore, it is important to ensure sufficient chlorine levels at all points to prevent biofilm formation and guarantee the microbial quality of water.

Despite these many considerations, appropriate microbial levels can be assumed in all public mains. However, the responsibility of suppliers ends when water arrives at the consumer. In private networks, the same concern for water quality control does not always exist. In institutions, such as hotels or hospitals, the private network is complex, and factors promoting bacterial regrowth may exist. Some networks have end points at which water accumulates or recirculates. Both conditions produce an increase in the residence time, causing a loss of disinfectant and subsequent bacterial regrowth [39]. Thus, in some drinking water systems, deficiencies can promote colonization and multiplication of frank or opportunistic pathogens. For example, in the drinking water systems of big buildings, such as hospitals, especially in hot water conduction systems, colonization by Legionellaceae is a serious hazard, and strategies are needed to control bacterial growth. Routine and emergency treatments exist to control pathogens in large buildings. Routine treatments maintain a minimum disinfectant concentration in all water circuits. In hot water circuits, a temperature above 50ºC is maintained in the most distal point. In an outbreak, such as Legionnaire's disease, several shock treatments consisting of hyperchlorination and heat are performed. Although public health authorities promulgate different rules in each country, they are essentially the same. Correctly performed shock treatments can stop the contamination. However, if routine treatments are not rigorous, the public health hazard will likely reappear.

High temperature, chlorination, and more recently silver and copper ions are the current tools used in routine treatment of institutional drinking water systems to prevent bacterial proliferation.

Objectives.

- Evaluate the response of biofilms to different environmental factors, such as dissolved organic carbon, microbial load, and free chlorine on biofilm development.

- Evaluate the effect of different episodes of deficient chlorine depletion on biofilm growth and the subsequent impact on microbial load in water.

- Evaluate the applicability of new disinfectants or disinfection strategies against biofilms in drinking water systems.

Overall summary of results and general discussion.

Three published studies are included in this PhD thesis, all of them related to the microbial dynamics of drinking water systems:

- Effect of chlorine, biodegradable dissolved organic carbon and suspended bacteria on biofilm development in drinking water systems. Codony F, Morato J, Ribas F, Mas J. Basic Microbiol. 2002;42(5):311-9.
- Role of discontinuous chlorination on microbial production by drinking water biofilms. Codony F, Morató J, Mas J. Water Res. 2005 May;39(9):1896-906.
- Assessment of bismuth thiols and conventional disinfectants on drinking water biofilms. Codony F, Domenico P, Mas J. J Appl Microbiol. 2003;95(2):288-93.

The response of biofilms to different environmental factors, such as dissolved organic carbon, microbial load, and free chlorine, was evaluated in the first paper. A lack of disinfection was demonstrated as being key to the fast formation of biofilms. On the other hand, microbial load and dissolved organic carbon do not contribute to increase microbial levels on surfaces if exist free chlorine.

In the second paper we examined the effect of different episodes of deficient chlorine depletion on biofilm growth and the subsequent impact on microbial load in the water. We demonstrated that successive episodes of chlorine depletion increase microbial resistance to disinfection, and this major resistance is related to an increased microbial load in the water column.

As a rule of thumb, the first two papers demonstrated that chorine depletion in the water supply is key to microbial proliferation throughout the system. In developed countries, common management practices ensure that, in most cases, necessary chlorine levels are maintained at appropriate levels from the production point to the end customer. However, practical experience has shown that, despite of the implementation of good management practices, free chlorine levels can become low for short time periods (i.e., hours or days). These episodes do not directly impact microbiological or chemical water quality, at least from a legal point of view.

On the other hand, in private systems, the water quality can change easily due to differential flow, high residence times, and structural deficiencies. Also, in these cases the poor chlorine level or absence of disinfectant can lead to quality problems. In hot water distribution systems, water recirculation and high temperatures makes it impossible to maintain appropriate chlorine levels throughout the pipeline.

Past and current concerns about the microbiological quality of water have mainly focused on controlling faecal pollution because it can be directly related to acute diseases. By monitoring the levels of some microorganisms, such as *E. coli,* the faecal pollution can be monitored. The absence of *E. coli* in 100 mL has been used as an indicator of good quality. Complementary indicators, such as intestinal Enterococci and *Clostridium pefringens*, have also been used. Faecal pollution control is accompanied by a microbiological process control focused on monitoring the levels of total coliforms and controlling changes in heterotrophic plate counts (fixing in some cases an upper level of 100 ufc/mL).

Both approaches have been useful and key in supplying safe potable water, but are based on an incomplete set of premises: pathogens are from a faecal source, pathogens can enter the network but have a low survival rate, And normal ecosystems do not include pathogens or microorganisms that need to be monitored because their presence could be related to conventional faecal pollution indicators.

During the last two decades, our understanding of microbial dynamics in drinking water has improved, the role of biofilms as a pathogen reservoir is accepted; and in most cases these pathogens are known to not be from a faecal source (i.e., Legionellae, Mycobacterium, Para-Chlamydia, and other emerging pathogens). The role of amoebas in the water network is also important to note. In the last few years, increasing evidence has shown the active responsibility of amoebas in pathogen survival, especially species from a faecal source.

In all of these scenarios, alternative disinfection approaches are needed, not only for the distribution of safe water, but for the control of biofilm growth in cases of a direct health risk (i.e., Legionellae presence in a network).

In the third paper, we evaluated the applicability of a new family of disinfectant called bismuth thiol (BT), which represents a new generation of antibacterial agents. These agents are bismuth based, but their antibacterial activity has been enhanced markedly in combination with certain lipophilic thiol compounds. BTs exhibit more persistent residual effects than chlorine despite their relatively slow action. BTs could be especially useful for artificial water ecosystems in which it is very difficult to maintain a constant level of free chlorine, such as hot water systems or cooling towers.

In summary, chlorine depletion greatly impacts biofilm development, and deficient disinfection leads to an increase in biofilm development and subsequent increase in the microbial load in the water column. As biofilms develop resistance to chlorine disinfection, new alternatives are needed. BTs have demonstrated an interesting potential, especially in hot water systems.

Conclusions

- An adequate level of chlorine (~0.5 mg/l) is enough to ensure the absence of biofilms, even after microbial contamination events or the input of organic carbon into the system.

- Reducing chlorine levels, even without adding carbon or increasing the suspended microbial load, allows the development of an attached community in approximately 2 weeks.

- The release of microbial cells into the water phase increased 10-fold in the absence of chlorine.

- Chlorine-dependent changes in microbial levels in the water phase were significantly higher when expressed as HPC counts rather than total cell counts.

- Several episodes of deficient disinfection could prompt an increase in biofilm resistance to disinfection.

- Subsequent episodes of chlorine depletion may accelerate the development of microbial communities with reduced susceptibility to disinfection in real drinking water systems.

- BTs have more persistent residual effects than chlorine and hyper-heating in water systems.

- BT efficiency increased with temperature but, like copper–silver ions, its action is relatively slow. The combination of bactericidal and residual effects may prevent slime build-up in hot water systems.

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Annexes

Article 1

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Article 2

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Article 3

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