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*Review*

# Biofilm Formation in Water Distribution Systems

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**Abstract:** A biofilm is a biologically active matrix attached to the surface of cells and their extracellular products. As they are a mixture of many microorganisms, the microbiological activity of biofilms varies according to their position in the aggregate. With particular emphasis on drinking water distribution systems, this review focuses on the process of biofilm formation, associated bacteria, chlorine resistance of bacteria, and the predominant surface materials. We have compiled studies on the bacteria in drinking water distribution systems and their interactions with biofilm formation on different materials, and we also analysed the chlorine-resistant bacteria and their problems in the water networks. The materials used in the drinking water network are significantly affected by the disinfection method used to produce the biofilm that adheres to them. Some studies propose that the material is inconsequential, with the disinfection process being the most significant factor. Studies suggest that materials based on plastics (such as PVC and HDPE) tend to be more effective in controlling biofilm formation or removal than those based on metals (such as stainless steel), which have been found to be less effective in some instances. Chlorine-resistant strains are becoming more and more common in drinking water networks, resulting in the occurrence of diseases such as typhus and cholera.

**Keywords:** DWDS; pipes; PVC; HDPE; *Pseudomonas*; chlorine-resistant bacteria (CRB)



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## 1. Introduction

Access to safe and controlled drinking water remains inadequate despite its essential role in sustaining life. Waterborne bacterial infections, such as gastroenteritis, are a common cause of preventable illness and death, affecting many who lack safe water sources. Additionally, chemical contaminants present significant health risks, resulting in vomiting, skin diseases, lung irritation, dizziness, and, in extreme cases, fatalities. Fully purifying drinking water is not necessary, as it may contain harmless and varied microorganisms. These are frequently derived from dispersed and detached biofilm cells and fragments. However, consuming contaminated drinking water or participating in unsafe water activities such as swimming, sailing, and other water sports can lead to waterborne diseases caused by bacteria and viruses. Land-based activities, such as land use and the disposal of faecal waste, are the primary factors contributing to the spread of pathogenic species in water. In the absence of adequate treatment, waterborne diseases will remain a significant public health concern [1]. The presence of biofilm in drinking water systems is the root cause of the spread of enteric pathogens and the contamination of drinking water. Biofilm is the primary cause of the increase in microbial contamination in drinking water systems. Human health is at risk due to three factors: ingestion or inhalation of water droplets while showering and skin contact during swimming or showering [2].

Drinking water treatment plants deliver microbiologically safe drinking water to consumers through numerous miles of pipelines. In several instances, the pipes within the water distribution system are coated with biofilm, which, according to the scientific community, may impact water quality by introducing bacteria into the drinking water.

The design and ongoing monitoring of drinking water distribution systems guarantee the provision of high-quality drinking water, thereby preserving the overall physical health of the population [1].

A plethora of research studies have been conducted on water treatment methods, all of which have contributed to enhancing the quality of purified water. High-quality treated water is distributed through the water distribution system, ensuring its availability at the taps of the population. The primary aim of water utilities is to provide satisfactory drinking water not only at the treatment point but also at the taps. It is widely agreed that this should be the ultimate objective. A drinking water distribution system (DWDS) should act as a defensive measure in conjunction with continuous operation and maintenance to halt the growth and spread of microorganisms that infiltrate the drinking water system before it reaches consumers. The water distribution network facilitates the delivery of properly treated drinking water to the population. The treated drinking water undergoes various forms of stress by the time it enters the populace via the distribution system: physical stress (particulate matter), microbial stress (living biomass), and nutrient stress. The biological and physicochemical processes within the drinking water distribution network led to decreased drinking water quality as compared to the water quality at the initial treatment stage. Microbial interactions significantly contribute to the occurrence of issues in DWDSs. These issues primarily involve the development of biofilm on pipe walls, nitrification, biocorrosion, and pipe material degradation in the network. These conditions alter both the taste and odour of the distributed water and create favourable conditions for the proliferation of opportunistic pathogenic microbes. Understanding the microbial ecology of drinking water distribution necessitates the creation of innovative and practicable control measures to guarantee the provision of safe and high-quality drinking water to the end user [3].

Biofilm formation and emergence in drinking water networks can pose severe health risks by the emergence of pathogenic microorganisms, notably bacteria. The emergence of pathogenic bacteria is a significant issue. Preventing the emergence of harmful microbes from the distributed water supply system is a challenge. Biofilm formation can be prevented by using disinfectants [4,5], ensuring proper hydrodynamic conditions [6,7] and using appropriate pipeline materials [8]. It is also essential to maintain the quality of water throughout the transport networks, including the flow between individual households. The ingestion of chemically or biologically polluted potable water may lead to various health issues for the human body, particularly among vulnerable segments, such as infants, pregnant women, the elderly, and individuals with autoimmune ailments [1].

The aim of this work was to provide an overview on biofilm formation, biofilm investigation, and control with specific emphasis on DWDS. This paper updates the novel methods on bacterial biofilm analysis and provides researchers with an up-to-date summary of recent discoveries regarding the impact of chlorine resistance on biofilm formation. Additionally, the study aimed to delineate the predominant materials present in drinking water networks and their correlation with biofilm development.

## 2. Biofilm

Biofilms can be found in nature, in living organisms, in industrial environments, and in water distribution systems. In the food industry, the presence of biofilm layers on production surfaces and equipment can be a significant hygiene problem. Biofilm can also be formed by pathogenic microbes, which can cause not only industrial hygiene problems but also serious epidemiological problems [1,9–14].

Biofilms in drinking water distribution systems cause different problems in water quality (discolouration, changes in odour and taste) and safety [15,16].

There are many definitions of biofilm in the literature, but they all essentially state a very similar terminology, according to which a biofilm is a biologically active matrix attached to the surface of cells and their extracellular products (EPS—extracellular polymeric substances) [17,18]. Microorganisms not only adhere but also multiply, and the organic

polymers they produce play a role in the formation of the biofilm [19]. EPS formation depends on the composition of the mature matrix, from the forming microbes and their physiological state, as well as the available nutrients [19–21]. Metabolism changes within the biofilm, with anaerobic microorganisms close to the surface and aerobic microorganisms in the aqueous phase (Figure 1 [22]). Within the matrix, there are capillaries and channels through which the cells absorb nutrients [22].

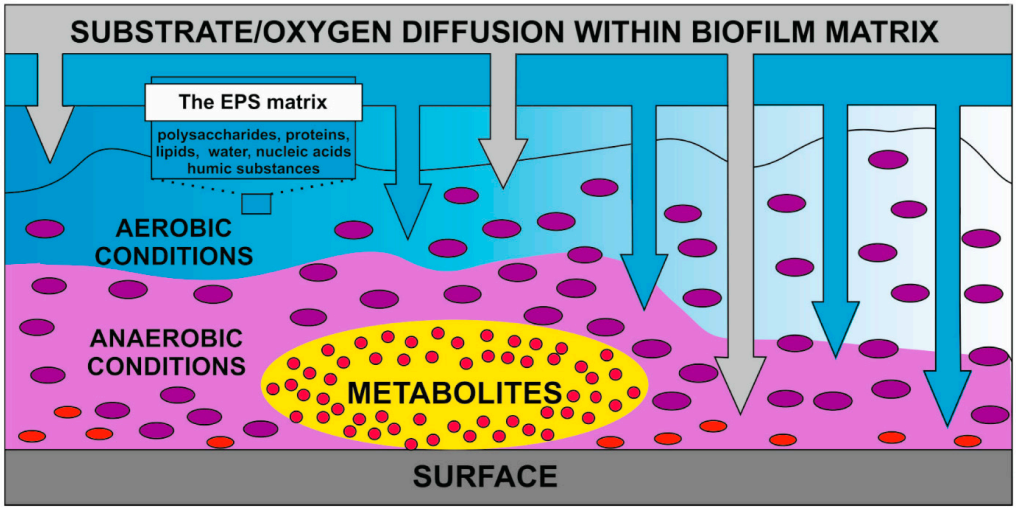


**Figure 1.** Biofilm composition [22].

Biofilm formation in general has been frequently reported in the literature, and the stages and process of biofilm formation in DWDSs occur in a similar way [23,24].

Microorganisms prefer to attach to a solid surface because it provides greater protection [20] and a better supply of nutrients than the planktonic state [25]. Biofilms are more likely to form in flowing, nutrient-poor systems (e.g., a drinking water distribution system) because the starving cells can obtain a continuous supply of nutrients by adhering to the surface [26]. Flemming and coauthors [27] reported that over 95% of the biomass in DWDSs exists in the form of biofilm, with only 5% detectable in planktonic form.

During biofilm formation, proteins, carbohydrates, and other organic/inorganic molecules (rich in nutrients) adhere to the surface of equipment and piping systems, and by diffusion or turbulent flow a 'conditioning film' is formed, which changes the electronic charge and hydrophobicity of the surface in a way that is beneficial to the biofilm [28]. If a flowing aqueous phase is present in the system, the nutrient supply of the conditioning layer is much better than that of the aqueous phase [28]. Once the conditioning layer is formed, cells can adhere, but this depends largely on the nutrient composition and other environmental parameters [29,30]. In some microbes, different proteins promote adhesion. For example, according to some studies, albumin promotes the adhesion of *Listeria monocytogenes* [28], but inhibitory effects can also occur; for example, Helke and coauthors [31] also reported the inhibitory effect of casein on *Listeria monocytogenes*. The formation of the conditioning layer is also strongly influenced by the material and roughness of the surface [11,32]. It also provides a protective function against adverse physical and chemical environmental effects: dehydration, thermal effects, mechanical cleaning, chemicals, disinfectants (e.g., chlorine) [33].

## 3. Bacteria in DWDS Biofilms

Bacteria are the most studied microorganisms in biofilms. The aim of this chapter is to collect commonly used techniques for biofilm monitoring, diversity mapping, and identification, and to list the main bacteria commonly encountered through some studies found in the literature, giving examples of some of the methods (Figure 2). The methods

presented here are generally applicable to biofilms, but they are also suitable for biofilm monitoring in drinking water networks.

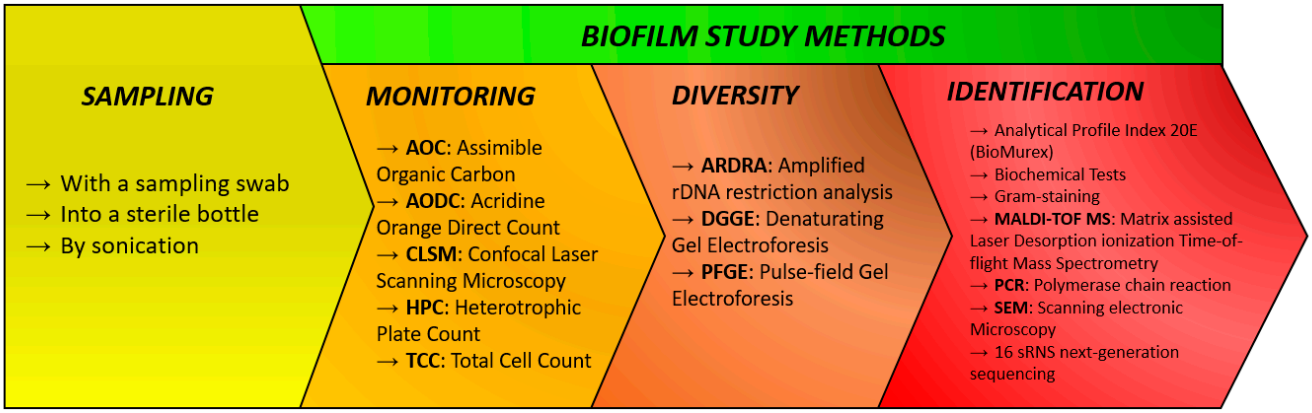


**Figure 2.** Commonly used methods to study biofilm.

The bacterial composition of drinking water was investigated in several studies, using many methods [34–40]. The methods were presented in one or more studies. Hussain and coauthors [34] collected a total of 50 samples from urban wells, tubewells, springs, hand pumps, and taps at different locations in a city of 500,000 citizens in Pakistan. They identified isolates from the samples with traditional microbiological plating techniques: phenotypic studies, morphological studies, biochemical tests (carried out using the Bergey Manual and the online software ABIS 7 and the api*web*$^{TM}$ identification software for API20E). Based on the identification results, 79 strains were identified, of which 82% were Gram-positive. Most strains were rod-shaped (92.5%). Additionally, 10% were Gram-positive bacilli, and 7.5% were Gram-positive cocci. The Gram-positive strains were then tested for spore-forming ability. Most Gram-positive strains (64.2%) had spore-forming characteristics. Among the bacteria found at the different sampling sites, several pathogenic strains were isolated even in tap water, such as *Aeromonas hydrophila*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* sp., and *Vibrio parahaemolyticus* [34].

Zhang and coauthors [41] also investigated biofilm formation in drinking water networks, specifically the occurrence of antibiotic-resistant bacteria (ARB) species; ARB concentration and ARB percentage were detected in a real DWDS for one year. The bacteria were resistant to tetracycline, sulfamethoxazole, clindamycin, and norfloxacin. The bacteria were detected and identified using the heterotrophic plate count (HPC) method, and the effect of biofilm detachment was simulated using a bacterial ring reactor. The result showed that the concentration and relative amount of ARB in biofilms in effluent water were higher than those in biofilms in influent water. Using high-throughput sequencing, the relative abundance of some populations (e.g., *Acinetobacter*, *Sphingomonas*, and *Bradyrhizobium*) was higher in the effluent than in the tap water. These common populations of biofilm and tap water had more antibiotic-resistant species. The results showed that the amount of ARB increased in the tap water, presumably due to biofilm deposition. *Pseudomonas aeruginosa*, *Enterococcus* spp., and *Staphylococcus* spp. appeared, and these were the 3 most common and most identified species from drinking water that affect human health. *Sphingomonas* spp. and *Acinetobacter* spp. were also identified.

The importance of clean and good-quality drinking water is emphasised in the field, and therefore, the study of biofilms and bacteria in drinking water is of paramount importance [3,42–46]. This is also the case in developed countries. In Scotland, a study investigated the presence of *Helicobacter pylori* in biofilms formed on the inner surface of cast iron pipes [47]. *Helicobacter pylori* is one of the most common causes of chronic bacterial infections in humans and is a major cause of chronic gastritis acquired in early childhood, as well as duodenal ulcers and gastric cancer [47,48]. In a Swedish research study, samples of the biofilm have been taken with a swab of the inner surface of the removed section (im-

mediately after removal) during routine pipe section replacement [47]. Subsequent analysis for the presence of *Helicobacter* DNA by nested PCR gave a positive result, i.e., *Helicobacter pylori* was present. These data provided the first evidence that *Helicobacter* is present in biofilms in water supply systems anywhere in the world. In another study [49], researchers analysed municipal water and treated wastewater and well water from all 25 counties in Sweden for the presence of *Helicobacter* spp. DNA. They used immunomagnetic separation to concentrate the bacteria, followed by culturing, Gram staining, and urease tests. Two highly sensitive polymerase chain reaction (PCR) assays targeting adhesin and 16S rRNA genes of *Helicobacter* spp. were performed. The results showed that 9 out of 24 private wells, 3 out of 25 municipal tap water samples, and 3 out of 25 wastewater samples tested positive for both PCR assays. Notably, the positive municipal and wastewater samples were from the same counties.

The importance of testing drinking water is particularly important in developing countries, where many areas still have poor-quality drinking water, and pathogenic bacteria, including *Helicobacter pylori*, are emerging. Bunn and coauthors [48] sampled water catchments to detect the presence of *Helicobacter pylori* and demonstrated that its presence is associated with poor water quality. Samples were incubated on different types of culture media. Colony DNA was purified from the cultured bacteria using the DNAce Clinipure kit. Detection of *Helicobacter* was performed by nested PCR. Based on the results, it was concluded that a decrease in water purity is associated with an increased rate of *H. pylori* colonization. The presence of amplifiable *H. pylori* DNA from water containers further supports the view that *H. pylori* is transmissible. These results provide evidence for the presence of *H. pylori* in the water chain, which may be present in the biofilms of all water containers.

Biofilm testing often involves the construction of a model drinking water system that attempts to mimic the conditions in the drinking water system on a smaller scale. These models use water from the local drinking water network and test the tap water used. In one of these studies [50], a drinking water distribution model was run at 10–15 °C (because the national average drinking water temperature in the area is around this temperature), using stainless steel pipes, and the inside of the pipes was sampled at 140 locations. The model was built up of removable segments and was run for 1 year to develop a quasi-permanent biofilm layer. The microbes were quantified using total microscopic counts (AODC) and heterotrophic plate counts (HPC). They did not specifically quantify the bacteria present in the biofilm but proposed different techniques to quantify the microbes: AODC and ATP technologies were used to monitor the biofilm over a year, and confocal laser scanning microscopy (CLSM) was used to visualize the biofilm structure. To monitor microbial diversity and dynamics in the changing dynamics of biofilms formed on the inner surfaces of the tube, denaturing gradient gel electrophoresis (DGGE) was used after the initial mapping of microbial populations. DGGE results are usually available quickly, and this technique provided a profile of the entire community.

In another constructed model [51], bacterial tracking and identification were investigated using standard heterotrophic plate count and 16S rRNA next-generation sequencing over a total of 7 years. The model was sampled from the inner surface of the tubes, and the results showed that the following bacterial genera were present in the biofilm: *Acinetobacter*, *Legionella*, *Enterobacter*, *Mycobacterium*, *Pseudomonas*, and *Staphylococcus*, with a predominance of *Proteobacterium* taxa.

Similarly, in a constructed DWDS model [51], pipes were constructed from a PVC-U model, and the entire inner surface of the pipe was mapped for biofilm formation. The pipe surface was divided into 3 major parts and each part into even smaller parts (Figure 3).

In the study, the model was run on drinking water and consisted of removable segments. After removing the segments, the samples were collected by swabbing and sonication. Scanning electron microscopy (SEM), DNA extraction, and Illumina 16S rRNA sequencing technologies were used to identify bacteria, and an ATP assay was used to monitor activity. The results showed that the biofilm was unevenly distributed across the

16 sections and 3 sections of the tube surface. The bacterial communities in each section were dominated by *Proteobacteria*. At the genus level, *Nitrospira* spp., *Terrimonas* spp., and *Hyphomicrobium* spp. dominated the sections. *Gaiella* spp. and *Vicinamibacter* spp. dominated in S-I, *Blastopirellula* spp. and *Pirellula* spp. in S-II, and *Holophaga* spp. and *Phaeodactylibacter* spp. in S-III. The methods of swabbing and tube sectioning were also compared, and the results showed that the sampling strategy significantly influenced the resulting biofilm bacterial community. A consistent, multi-stage swab-sampling strategy was proposed for future biofilm sampling [52].

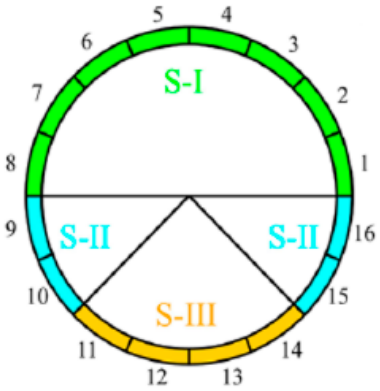


**Figure 3.** Cross section: Great example of how to sample a pipe covering the entire inner surface [52].

Different microscopical methods [50,52] and molecular methods [48,49,51,52] have been applied for the isolation and identification of microorganisms from biofilms. A further method for identifying bacteria in biofilms is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). This method is also commonly used to identify bacteria, especially in biofilms [49,53–58]. Researchers often studied the bacterium *Pseudomonas aeruginosa* and its monitoring of biofilm formation using MALDI-TOF MS and scanning electron microscopy (SEM), comparing the efficiency and applicability of these methods. *Pseudomonas aeruginosa* itself is a pathogen of clinical importance, and its occurrence in drinking water is extremely common. In a specific investigation [53], biofilms were grown in polypropylene tubes with glass plates and sampled at different times. Two separate MALDI-TOF experiments were carried out: one simultaneously sampled biofilm from the glass plate and the inner surface of the polypropylene tube, and another sampled biofilms formed on different materials individually. The biofilm's morphological development was analysed using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The molecular findings revealed that MALDI profiling can effectively differentiate between various biofilm stages and detect the release of biofilm cells during the dispersion phase, initially observed on the polypropylene surface. Moreover, the study suggested that MALDI profiling can be a valuable tool for diagnosing and predicting clinical biofilm formation as well as for ongoing monitoring purposes.

## 4. Biofilm on Different Materials in DWDS

Most scientific papers discussing or even mentioning biofilm highlight the aesthetic problems (colour, odour), public health risks (waterborne diseases), and technical problems (corrosion of pipe materials) associated with biofilm [46,59,60]. Biofilm adhering to materials can be affected by ageing, stressed, or poorly functioning and maintained distribution systems, resulting in a deterioration of the quality of piped drinking water. Water quality can fall below acceptable levels and pose a serious health risk [61]. The piping systems in drinking water networks can be made of different materials which may have different effects on biofilm formation. The materials used in drinking water networks vary from country to country, but there are some common ones that are quite widespread in Europe: asbestos cement (AC), cast iron (CI), polyvinyl chloride (PVC-U/PVC-C), polyethylene (PE), stainless steel, and concrete [60]. High-density polyethylene (HDPE) and polyvinyl

chloride (PVC) are starting to replace concrete in drinking water networks because they are cheaper, thinner, lighter, and less corrosive [46].

Stainless steel pipes are also commonly used in networks. In a study [50], a model drinking water network was constructed using stainless steel pipes, and biofilm formation was monitored at low nutrient levels. Despite the low nutrient availability, significant biofilm growth was observed during the colonisation phase. The results also showed that the diameter of the pipe is important. The electropolished stainless steel exhibited the smoothest surface among the treatments and notably fewer bacterial cells, along with a lower occurrence of early-stage biofilm formation compared to other treated surfaces [62]. Bacterial populations could be reduced during processing by increasing the use of materials that are resistant to bacterial contamination. These discoveries hold the potential to guide equipment manufacturers and processors in selecting materials and finishes that demonstrate resilience against bacteria and the formation of biofilms, consequently strengthening food safety measures.

In the study by Zhou and coauthors [63], biofilm formation was investigated in models of drinking water networks constructed of stainless steel and adjacent copper. The biofilm formation of chloraminated and chloramine-neutralised water was studied in models constructed with both types of materials. Biofilm formation was influenced by the type of biofilm disinfectant and the material of the pipe. Comparing the two materials, a higher bacterial density was found on the surface of the stainless steel pipe than on the surface of the copper pipe. Copper pipes are one of the most notoriously resistant to contamination due to the toxicity of copper ions to microorganisms, particularly bacteria in the biofilm. In the case of water containing chlorine at a concentration of 0.6 mg/L, it was found that the concentration of bacteria present on the surface of copper pipes has been halved within 16 days. The presence of chloramines or chlorine alone does not completely prevent biofilm formation, but suspended heterotrophic bacteria can reduce their numbers. Compared to chlorine, chloramine treatment was more effective in reducing biofilm formation in stainless steel, but especially in copper. The pipe materials tested affected bacterial accumulation with both chlorine and chloramines. Compared to stainless steel, fewer bacteria were bound to the inner surface of the copper pipe by disinfectants containing chloramines or chlorine. The combination of copper pipe and chloramines as a disinfectant was the most effective, resulting in reduced bacteria levels.

Manuel and coauthors [64] also observed biofilm formation on different materials: HDPE = high-density polyethylene, PEX = cross-linked polyethylene, PP = polypropylene, and PVC = polyvinyl chloride. An artificial drinking water system was constructed, adhesion sections of the listed materials were installed, and biofilm formation on their surfaces was studied in two different operating modes, flowing and standing water. Based on the results, it was concluded that the different surface materials tested (PVC, HDPE, PEX, and PP) had no effect on bacterial accumulation in either flow or stagnant conditions. Operation under continuous flow (0.8–1.9 Pa) or standing water had a significant effect on biofilm formation: biofilm growth was lower in standing water. Using mass balances and an asymptotic biofilm formation model, the bacterial growth rates formed on PVC and HDPE surfaces under turbulent flow conditions were similar for both materials and much lower than the specific growth rate, i.e., the least biofilm formed on these two materials. Overall, biofilm formation was independent of the material but was significantly influenced by the mode of operation.

Biofilm formation was monitored for 7 years in a model of a drinking water network constructed at the municipal site in a city of 50,000 inhabitants where the drinking water supplying the city was circulated [51]. The pipe system was made of PVC-U (unplasticized polyvinyl chloride), PE-HD (high-density polyethylene), and cast iron. After 7 years of the model installation, biofilm and water samples were taken from the research collectors. Over the seven years, a large number of bacteria and other microorganisms adhered to the surfaces. In terms of materials, the following genera of bacteria were present in the biofilm adhering to the surface of the cast iron pipes: *Acinetobacter*, *Legionella*, *Enterobacter*,

and *Mycobacterium*. In the biofilm formed on PE-HD and PVC-U surfaces, the classes *Acinetobacter*, *Legionella*, *Mycobacterium*, *Pseudomonas*, and *Staphylococcus* were identified. The *Proteobacteria* taxon dominated on the PE-HD and PVC-U surfaces, while the *Nitrospirae* taxon dominated on the cast iron surface. It was concluded that plastic pipes provide a more favourable environment for potentially pathogenic taxa than cast iron.

In the study by Learbuch and coauthors [60], biofilm formation was investigated on six commonly used materials under worst-case conditions (25 °C, unchlorinated water) with tap water for sixteen weeks: copper, PVC -C, PE-Xb, PE-Xc, PE-100, and PVC-P. First, 950 mL of tap water was poured into a bottle, and a 150 $cm^2$ material piece was placed. This sampling method is quite common [65–67]. The water was dechlorinated, and the flasks were changed weekly. The flasks were kept at 25 °C, and biofilm formation was monitored. Twelve dominant classes of bacteria were identified on a total of 6 different materials over the 16 weeks. Overall, *Betaproteobacteria* were present in significant numbers on each surface, followed by *Alphaproteobacteria*, and then *Actinobacteria* were the third-most abundant.

When testing the materials, PVC-P had the highest biomass, while PVC-C had the lowest, and PE was in between. The main conclusions drawn from the results of the study were that the type of pipe material used in drinking water systems influences the biomass concentration, the number of specific microorganisms, and the composition of the bacterial community. PVC-P showed high values for both bacterial community composition and biomass concentration compared to other materials. The study found that PVC-C is the most beneficial material for controlling microbial growth in drinking water systems because it is the least abundant on the surface. Compared to the two studies described above [60,64], the lowest biofilm formation occurred on PVC material.

Overall, biofilm formation depends both on the material of the pipe in the DWDS and the disinfectant applied for inhibiting biofilm formation [60,64,65,67–70]. In some cases, studies and results are contradictory, as occasionally the material is not important, only the disinfectant treatment [64]. However, there are results showing that plastic-based materials are more effective in controlling biofilm formation and removal [60,64], in contrast to cases where metal-based materials have been shown to be more effective [51,71]. This is an issue worthy of further research, as biofilm formation is influenced by a number of other factors.

## 5. Microbial Chlorine Resistance in DWDS

The removal of biofilm in drinking water distribution systems is based on both preventive and restorative methods, such as reducing the nutrient content of the tap water implementing various disinfection measures or flushing and replacing the water. However, these procedures often fail, primarily because of the cohesive nature of the biofilm, attributed to the physicochemical properties of the exopolymeric matrix. Effective cleaning procedures must disrupt the matrix and/or modify the properties of bacterial biofilms [72].

Chlorine and chlorine-based methods are widely used for the disinfection of water in DWDSs for both households and industries. These methods are popular due to their easy accessibility, affordability, and safety. Chlorination is the prevalent approach to combat biofilm. Chlorine has an exceptionally broad-spectrum effect against most microorganisms. Additional factors contributing to the widespread use of chlorination include its ability to inactivate a significant proportion of microbiological populations through chlorine residues, as well as its powerful sterilization ability, low investment, and operating costs, comparatively lower organic carbon reactivity when compared to other disinfectants, and the long-term maintenance of biostability in treated water. A chlorine-based disinfection process is advantageous due to its lack of bacteriostatic effect and need for continuous application, in contrast to UV and ozone treatments [33,73,74].

### 5.1. Problems with MCR

However, the efficiency of the chlorination method has declined due to the emergence of microbiological chlorine resistance (MCR), resulting in an increased risk of waterborne diseases among the population. Chlorine-resistant strains are increasingly prevalent in

drinking water networks, leading to the occurrence of diseases including typhus, cholera, shigellosis, salmonellosis, giardiasis, cryptosporidiosis, campylobacteriosis, and Hepatitis A virus infection [75]. MCR is a state in which the previously established concentration of chlorine or chlorine-based disinfectant/procedure no longer has a destructive effect on the microorganism during drinking water and wastewater treatment. This state encompasses the survival of microorganisms following chlorine disinfection and their subsequent or repeated growth despite the physiological and genetic destructive effects of chlorine. The mechanisms of MCR comprise in situ cell aggregation, clumping, and structural modification of the microbial cell surface, EPS production, the formation of resistant spores due to re-proliferation, and good adhesion to surfaces within the biofilm matrix [63,72,74,76].

MCR poses several technical problems in both residential drinking water and wastewater treatment. These include issues with management and technical aspects related to inadequate chlorine dosing, problems due to fluctuating flow rates, hazards arising from unstable free chlorine residuals, improper design, and process selection, as well as a lack of quality control in the chosen process. The aforementioned issues collectively contribute to the emergence of pathogenic microorganisms such as protozoa, fungi, and viruses in drinking water. As a consequence, the potential of MCR spreading globally is a concern. Currently, scientific evidence related to MCR is rare and the public health risks are not studied in depth, despite numerous articles discussing the issue of chlorine resistance [74].

Several studies have focused on researching the chlorine resistance of microorganisms present in biofilms [72,76,77]. Various microbial communities, predominantly bacteria, are examined using different concentrations of chlorine-based disinfectants. During one study [77], different chlorine concentrations were applied, and five bacterial strains were tested: *Sphingomonas* sp., *Acidovorax defluvii*, *Acinetobacter* sp., *Bacillus cereus*, and *Microbacterium laevaniformans*. During the study, the biofilm-forming ability of the bacteria was also examined. It was observed that the bacterial strains were able to form a biofilm independently. Consequently, the biofilm formation was solely attributed to one type of bacterial strain. The biofilm-forming power of the bacteria was analysed and ranked in order of strength. *Acinetobacter* sp. Demonstrated the strongest biofilm-forming ability followed by *Sphingomonas* sp., *Bacillus cereus*, and *Microbacterium laevaniformans*, with weaker biofilm-forming ability compared to *Acinetobacter* sp. *Acidovorax defluvii* proved to be the weakest biofilm former. Bacterial strains were ranked according to their resistance to chlorine, and it was observed that *Bacillus cereus* exhibited the highest resistance, followed by *Sphingomonas* sp., then *Microbacterium laevaniformans* and *Acidovorax defluvii*, and finally *Acinetobacter* sp. [77].

Another investigation by Zhu and coauthors [76] examined the chlorine resistance of a multispecies biofilm. This study closely parallels the one described above, as it employed identical chlorine concentrations and bacterial strains: *Sphingomonas* sp., *Acidovorax defluvii*, *Acinetobacter* sp., *Bacillus cereus*, and *Microbacterium laevaniformans*. However, it is noteworthy that in this instance, six different groups of bacterial strains were formed, each comprising four or five mixed bacterial strains, creating six distinct biofilms. The individual groups were composed of the following bacteria:

- Group I: all five strains of bacteria;
- Group II: without *Acidovorax defluvii*;
- Group III: without *Acinetobacter* sp.;
- Group IV: without *Bacillus cereus*;
- Group V.: without *Microbacterium laevaniformans*;
- Group VI.: all except *Sphingomonas* sp.

Resistance to chlorine significantly increased in the biofilm group containing *Bacillus cereus*, while it decreased in the group containing *Microbacterium laevaniformans* (but *not B. cereus*). Resistance to chlorine was not analysed per bacterial strain, but the effect of the various concentrations was observed uniformly for all six groups. The communities demonstrated significant resistance to low chlorine concentrations (<2 mg/L chlorine), with the action mechanisms within the biofilm working well (e.g., EPS formation). However, the

activities within the community were significantly weakened at higher chlorine concentrations (2–4 mg/L). No significant difference was observed compared to concentrations of 2–4 mg/L when the chlorine concentrations exceeded 4 mg/L [76]. The comparison of the two tests shows that *Bacillus cereus* has a high chlorine resistance.

Biofilm removal using chlorination should be compared with alternative techniques to determine its effectiveness. Within an artificially created drinking water network, a biofilm in drinking water was matured for two months on the inner surface of a pipe with a high polyethylene content, and the efficacy values of two biofilm removal methods were compared. In the case of one method, a significant level of chlorine disinfectant was applied by increasing the hydrodynamic shear stress on the wall of the drinking water network. The other method involved exerting continuously increasing pressure on the inner surface of the HDPE to mechanically remove the biofilm. Based on the results, more than half of the bacteria were removed with both procedures. During exposure to increasing hydrodynamic shear stress, biofilm volume decreased. With chlorination, 75% of the cells could be removed and the volume of the biofilm was also reduced [72].

### 5.2. Chlorine-Resistant Bacteria

Bacteria that are highly resistant to chlorine disinfection, capable of survival or even reproduction, are classified as chlorine-resistant bacteria (CRB). Chlorine disinfection does not fully control the risks of CRB, such as pathogenicity, antimicrobial resistance, or microbial growth. In most cases, the attention of researchers is focused on pathogenic and antibiotic-resistant bacteria, and in several locations, the presence of non-pathogenic bacteria is allowed to a certain extent in drinking water networks [33].

Several researchers [33,72,76,78,79] have confirmed the presence of the following CRB bacteria in drinking water networks: *Mycobacterium*, *Pseudomonas*, *Staphylococcus*, *Acinetobacter*, *Bacillus*, *Acidovorax*, and *Sphingomonas*. Subsequent tests are required with these bacteria to determine the chlorine concentration at which their presence in drinking water networks can be minimised. The most common CRBs are shown in Figure 4.

It is also worth dealing with the presence of non-pathogenic CRBs in drinking water networks, as these bacteria can also cause problems. Their presence can lead to pipeline corrosion, increased biofilm formation, nitrification, and affect the sensory quality of drinking water (i.e., colour, smell, taste). Additionally, the presence of CRBs in the biofilm can also lead to the appearance of pathogens in the biofilm, they are better able to adhere to surfaces as they are protected against the disinfectant [33].

The chlorine resistance mechanism of CRB is non-specific (while it is specific for antibiotic resistance). As a rule, chlorine reacts with various cell components, such as the cell wall or cell membrane, resulting in damage, and the inactivation of microorganisms. Consequently, the primary source of chlorine resistance can be attributed to the permeability barrier and chlorine consumption capacity of the biofilm matrix (cells and EPS). A protective permeation barrier formed by cell membranes, cell walls, spore membranes, and EPS serves as a defence mechanism against chlorine. Additionally, chlorine disinfection triggers the activation of various functional genes, including those responsible for managing oxidative stress, facilitating DNA repair, secreting antioxidant enzymes, regulating pore proteins, and repairing cell walls [33]. The development of chlorine resistance in bacteria can be attributed to three primary factors: increased efficiency of the efflux system, activation of the bacterial self-repair mechanisms, and an increased capacity to absorb nutrients [75].

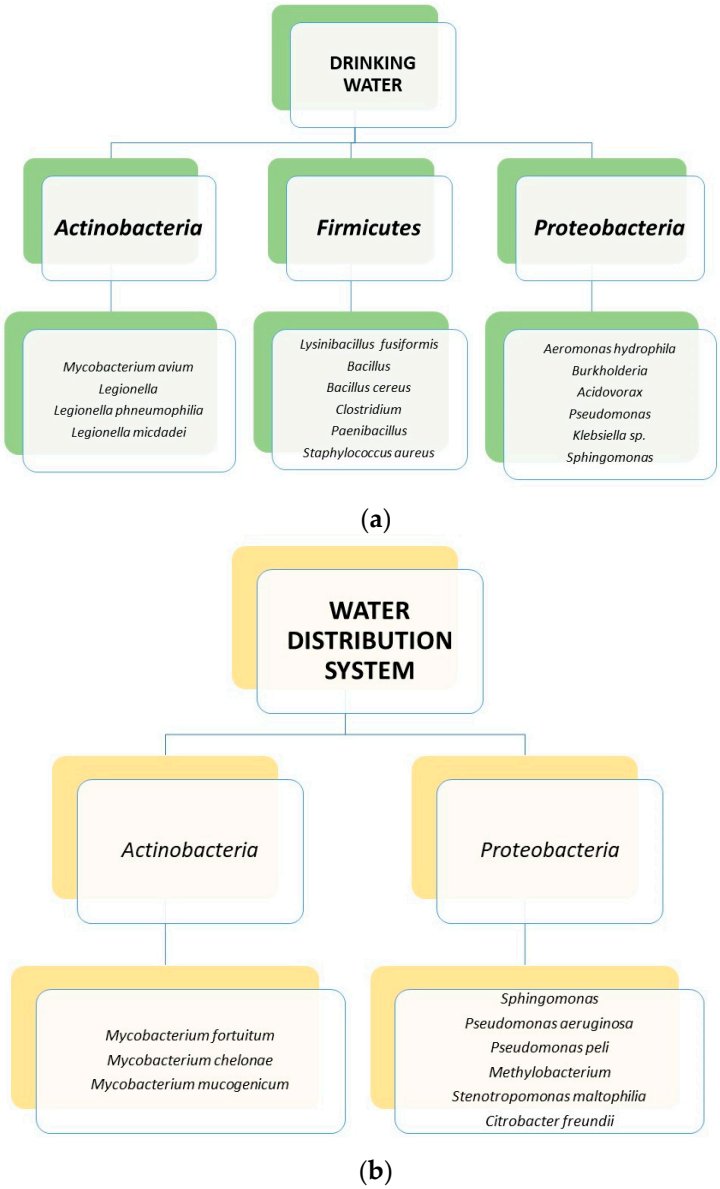


**Figure 4.** The most common CRB in drinking water (**a**) and in water distribution systems (**b**) [33,76,78,79].

## 6. Conclusions

Biofilm formation poses a significant challenge in drinking water networks due to the potential release of waterborne opportunistic pathogenic or pathogenic bacteria, carrying a severe health hazard. Additionally, it can cause biocorrosion and pipe material degradation in the network.

Biofilm formation in drinking water distribution systems similarly to biofilm formation in general is a complex process influenced by various factors, such as available nutrients, the species of biofilm-forming microbes, their metabolism, and EPS production capacity.

There are many technical solutions for studying biofilms. Most of them work with model systems that mimic the conditions found in real drinking water networks. Many studies are based on the examination of sample surfaces used in DWDSs (many of them operate under static conditions). They provide low-level information about the conditions found in real networks connected to parameters depending, e.g., on the influence of fluid flow, but can be well used to study, for example, the biofilm formation of certain microorganisms and the behaviour of microbial consortia. Constructed model DWDSs are closer to real systems. There are very few studies in the literature that examine real drinking water networks, as the piping systems used in daily life can easily become contaminated

even during a poorly performed sampling procedure, which is why it is rarely allowed by water authorities. Under such real conditions, the biofilm formation of certain microbes after deliberate inoculation cannot be studied, but it is possible with the DWDS dynamic or static surfaces model systems. The sampling strategy significantly influenced the resulting biofilm bacterial community.

The materials used to construct piping networks for drinking water can have varying effects on biofilm formation. Different materials are used in different countries. Biofilm formation depends both on the material of the pipe in a DWDS and the disinfectant applied for inhibiting biofilm formation. In certain research studies, findings are inconsistent, as some posit that the material is unimportant, with the disinfectant process being paramount. However, research indicates that plastic-based (PVC, HDPE) materials are more effective in controlling biofilm formation in comparison to instances where metal-based materials (stainless steel) have been found to be less effective.

Biofilm removal in drinking water distribution systems (DWDSs) utilises both preventive and restorative methods, including reducing nutrient content, implementing disinfection measures, flushing, and replacing the water. Chlorine and chlorine-based methods are commonly used to disinfect water in both household and industrial settings, owing to their accessibility, affordability, and safety. Chlorination is the preferred method to combat biofilms, as chlorine has an exceptionally broad-spectrum effect against most microorganisms. However, chlorine-resistant strains are becoming more and more common in drinking water networks. MCR refers to the point at which the concentration of chlorine or chlorine-based disinfectants/procedures that were previously effective in destroying microorganisms during drinking water and wastewater treatment are no longer effective. There are three main reasons behind the emergence of chlorine resistance in bacteria: increased operation of the efflux system, activated bacterial self-repair system, and increased ability to absorb nutrients.

Although there are numerous techniques available to control microbial growth in drinking water, it is not possible to completely eliminate biofilms in DWDSs. Therefore, it is crucial to continuously study biofilm formation in the future to better understand the processes behind their formation and control, as well as the effectiveness of different biofilm management approaches to prevent, limit, and control the spread of biofilms within the DWDS.

To prevent biofilm formation in DWDSs, innovative and effective strategies and approaches are required including the development of biofilm-resistant materials and residue-free chemical treatments. Additionally, new in situ methods must be developed for examining distribution systems to provide more realistic information about the biofilms formed in DWDS systems.

Further research is required to identify the related microbial species, quantify the direct biomass, explore the generation mechanisms, and find effective control methods. To ensure more reliable comparisons of results, it is necessary to develop standard test methods as soon as possible.

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