Understanding, Monitoring and Controlling Biofilm Growth in Drinking Water Distribution Systems

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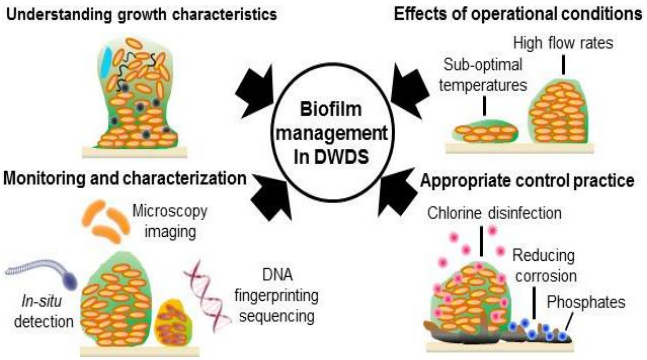
ABSTRACT: In drinking water distribution systems (DWDS), biofilms are the predominant mode of microbial growth with the presence of extracellular polymeric substance (EPS) protecting the biomass from environmental and shear stresses. Biofilm formation poses a significant problem to the drinking water industry as a potential source of bacterial contamination, including pathogens and in many cases also affecting the taste and odor of drinking water and promotes corrosion of pipes. This article critically reviews important research findings on biofilm growth in DWDS, examining the factors affecting their formation and characteristics, as well as the various technologies to characterize, monitor and ultimately, to control their growth. Research indicates that temperature fluctuations potentially affect not only the initial bacteria-to-surface attachment but also the growth rates of biofilms. For the latter, the effect is unique for each type of biofilm-forming bacteria – ammonia oxidizing bacteria for example, grow more developed biofilms at typical summer temperature of 22°C compared to 12°C in fall , while the opposite occurs for the pathogenic *V. cholera*. Recent investigations have found formation of thinner yet denser biofilms under high and turbulent flow regimes of drinking water, in comparison to the more porous and loosely attached biofilms at low flow rates. Further, in addition to the rather well-known tendency of significant biofilm growth on corrosion-prone metal pipes, research efforts also found leaching of growth-promoting organic compounds from the increasingly popular use of polymer-based pipes. Knowledge of the unique microbial members of drinking water biofilms and importantly, the influence of water characteristics and operational conditions on their growth, can be applied to optimize various operational parameters to minimize biofilm accumulation. More detailed characterizations of the biofilm population size and structure are now feasible with fluorescence microscopy (epifluorescence and CLSM imaging with DNA, RNA, EPS, protein and lipid stains) and electron microscopy imaging (ESEM). Importantly, thorough identification of microbial fingerprints in drinking water biofilms is achievable with

DNA sequencing techniques (the 16S rRNA gene-based identification), which have revealed prevalence of previously undetected bacterial members. Technologies are now moving toward *in situ* monitoring of biomass growth in distribution networks, including the development of optical fibres capable of differentiating biomass from chemical deposits. Taken together, management of biofilm growth in water distribution systems requires an integrated approach, starting from treatment of water prior to entering the networks, to potential implementation of 'biofilm-limiting' operational conditions and finally, to the careful selection of available technologies for biofilm monitoring and control. For the latter, conventional practices, including chlorine – chloramine disinfection, flushing of DWDS as well as nutrient removal, and emerging technologies are discussed with their associated challenges.



TOC

INTRODUCTION

Safe drinking water is a basic need and its provision has been a top priority issue world-wide. The main challenge to the drinking water industry is to deliver a product that is microbiologically and chemically safe, as well as aesthetically pleasing. While disinfection practices remove the majority of microorganisms found in raw water, the treated water is not sterile and low levels of microorganisms persist in the water when entering the distribution networks. Studies on drinking water distribution systems (DWDS) have indicated that more than 90% of the total biomass resides in matrix-enclosed microbial colonies on pipe walls called biofilms, with only up to 5% of the biomass suspended in the bulk water.[1] Biofilms are ubiquitous and persistent microbial communities growing on surfaces, capable of continuous shedding of cells that promotes the spread of microorganisms. Biofilms in DWDS range from a few tens of micrometers to a few mm.[2, 3] The biofilms consist of complex and functionally organized microbial communities composed of cells embedded in a gelatinous matrix of biological origin comprised of extracellular polymeric substances (EPS).[4] The EPS matrix is responsible for the integrity of the three dimensional structure of biofilms, gluing cells together and onto surfaces. The EPS also provides protection for the microbial community from adverse environmental conditions. Recent studies revealed that microorganisms can also dwell in loose deposits, such as particulate matter that accumulates at the bottom of the pipes or on suspended solids that are transported through the network.[5-7]

DWDS harbor biofilms even in the presence of disinfectants,[8] potentially affecting the turbidity, taste, odor and color of the water,[9] and in many cases, promoting the decay of residual disinfectants.[10] Growth of biofilms therefore necessitates increased levels of disinfectant agents to improve the disinfection outcome, which can negatively impact the chemical and aesthetic quality of drinking water. Biofilm growth in distribution systems could also increase flow resistance,[3] affecting the network's hydraulic efficiency in the long

run. Moreover, biofilms in many cases secrete acid metabolites that corrode concrete and metallic pipes.[11-13]

Posing a major health threat, biofilms have been known to harbor pathogenic microorganisms,[14-16] potentially releasing them into the water flow through the natural shedding cycle of biofilm.[17] The consumption of contaminated water has been known to cause a wide range of diseases and health problems, particularly affecting infants, young children, the elderly and the immune-compromised population.[18] Examples of pathogens found in DWDS include *Vibrio cholerae* (causes cholera), *Salmonella typhimurium* (typhoid fever), *Escherichia coli, Giardia lamblia* and *Cryptosporidium parvum* (gastroenteritis), *Naegleria fowleri* (amoebic meningoencephalitis), *Mycobacterium avium* (pulmonary infections) and hepatitis viruses.[18-20] In the USA alone, waterborne infections are responsible for over 40,000 hospitalizations, costing the economy $970 million per year.[21]

A core yet still debated issue is the development and impact of biofilms in DWDS, that is, how the characteristics of water and operating conditions of DWDS determine the traits of the growth and subsequently, their roles on corrosion, degradation of disinfectants as well as in facilitating proliferation of pathogens.[16] The knowledge is key for appropriate monitoring and control strategy of biofilm growth in the distribution system. In this *Review*, we describe the occurrence and characteristics of biofilms in DWDS and how the growth is potentially affected by the various operational conditions, including their fluctuations. The choice of pipe materials, flow rate variations, and quite often, changes in temperature and pH, affect biofilm formation, including the initial stage of microorganism attachment onto pipe surfaces. The *Review* further describes a range of technologies that have been available or that are currently being developed for potential use in the monitoring and characterization of drinking water biofilms. Finally, practices to control biofilm development are discussed, including the emerging catalysis and biometabolic based technologies. The *Review* is expected to provide

insights into the susceptibility of water distribution systems to biofilm growth, featuring potential for manipulation of operational parameters as well as the selection of the right technologies for the challenging issue of biofilm monitoring and control.

Occurrence and characteristics of biofilms in water distribution systems

In comparison to their planktonic, 'free-living' counterparts, biofilm microbes in general show increased protection against a range of stressors. In the case of drinking water biofilms, these include resistance against disinfectants,[22, 23] shear stress conditions,[24] thermal stresses and predators.[25, 26] The increased resistance of bacteria within biofilms is in part due to the EPS matrix that they produce. EPS can retain and store compounds including nutrients to provide food reserves for microbial members during starvation period,[27] as well as bind and inactivate disinfectants such as chlorine and chloramines.[28] Further, specific EPS components, such as the Psl exopolysaccharides formed by *Pseudomonas aeruginosa* biofilms have been known to increase the elasticity and cross-linking within the matrix, which in addition to increase protection against shear stress, is thought to facilitate formation of microcolonies.[29]

Biofilms form through a series of developmental stages (Figure 1), which are generally controlled genetically in response to environmental cues and signals. Of the genetic factors controlling biofilms, one of the better studied systems include the intra- and inter-species cell-to-cell communication systems called quorum-sensing (QS), which are responsive to changes in cell population density or local diffusion parameters. Quorum sensing bacteria produce and detect diffusible signal molecules, called autoinducers, enabling cells to sense, communicate with other cells and subsequently adjust to changing physiological needs under different growth conditions and to do so in a coordinated, population level response. Two of the best described quorum-sensing systems in bacteria are the acylated-homoserine lactone

(AHLs)[30] system present in many Gram-negative species and the peptide-based signaling system present in many Gram-positive species.[31] Quorum sensing has been shown to influence biofilm formation by controlling EPS synthesis in *V. cholerae*[32] and by controlling cell aggregation in *Serratia marcescens* (*liquefaciens*).[33] Referred to as diffusion sensing, QS is thought to play a role on biofilm development in specific geometric configurations of the DWDS, such as small diameter pipes or dead-end pipes, whereby QS molecules bounce off neighbouring surfaces, thus triggering QS-mediated gene expression, even with only presence of low cell density.[34] In high velocity regions on the other hand, it is still unclear as to whether QS molecules could accumulate to the required threshold concentration to play a role in biofilm formation.[35] Nonetheless, bacterial isolates from drinking water have been shown to produce QS signals as well as QS quenching molecules, suggesting that these signalling systems are active in DWDS.[36]

In addition to QS, biofilm formation is genetically controlled by bis-(3',5')-cyclic dimericguanosine monophosphate (c-di-GMP) signaling. C-di-GMP is a highly conserved secondary messenger molecule that controls the transition from a free-living, motile lifestyle to a biofilm mode in many bacteria.[37] Cells adjust their c-di-GMP levels in response to environmental cues and intracellular signals. High concentrations of c-di-GMP tend to promote cell attachment to surfaces, biofilm formation, EPS production, and attenuation of motility and virulence, while low concentrations of c-di-GMP promote planktonic growth, activate motility, induce biofilm dispersal and repress EPS production (note that the 'high' and 'low' c-di-GMP thresholds are unique for different bacterial strains).[38-40] For example, upon sensing nutrient limitation, *e.g.* depletion of carbon, nitrogen or oxygen sources, intracellular c-di-GMP level decreases in *P. aeruginosa*, resulting in rapid dispersal of the

biofilm.[41] The quantification of c-di-GMP can be achieved by organic extraction and LC-MS/MS analysis[41] or can also be performed semi-quantitatively using a bio-reporter strain.[42]

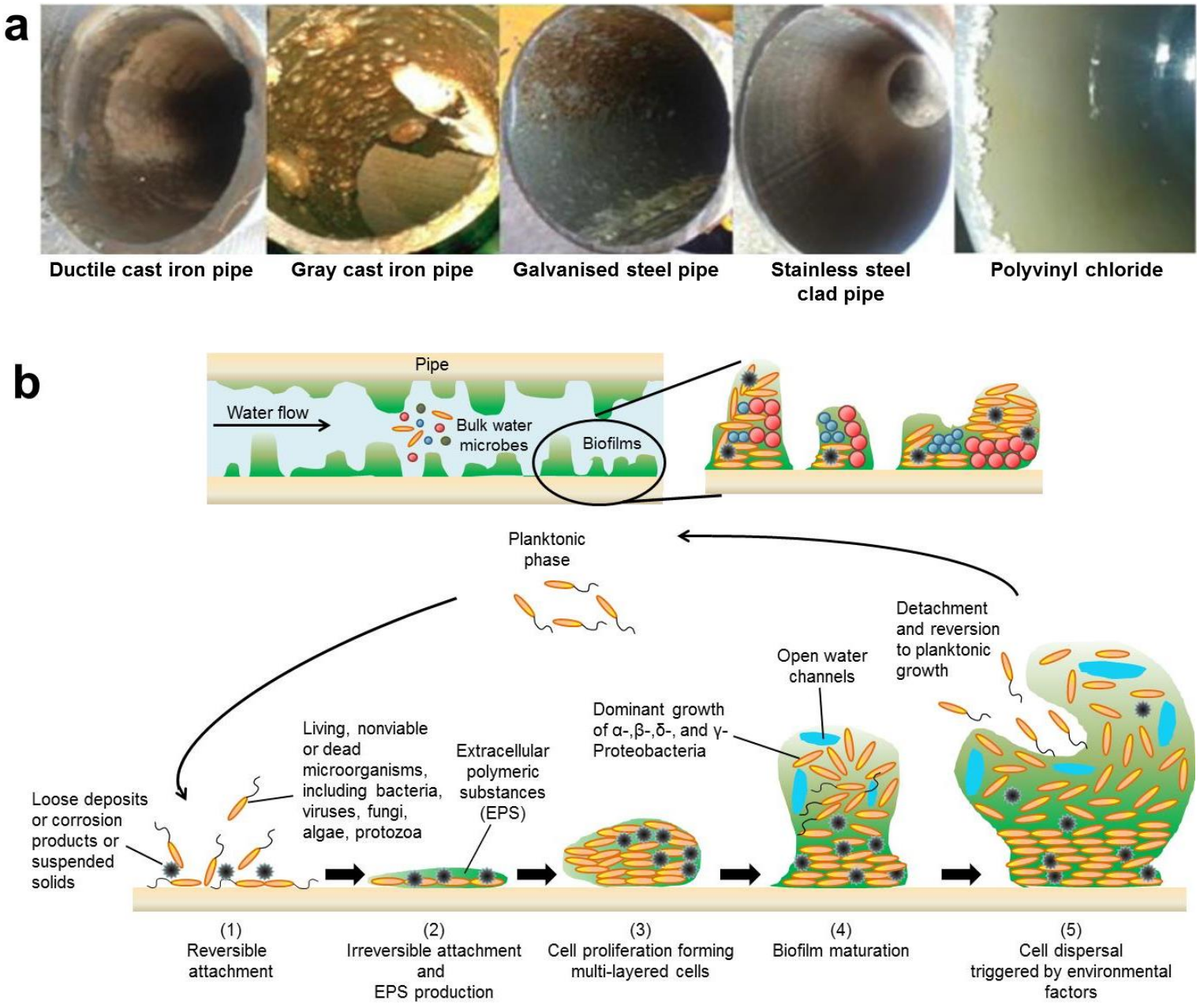


Figure 1. (a) Biofilm growth on different pipe materials. Reprinted with permission from Ren *et al.*[43] Copyright (2015) Springer. (b) Biofilm life cycle in DWDS.

Biofilms in water distribution systems are mainly comprised of water from the gelatinous matrix, which can occupy up to 99% of the total volume, while microorganisms in fact represent only 2–5% of the volume.[44-46] EPS accounts for 50–90% of the total organic carbon in biofilms[47, 48] and is generally comprised of polysaccharides and proteins as the major components (75-89%),[49] with varying amounts of nucleic acids, lipids, phospholipids and humic substances.[50] Inorganic particles such as corrosion products, suspended solids and sand, may also be incorporated in biofilms, increasing its mechanical strength and biomass accumulation.[51] According to Characklis and Marshall,[52] bacteria are generally the dominant members of biofilm microbial communities in DWDS due to their high growth rates, relatively small size, adaptation capabilities and ability to produce EPS. Viruses, filamentous fungi, algae and protozoa may also be present in drinking water biofilms.[15, 53, 54] These 'secondary' microorganisms, in particular, viruses and protozoa could rapidly attach and persist in existing drinking water biofilms,[17] while the involvement of filamentous fungi in biofilms has not yet been satisfactorily established.[55] Protozoan opportunistic pathogens including *Acanthamoeba* (causing keratitis mostly from contact lenses stored or washed in tap water) and *Naegleria* (encephalitis *via* nasal washes) are regularly found in DWDS, probably mainly in reservoirs. *Cryptosporidium* (protozoa responsible for gastrointestinal diseases) was reported to be present in some DWDS, potentially introduced *via* faecal contamination.[16]

In DWDS, true monospecies biofilms are rare. Diverse members of the bulk water microbes are known to have capabilities to produce EPS and/or molecules required for cell-to-cell communication, facilitating their initial attachment, subsequent colonization and biofilm formation on pipe surfaces, even in chlorinated drinking water.[56] Clear examples of such microbes include members of the *Pseudomonas, Janthinobacterium* and *Methylophilus* genera, with their high initial affinity and subsequent growth on high density polyethylene

pipes.[56] In many cases, the co-presence of microorganisms has been shown to enhance biofilm formation. For example, Min and Rickard[57] reported that co-aggregation of bacteria promotes biofilm development by facilitating attachment to the partner species. Further, Simoes *et al.*[58] investigated the role of species-to-species interactions in the formation of mixed-species drinking water biofilms, and observed a range of synergistic interactions. *Acinetobacter calcoaceticus* for example, was found to co-aggregate with bacteria commonly found in drinking water, such as *Burkholderia cepacia* and *Mycobacterium mucogenicum* to form biofilms and interestingly, no bacterial co-aggregation was observed in its absence. The results suggest the 'bridging' function of *A. calcoaceticus* in drinking water biofilm formation.[58] Similarly, despite its inability to attach to solid surfaces, *Escherichia coli* PHL565 was able to form mixed biofilms with 'adhesive' bacteria, such as *Pseudomonas putida* MT2.[59]

Research efforts have revealed wide variation in the identity and composition of microbial communities in drinking water biofilms,[8, 60] which are also noticeably different when compared to the corresponding planktonic population in bulk water.[8, 61, 62] The latter suggests that only specific members of the free-living bulk water microbes are capable of attaching to pipe surfaces and form biofilms. Proteobacteria, particularly those belonging to the α, β, γ and δ subclasses, have been found to dominate biofilms in DWDS (Figure 1b),[8, 62, 63] suggesting that these microorganisms are well suited to survive in potable water supplies. The proportion of the bacterial subclasses in the biofilm varies widely depending on the pipe material,[64, 65] biofilm age,[66] phosphate treatment[67] as well as disinfection practices.[67, 68] Two separate studies revealed that α-Proteobacteria such as *Sphingomonas* and *Hyphomicrobium* predominate in water with low chlorine residuals (<0.02 mg/L), and in chloraminated water,[68] whereas β- and γ-Proteobacteria flourish with increased chlorination.[69] Within the class of β-Proteobacteria, examples of predominant bacterial genera include *Janthinobacterium,*

206 *Methylophilus, Burkholderia, Nitrosomonas* and *Alcaligenes*.[2, 26, 30] A number of pathogens
207 and opportunistic pathogens belonging to γ-Proteobacteria subclass have been particularly
208 found in water distribution systems, which are thought to also exist as members of drinking
209 water biofilms: (1) the faecal bacteria *Escherichia coli* of which a few strains are pathogenic,
210 (2) the opportunistic pathogens 'non-tuberculous mycobacteria' (NTM) such as
211 *Mycobacterium avium* and *M. kansasii* can cause serious pulmonary and lymphatic disease,
212 with at least 20,000 reported cases in the USA alone in 2010, (3) the opportunistic pathogen
213 *Pseudomonas aeruginosa* can infect eyes, ears and skin and its transmission in hospitals has
214 been implicated to result from water source, and (4) the opportunistic pathogen *Legionella*
215 *pneumophila* that causes Legionnaire's disease (pneumonia) with 8,000 – 10,000 cases in the
216 USA alone in 2008.[16, 70] Some pathogens, including *M. avium* and *L. pneumophilla* can even
217 proliferate within various amoebas in biofilm.[16, 71] Other γ-Proteobacteria pathogens and
218 opportunistic pathogens found in DWDS include *Enterobacter*, *Acinetobacter, Klebsiella*,
219 *Aeromonas*.[67, 72, 73] Note that the occurrence of such hygienically-relevant microorganisms in
220 distribution systems is different to that of in the drinking water installations. In temperate
221 climates for example, the opportunistic pathogens NTM, *P. aeruginosa* and *L. pneumophila*
222 only have a minor role in DWDS in comparison to the drinking water installations in
223 buildings.[74] Additional bacteria found in biofilms in DWDS include members of
224 *Actinobacteria, Chloroflexi, Bacteroidetes, Nitrospirae, Firmicutes, Verrucomicrobia* and
225 *Acidobacteria*.[62] Mixed community biofilms display enhanced protection against
226 environmental stresses, that renders them significantly more stable than the monospecies
227 systems.[75, 76]

228 Advances in molecular biology technique, such as the 16S rRNA gene-based identification
229 (discussed in later section) have allowed detection of water relevant 'viable but non
230 culturable' (VBNC) bacteria,[77, 78] which is thought to also reside in drinking water biofilms.

231 A number of relevant pathogenic bacteria, such as *E. coli*, *L. pneumophila*, *Listeria*
232 *monocytogenes* and *P. aeruginosa*, have been reported to enter starvation mode or a
233 physiologically viable but non-proliferating state as a response to adverse environmental
234 conditions such as unfavourable temperatures, chlorination, pH fluctuations, nutrient
235 depletion, and oxygen stress.[79, 80] The potential presence of pathogenic 'viable but non
236 culturable' (VBNC) bacteria in drinking water biofilms is a threat to public health due to their
237 ability to regain virulence under favourable growth conditions.[80, 81] Undetectable by
238 conventional culturing methods, the population density of VBNC bacteria are often
239 underestimated.

240 Finally, recent studies have indicated that biofilms may also serve as reservoirs for the
241 spread of antibiotic resistance genes (ARGs), most likely as a result of the high cell density
242 and close cell-to-cell proximity and consequently, the increased likelihood of gene transfer
243 within bacterial populations. Engemann et al.[82] found that tetracycline resistance genes readily
244 migrated into biofilms, suggesting biofilms as long-term reservoirs for ARGs. Antibiotic
245 resistant bacteria (ARB) and ARGs in drinking water are increasingly considered as
246 contaminants since they may greatly affect public health. ARB and ARGs in natural fresh
247 water systems can reach drinking water supplies and in turn, entering human. For example,
248 the *vanA* gene, which confers resistance to vancomycin, was detected in drinking water
249 biofilms in the absence of *Enterococci* (faecal bacteria thought to be the original carriers of
250 these genes), implying transfer of resistance genes from faecal bacteria found in wastewater
251 and surface water to naturally-present drinking water bacteria.[83] It is also possible that the
252 genes were part of the genome of VBNC bacteria.[83] Several studies have detected ARB in
253 drinking water systems. Faria et al.[84] detected *Staphylococcus* with resistance to multiple
254 antibiotics in drinking water samples. Xi et al.[85] detected ARGs and heterotrophic ARB in all
255 finished water (water that has passed through a water treatment plant that is, prior to entering

the distribution system) and tap water in several cities in Michigan and Ohio, with higher quantities of most of the ARGs and ARB in the tap water compared to those in the finished water. The latter suggested regrowth of the bacteria in the distribution systems.

Biofilm growth is affected by water characteristics and operational conditions of the distribution systems

Growth of biofilms in DWDS is a complex phenomenon, consisting of a number of interconnected growth stages (Figure 1b). Biofilm development typically starts with the formation of conditioning films composed of macromolecules (such as polysaccharides, lipids, proteins and humic substances found in drinking water and/or secreted by microorganisms) on surfaces, and the subsequent initial attachment/adhesion of microorganisms on the films.[86, 87] Note that the formation of a conditioning film is particularly important in nutrient-depleted environment, such as drinking water, where the accumulation of organic molecules at surfaces create a relatively nutrient-rich local environment. This is followed by the formation of microcolonies with generation of EPS and quorum sensing molecules. Upon reaching a maturation stage, biofilms undergo a dispersal phase, releasing single cells into the bulk water to form new colonies elsewhere, thus completing the biofilm life cycle. The microbial composition of biofilms changes rapidly prior to reaching maturity and up to this stage, it remains unclear as with the stability of mature biofilms.[16] Herein, comprehensively established from numerous drinking water biofilm studies, we found that the development of biofilms in the distribution system is likely to be affected by a number of inter-related factors (Figure 2).

Organic and inorganic matter as nutrients for biofilm growth in water distribution systems

Organic matter content in water distribution systems is fuel for biofilm formation, with most aquatic microorganisms metabolizing biodegradable organic matter (BOM) for energy sources (dissimilation) and for the production of cellular materials (assimilation). Studies have correlated the structural and physicochemical characteristics of organic matters to their biodegradability or in other words, their bioavailability. Unsaturated aliphatic compounds, such as simple carbohydrates, low molecular weight proteins and organic acids are in general more accessible for microbial degradation compared to the more hydrophobic aromatic compounds, including aromatic carboxylic acids, phenolic compounds and humic substances.[88-91] Sun *et al.*[90] reported a positive correlation between the percentage of aliphatic carbon, indicated by H-to-C ratio of organic matters with their bioavailability. Significantly influencing the species of microbial growth, the levels of BOM are unique to each distribution system depending on the water source and the capability to identify which carbon species are present is still not so well-developed in the water industry.[16] Regardless, oxidative drinking water treatments, such as ozonation[92, 93], UV radiation[94-96] and a combination of UV/H_2O_2 treatment[97], have been suggested to increase the bioavailability of organic matters in distribution systems due to alteration of their chemical structures. Reduction of BOM contents, typically by biological filters, is therefore necessary prior to the water entering the distribution system to control the subsequent biofilm growth and development.

Biofilm growth is also significantly influenced by the presence of inorganic nutrients, such as phosphorus, even in distribution systems with high organic matter contents.[98-100] Growth of bacteria in drinking water needs phosphorus,[99, 101] as it is indispensable for cellular metabolism, *e.g.* for the formation of high energy compounds such as ATP, as a building block in DNA, RNA and phospholipid biosynthesis, as well as in post-translational control of protein activity although it is absent in nascent proteins.[102] Despite its growth enhancing

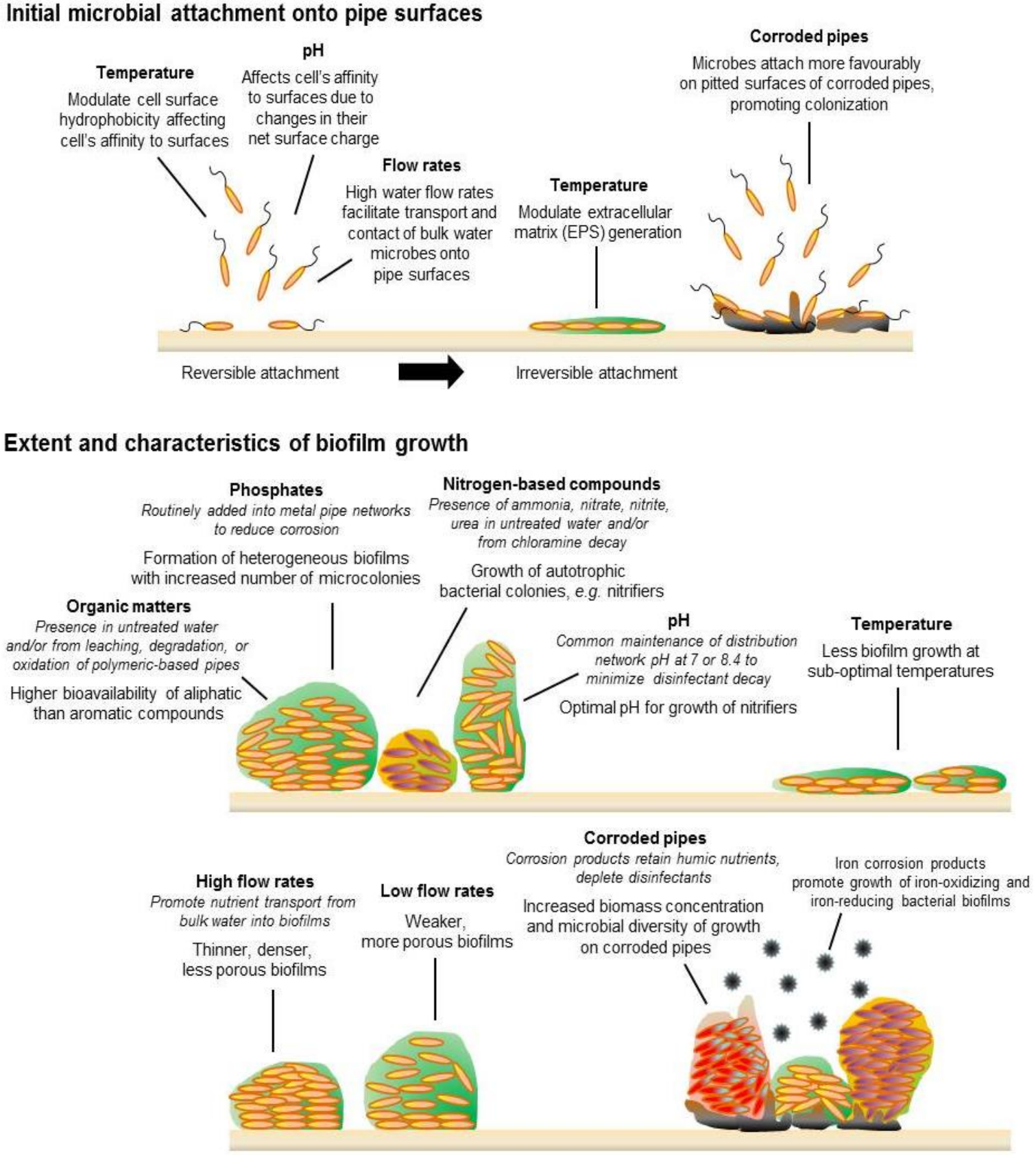
306 effect, phosphorus in the form of phosphate is still routinely added to water distribution
307 systems to passivate metal surfaces, whereby it forms stable complexes with corroded surface
308 metals,[103] which limits further corrosion. Changes in the biofilm structure and microbial
309 community have been reported following phosphate (or phosphoric acid) addition.[104, 105] A
310 clear example is given by Fang *et al.*[106] who observed the formation of thicker, more
311 heterogeneous biofilms with higher number of micro-colonies upon phosphate treatment.
312 Batté *et al.*[67] reported a significant increase in γ-*Proteobacteria* within biofilms, which
313 potentially includes common pathogens. The work however, further reported no change in the
314 bacterial counts following addition of a relatively high concentration of phosphate (500 µg/L)
315 to systems with already established biofilms.[67] This suggests that the phosphorus was not
316 stimulating growth under the conditions tested. The addition of phosphate to distribution
317 systems naturally containing growth-optimal phosphorus concentrations is therefore expected
318 to have no impact on microbial growth. In water with low phosphorus content, both
319 planktonic and biofilm growth have been reported to increase with 1 to 300 µg/L phosphate
320 addition.[107-109] Biofilms have also been shown to elevate their EPS production in response to
321 phosphorus limitation,[110] which appears to serve as protective mechanisms against the growth
322 inhibiting effect of phosphorus limitation.

323 Interestingly, studies have shown that addition of phosphate to highly corroded distribution
324 systems is in fact unfavourable for biofilm development. Appenzeller *et al.*[111] reported that
325 phosphate modifies the properties of iron corrosion products, reducing their bioavailability
326 and in turn, rendering the pipe surface less favourable for microbial colonization.[112] Further,
327 the disinfection efficiencies of chlorine and monochloramine treatments were found to
328 increase with phosphate addition, and were attributed to the reduction in EPS production as a
329 result of phosphate treatment although the cell number increased.[106]

330 Another key inorganic nutrient affecting biofilm development is nitrogen, a building block
331 for proteins and genetic materials (DNA and RNA). A major class of microorganisms that
332 form biofilms in DWDS are the autotrophic nitrifying bacteria or nitrifiers, which utilize
333 nitrogen-based compounds such as ammonia, nitrate, nitrite and in some species, urea, as an
334 energy source,[113] with ammonia as the preferential compound for biomass production.[114]
335 Ammonia is often present in untreated water and is also released during chloramine decay.[115]
336 Ammonia also forms from reactions of nitrate with metal surfaces in distribution systems.[116,
337 117] As with phosphorus, it was reported that the water's nitrogen content could modulate the
338 composition of microbial communities in biofilms. Biofilms with predominantly autotrophic
339 bacteria tend to form at high nitrogen-to-carbon ratios, whereas low nitrogen-to-carbon ratios
340 promote growth of heterotrophic bacteria.[118, 119] A modelling work by Zhang *et al.*[120] (based
341 on the work of Verhagen and Laanbroek[119]) predicted that autotrophic bacteria will flourish
342 above the critical nitrogen-to-carbon ratio of around 10, while their presence is expected to be
343 negligible at nitrogen-to-carbon ratio of 0.1. The work also predicted co-existence of
344 heterotrophic and autotrophic bacterial population at between 0.1 to 10 nitrogen-to-carbon
345 ratios. Unlike heterotrophic bacteria that degrade complex organic matters as a carbon source,
346 autotrophic bacteria are capable of synthesizing their cellular constituents using carbon
347 dioxide as carbon source.
348 Finally, trace metals such as iron and copper are also known to affect biofilm development
349 in DWDS. Iron is essential for almost all bacterial growth and development, but at high
350 concentrations can be toxic to the cells.[121] Growth in biofilms is often associated with
351 expression of iron acquisition genes, suggesting that iron is a limited resource in biofilms.[122,
352 123] Further, several studies reported that iron sequestration can inhibit biofilm formation,[124]
353 while others have reported that addition of iron can induce dispersal from biofilms.[125] Copper
354 on the other hand, is reported to enhance bacterial aggregation at toxic levels, which is

thought to act as protective responses to stress.[126, 127] Presence of copper in drinking water has also been shown to induce VBNC state on the opportunistic pathogen *P. aeruginosa.*[81]

Thus, it is clear that biofilm growth in the DWDS can be controlled by removal of not only the biodegradable organic matter (BOM), but also by limiting the amount of inorganic nutrients, including nitrogen, in the bulk water prior to entering the distribution system. In some countries and even parts of the US, such nutrient limitation has been a common practice for decades. Bulk water pretreatment could be performed in the case of high nitrate-containing ground water through ion-exchange processes, reverse osmosis and even biological denitrification. In regard to the routine practice of phosphate addition to distribution systems, an appropriate monitoring strategy (as later discussed) is necessary to anticipate potential biofilm growth, particularly in systems with initially low phosphate bulk water content.



370

371 **Figure 2.** Effect of water characteristics and operational conditions of DWDS on biofilm

372 formation.

373 ***Influence of water temperature fluctuation in distribution systems on biofilm development***

374 DWDS, while they are commonly buried underground, are often subjected to temperature
375 fluctuations, in particular in multi-seasonal countries. For example, the temperature of
376 distribution systems in North America typically vary from around 22°C in summer, to 12°C
377 in fall and 6°C in winter,[128] while temperatures ranging from 6°C to 35°C[129] are not
378 uncommon in Australia. Such temperature fluctuations could significantly affect the initial
379 cell-to-surface attachment and subsequent formation of drinking water biofilms through a
380 number of innate mechanisms. Temperature affects expression of many genes that could
381 result in changes in the microbial ability to generate EPS as well as modification of the cell
382 surface hydrophobicity. Such temperature-dependent modulations have been observed in
383 bacteria, such as *Listeria monocytogenes*,[130-132] *P. aeruginosa*[133] as well as other bacteria,[134]
384 that have been found to form biofilms. *L. monocytogenes* for example, is reported to produce
385 EPS at 21°C and therefore adhering on surfaces and forming biofilm, but not at 10°C or
386 35°C,[130] as also observed by other studies.[131]

387 Relevant biofilm-forming bacteria, including *Acinetobacter*, *Agrobacterium radiobacter,*
388 *Alcaligenes, Arthrobacter* sp., *Corynebacterium* sp., *E. coli, P. aeruginosa*, *P. fluorescens*
389 and *P. putida*,[135] have been known to become more hydrophobic during the exponential
390 growth phase,[135] and temperature variations are reported to modulate cell surface
391 hydrophobicity in a growth phase-dependent manner. With decreasing temperature from
392 37°C to 8°C, Chavant *et al.*[136] observed a more prominent decrease in cell hydrophobicity
393 with stationary phase *L. monocytogenes* compared to exponentially growing cells.[136] It has
394 been suggested that bacteria modify their cellular membrane lipid composition as a function
395 of temperature, leading to changes in hydrophobicity,[137] and subsequently their affinity for
396 attachment to a particular substratum. A clear example would be where the hydrophobic
397 nature of *L. monocytogenes* cell surface at 37°C corresponds to a higher degree of initial

attachment onto hydrophilic surfaces (stainless steel) compared to hydrophobic surfaces (polytetrafluoroethylene, PTFE).[136] In contrast, at 8°C, the cell surface becomes hydrophilic and cell attachment was observed not only on the hydrophilic surfaces, but comparably also on the hydrophobic surfaces.[136] Despite potential differences in the initial bacteria-to-surface attachment, it has been frequently observed that over prolonged periods (days or months), there is generally no observable difference in the extent of biofilm accumulation on hydrophilic surfaces compared to those on hydrophobic surfaces.[138]

Microorganisms tend to form biofilms at a lesser extent at lower temperatures.[128, 136] This is primarily due to the prolonged lag time, the length of time before cells start to proliferate, and the reduced growth rate at sub-optimal temperatures.[139, 140] For example, *L. monocytogenes* forms three-dimensional biofilms at 37°C and 20°C on both hydrophilic (stainless steel) and hydrophobic (PTFE) surfaces, with only a monolayer of cells observed at 8°C.[136] In other drinking water relevant cases, temperature fluctuation does not appear to affect the presence of ammonia oxidizing bacteria (AOB) in chloraminated systems – the bacteria are capable to deplete monochloramine and generate nitrate.[128] However, less developed AOB biofilms are formed at 12°C compared to those formed at 22°C.[128]

Interestingly, some microorganisms form more developed biofilms at lower temperatures. Decreasing temperatures from 37°C to 25°C or 15°C were found to elevate the intracellular c-di-GMP level in the pathogenic *V. cholerae*, in turn, enhancing biofilm growth.[141] The cellular physiological responses was linked to 6 DGC genes, which encode for the synthesis of diguanylate cyclase enzymes involved in the formation of c-di-GMP. Mutants lacking the genes did not form biofilms in response to the temperature downshift.[141] In other studies, a temperature increase by 5ºC or more was found to induce dispersal of a pre-established *P. aeruginosa* (an opportunistic pathogen) biofilm, and the effects are also linked to changes in cellular c-di-GMP level.[142]

Taken together, the findings demonstrate the clear influence of temperature on the affinity of relevant biofilm-forming bacteria to unique types of surfaces as well as on the growth of biofilms. An understanding of the temperature-dependent susceptibility of water distribution systems to biofilm formation will allow for prompt implementation of appropriate biofilm monitoring and control strategies. It is noteworthy to mention however, that in general an increase in temperature leads to higher rates of disinfectant degradation,[143] which in turn, increases disinfectant demand. Applications of higher doses of disinfectant are therefore necessary, in particular during warmer temperatures, to maintain the microbiological quality of the water.

Effect of pipe materials on biofilm formation

A range of pipe materials have been used for the distribution of drinking water. The majority of the pipeline networks have been of iron (stainless steel and galvanized steel), copper or cement based materials, while polymer based materials such as, polyvinyl chloride (PVC) and polyethylene (PE) are becoming increasingly popular as they are easier to handle and install. The choice of pipe materials could affect development of biofilms in distribution systems. Polymeric pipes could be a source of biodegradable volatile organic compounds (VOCs) in drinking water,[144] due to leaching of polymer additives, polymer degradation as well as by-products of polymer oxidation. It has been shown that microorganisms could proliferate by metabolizing small molecular weight plasticizer, residual monomers as well as anti-oxidants, potentially promoting biofilm growth on pipe surfaces.[145, 146] Many studies however, have observed less growth and microbial diversity on polymeric pipes compared to those formed on corrosion-prone materials, including iron based pipes (Figure 1a).[147-150] In contrast to the 'smooth' surfaces of polymeric pipes, it is thought that the pitted surfaces of corroded iron pipes (old iron pipes can become severely encrusted with scale and rust

448 exceeding 10 centimetres in depth) protect biofilms from physical perturbation and/or
449 chemical disinfection, as well as promoting microbial attachment and colonization due to
450 greater surface area.[16, 138, 148, 151] Further, dissolved and solid iron corrosion products in
451 DWDS could support the growth of specific biofilm-forming bacteria. Iron-oxidizing
452 bacteria, such as *Gallionella* spp. oxidize ferrous iron to ferric iron,[152, 153] while iron-reducing
453 bacteria, such as *P. aeruginosa, P. fluorescens* and some members of *Bacillus spp.*[13, 154]
454 reduce soluble[155] or solid iron (III) species[156, 157] to iron (II) species.[158] Corroded pipe
455 material could retain nutrients, including carbon, nitrogen, phosphorus,[159, 160] for subsequent
456 utilization by biofilm bacteria. Corrosion products could also react with disinfectants,
457 depleting residuals particularly near pipe surfaces.[161] Indeed, an increase in microbial
458 concentration and diversity has been observed on biofilms formed on severely corroded
459 pipes.[162]

460 It is therefore clear that the right choice of pipe material would mean better management
461 of biofilm development in DWDS. Corrosion-prone materials, such as iron should be avoided
462 due to the growth-promoting effects of the corrosion products, including depleting
463 disinfectant residuals. Although polymeric-based pipes have less tendency to support biofilm
464 growth when compared to iron based pipes, countries such as Germany have been enforcing
465 certification systems that prohibit the use of growth-promoting polymers.[74] A range of
466 polymers, such as the 'without certificate' ethylene-propylene-diene-monomer (EPDM), have
467 been known to support microbial growth and cause contamination problems in practice.[74] The
468 implementation of these standards however, is difficult to monitor. The use of EPDM, for
469 example, is still common in drinking water systems.[74]

470

471

472

Flow rate variation in distribution systems affects biofilm growth

The hydrodynamic conditions in DWDS may dramatically vary between different locations, alternating from laminar to turbulent flow and *vice versa*. Flowing water affects biofilm development, giving rise to structurally-unique biofilm growth depending on the flow rate. During the initial cell adhesion and biofilm formation stages, high flow rates are reported to facilitate transport of bulk water microorganisms and their subsequent contact with surfaces as a result of convective diffusion.[163] Further, high shear force has been shown to boost EPS production in established biofilms and enhance cell-to-substratum adhesion.[164] Such enhanced EPS production (in particular polysaccharides) may further contribute to the mechanical stability of the growing biofilms and aid certain types of bacteria to remain attached to the surface.[165] Moreover, the nutrient transport rate from bulk water into the biofilm increases at high flow rates and in turn, stimulates further growth.[166, 167] Such enhanced growth was observed to be more pronounced on polymeric-based (polyethylene) pipes compared to those on copper pipes.[167] There is considerable evidence indicating that turbulent flow and high shear stress conditions promote the growth of thinner, denser, and less porous biofilms.[164, 168, 169] High flow rates however, also promote detachment of mature biofilms due to increased shear stress on the outer layers of the microbial communities.[166, 170, 171] Dispersed biofilms can compromise the microbiological quality of the drinking water. In contrast, at low flow rates, both the nutrient transport and shear effects are dampened,[166, 172] and this appears to result in the formation of more loosely attached and more porous biofilms.[164] Knowledge of the formation of distinct biofilm structures under different flow characteristics can be included as a factor when selecting the appropriate strategy for biofilm control, including the physical and chemical removal of biofilms, as later described.

Effective management of the distribution system hydraulics to avoid slow moving or even stagnant water pockets, will allow better control of biofilms. In places where water

498 consumption is low, stagnant water typically occurs, and is commonly associated with loss of
499 disinfectant residual and accumulation of sediment and debris. The presence of 'old' water
500 with low disinfectant residual is however inevitable in larger distribution networks with dead
501 ends and/or heavily looped designs.[16] The sedimentation and low disinfectant levels are likely
502 to promote extensive biofilm growth.[173, 174]
503
504 ***pH adjustment and fluctuations in distribution systems affects biofilm development***
505 Drinking water pH is often adjusted to facilitate optimum water treatment processes, to
506 minimize the decay of disinfectants or for corrosion control.[175] The growth of nitrifying
507 bacterial biofilms in distribution systems is most favourable at pH 7 – 8 with the actual
508 optimum pHs vary among different bacteria.[176] For example, *Nitrobacter* spp. grow optimally
509 at pH 7.2 – 7.6,[177] while *Nitrosomonas* spp. at pH 7.9 – 8.2.[177] When it occurs, nitrification
510 will most likely decrease the pH of the distribution system to 6 or less, particularly in poorly
511 buffered systems.[175] This pH fluctuation in distribution systems could in fact further promote
512 or inhibit nitrification through a number of known mechanisms[175]: (1) binding of H^+ at low
513 pH or OH^- at high pH to weak basic or acid groups of enzyme active sites,[178, 179] (2) pH
514 affects nutrient availability by governing the chemical equilibrium of the mineral carbon
515 source (CO_3^{2-} to HCO_3^- to CO_2).[178] At high pH, the mineral carbon will predominantly exist
516 as the insoluble and hard-to-metabolize carbonates,[178] (3) pH affects the concentrations of the
517 non-ionic ammonia and nitrous acid, which could inhibit nitrification.[178, 180] Free ammonia
518 dominates at high pH while nitrous acid dominates at low pH.[181]
519 The pH in distribution systems could also affect bacteria-to-surface interactions and in
520 turn, their initial attachment on pipes. At around pH 7, many biofilm-forming bacteria will
521 have a net negative surface charge due to presence of anionic groups (*e.g.* carboxyl and
522 phosphate) on cell surfaces.[182-184] Electrostatic repulsion could take place upon their

interaction with negatively-charged pipe surfaces[185, 186], for example, PVC pipes at around pH 7 (isoelectric point = pH 5.4).[187] A pH drop in distribution systems close to the isoelectric pH, due to growth of nitrifiers for instance, could reduce the bacteria-to-surface electrostatic repulsion and in turn, higher potential for bacterial attachment on PVC surfaces.

In summary, the pH of DWDS is conducive for biofilm formation. Even at pH below 7, biofilm could form due to the enhanced degradation rate of disinfectants as well as potential changes in pipe surfaces' net charge characteristics, rendering them more prone to bacterial attachment. This conveys the need for a surveillance strategy for the growth of biofilms.

Up to this stage, the current article has reviewed important research efforts to reveal how operational conditions of distribution systems affect biofilm growth, from the affinity and initial attachment of microorganisms onto surfaces, to the extent and characteristics of growth. The knowledge provides insights into the susceptibility of water distribution systems to biofilm formation, which signifies the need for water pretreatment and biofilm monitoring strategies. The knowledge will also allow potential tuning of operational parameters whenever applicable, to better manage the growth.

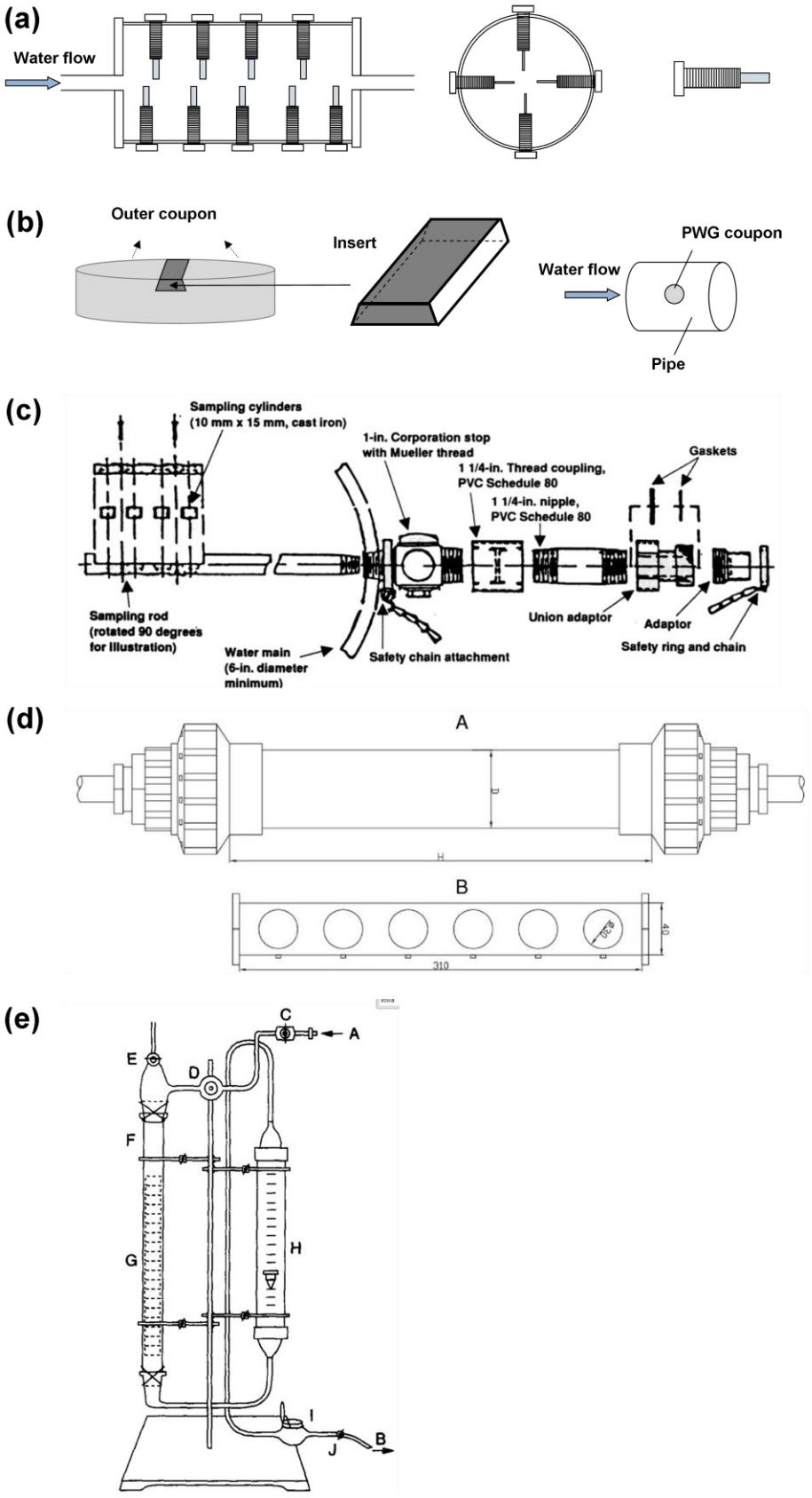
Characterization and monitoring of biofilms in water distribution systems

Continuous monitoring of biofilm growth in water distribution systems is essential to limit their potential adverse impact on the drinking water quality and safety. Conventionally, biofilms are extracted by scraping pipe surfaces followed by *ex situ* analyses of samples in laboratories. Biofilms are not uniformly distributed throughout the water distribution system and therefore, obtaining a representative sample is difficult. There are several devices that can be placed either directly into the flow or in a by-pass line, and used to assess biofilm growth in DWDS. Examples of these devices include (as shown in Figure 3): Corporation Sampling Device,[188] modified Robbins device,[189, 190] biofilm sampler,[191] Pennine Water

548 Group coupon,[192] and a column filled with glass cylinders.[193, 194] All of these devices contain
549 removable coupons of standardized size, which are exposed to similar conditions as those of
550 the pipe interior. These methods provide a standardized surface area, and to some degree
551 replicate conditions of the distribution system and simplify sample collection. Although these
552 biofilm sampling devices have been tested in the DWDS,[190, 191] they are still not widely used
553 as samples from DWDS often contain impurities that can complicate assessments of the
554 target biofilms. Appropriate measures are continuously developed to minimise contamination
555 during and post sampling, which includes the use of suitable sampling containers, transport,
556 and storage conditions of the samples.

557 Restricted access to DWDS often limits *in situ* characterization and monitoring of
558 biofilms. Quite recently, optical biofilm sensors have been developed allowing non-
559 destructive and continuous monitoring of biofilm formation, potentially applicable in the
560 water distribution system.[195] The small and flexible optical fibers are non-conducting and
561 chemically inert with its sensor tip uniquely mounted to probe the pipe's inner surface. One
562 of the earliest developed sensor detects backscattered light from biofilm deposits and
563 transmits the signal to a photo-detector. The technique however, is not suitable for thick
564 biofilms with more than 10^{10} cells cm^{-2} due to saturation of optical signals. Fischer *et al.*[196]
565 developed an optical fiber biofilm sensor that detects fluorescence emitted by the amino acid
566 tryptophan when excited by a UV source. An even more advanced optical fiber sensor
567 technology allows *in situ* discrimination of biological deposits from chemical fouling as well
568 as capability to evaluate the viability of the biomass.[197] The device is capable of measuring
569 fluorescence, light refraction, transmission, and scattering in real time simultaneously. Auto-
570 fluorescence of amino acids was used as an indicator of biomass, while chemical deposits
571 such as calcium carbonate or corrosion products can be clearly distinguished and monitored
572 from their light scattering signals.

573 Apart from the optical sensors, electrochemical techniques have also been used to monitor
574 biofilm growth and to detect the effect of bio-corrosion caused by microorganisms in real
575 time. A new electrochemical sensor (ALVIM) based on electrical phenomena induced by
576 living bacteria has been developed to give a fast and highly sensitive information on biofilm
577 formation, even at early stages of colonisation (*i.e.* only 1% of the probe surface covered by
578 bacteria).[198] ALVIM operate in two modes: (a) potentiostatic technique provides information
579 on the rate of biofilm development through the measurement of the cathodic currents of a
580 sample polarised at a fixed potential, and (b) intentiostatic technique gives a clear signal once
581 the biofilm covers a specific threshold of the surface through the measurement of the
582 potentials needed to sustain a fixed cathodic current during biofilm growth. The sensor has the
583 capability to monitor the attachment/detachment of biofilm following chlorine treatment and
584 therefore,[198] providing meaningful information to optimize treatment, *i.e.* the concentrations,
585 timing and duration of chemical additions.
586



587

588 **Figure 3.** Schematic diagrams of sampling devices for biofilm monitoring: (a) Robbins
589 device,[190] (b) Pennine water group coupon,[192] (c) Corporation sampling device[188], Reprinted

590 with permission from Donlan *et al.*[188], Copyright (1994) Elsevier, (d) Biofilm sampler,[191]
591 which consists of the coupon holder (B) and the pipe (A) in which the holder with coupons
592 were placed and (e) column filled with glass cylinders[194] (A, water supply; B, water
593 discharge; C, valve; D, pressure-reducing valve; E, valve; F, glass column; G, cylinders; H,
594 flow meter; I, water meter; J, valve), Reprinted with permission from Van der Kooij *et al.*[194],
595 Copyright (1995) Elsevier.

596

597 ***Microscopic characterization of biofilms***

598 A challenge in the monitoring of biofilm formation in the DWDS is the selection of
599 suitable technique(s) to estimate the population size (biomass quantification), its spatial
600 organization (structure) as well as the diversity of microorganisms present. The selection of
601 suitable technique is important as many metabolic processes in biofilms are associated with
602 the unique spatial organization of multiple microorganisms. For example, the anammox
603 process, which is responsible for ammonia metabolism, involves multiple organisms to
604 effectively convert ammonia to N_2.[199, 200] This is in part achieved by close spatial
605 organization of such organisms within the biofilm and this organization cannot be observed
606 by scraping or extracting the biofilm. Many characterization efforts are therefore focused
607 toward direct imaging of the sampled biofilms. During the initial stages of biofilm formation,
608 thin layers of biomass are typically visualized using epifluorescence microscopy with nucleic
609 acid staining (Figure 4a), which enables simple and relatively rapid *ex situ* monitoring of
610 biofilm development and enumeration of total cell counts. To seek relative quantification of
611 the viable population from dead biomass, the biofilm can be stained with a viability stain
612 such as the commonly used Live/Dead Baclight (from Molecular Probes, discussed later in
613 more detail).[201] More mature biofilms of more than 3 to 4 μm thick can be non-destructively
614 visualized using confocal laser scanning microscopy (CLSM), which allows optical

615 sectioning of biofilm structure.[202] Three-dimensional biofilm reconstruction can be achieved
616 using a range of reporter dyes to identify cells or matrix components. For example,
617 Calcofluor white[203] and FITC/TRITC-labelled lectins[204] have been used to target
618 polysaccharides in the EPS. FITC[205] has also been employed to stain amine-containing
619 compounds such as proteins and amino sugars. Nile red[203] has been used to stain lipids,
620 capable of differentiating polar and non-polar lipids due to its sensitivity to the degree of
621 hydrophobicity. For cell staining, nucleic acid specific stains, such as 4',6-diamidino-2-
622 phenylindole (DAPI), SYTO, Acridine Orange and propidium iodide (PI) have been used.[206,
623 207] Further, Fluorescence *in situ* Hybridization (FISH) have been used to visualize and
624 quantify the local organization of biofilm community, to elucidate interactions between
625 community members.[208, 209] FISH involves the use of fluorescently labeled probes that bind to
626 ribosomal RNA, which enables visualization of target microorganisms using epifluorescence
627 microscopy or flow cytometry.[210] FISH has been successfully used to characterise
628 microorganisms within biofilms[67, 192] and to detect pathogens in water samples, such as
629 *Legionella pneumophila,*[211] and viable *E. coli* cells.[212] Coupling of FISH with viability dyes
630 has already been used to indicate the presence and physiological status of very diverse
631 bacteria.[213, 214]

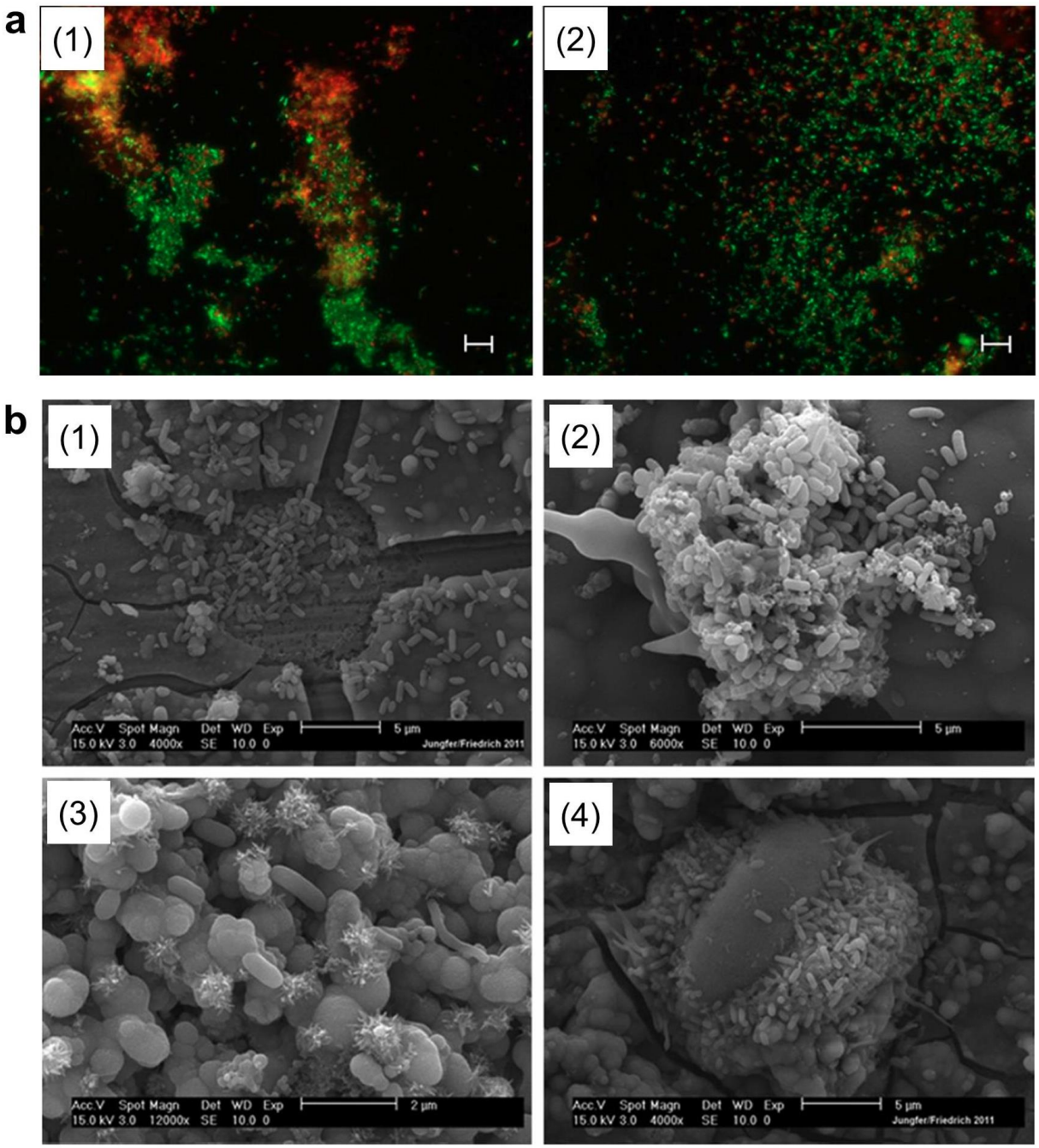
632 For mature biofilms of up to 2 mm thick, optical coherence tomography (OCT) offers high
633 resolution and relatively large imaging area without cell staining.[215] The current OCT
634 technology however, does not allow imaging at single cell spatial resolution. Sub-micron
635 structures of biofilms in water distribution systems have been increasingly investigated using
636 a 'biofilm friendly' environmental scanning electron microscopy (ESEM) technique that
637 currently has a much lower magnification compared to the conventional SEM. Typically used
638 to evaluate biofilm coverage and thickness,[216, 217] ESEM does not require dehydration of
639 samples, therefore enabling visualization of biofilm structure in their natural wet or partially

640 hydrated states without dehydration artifacts (Figure 4b).[218] Despite these advantages, ESEM
641 has inherent limitations, such as reduced resolution and increased beam damage at high
642 magnification due to the absence of metal coating. Obscured surface topography is also
643 common with presence of alternate dark and light areas as a result of differences in local
644 electric charge.[218, 219] Further, elemental composition mapping of macromolecules within
645 biofilm matrices (*e.g.* polysaccharides, proteins, lipids and nucleic acids) is feasible with
646 scanning transmission X-ray microscopy (STXM),[220] which can be used to generate a
647 detailed correlative map of biofilm structure and composition in the water distribution
648 system.
649
650 ***Measurements of active biomass***
651 It may be of interest to determine what fraction of the microbial community is active in the
652 distribution system, that is, distinguishing dead or recalcitrant biomass that persists from
653 biochemically active biomass. The latter may represent either a health risk – as reservoir for
654 pathogens, or whereby the active cells may contribute to inactivation of disinfectants such as
655 chloramine. Viability measures can be used to determine the efficacy of disinfection
656 regimens at different sites along the distribution network, to determine when and where in a
657 system that the disinfectant loses potency. Common techniques for biomass activity
658 estimation consist of biochemical tests that measure specific products of bacterial
659 metabolism. Example of such biochemical tests that are applicable to the drinking water
660 biofilms is the adenosine triphosphate (ATP) assay, which provides rapid and quantitative
661 information about the concentration of active biomass, either attached or suspended, with low
662 detection limits of 0.05 ng ATP L^{-1}.[221, 222] The analysis of ATP is based on the luciferase
663 catalyzed reaction of ATP with luciferin to produce a luminescent signal.[223] This signal is
664 proportional to the amount of ATP present, which correlates well to the number of viable

665 cells. Other measures of active biomass include the use of the earlier mentioned Live/Dead
666 Baclight staining to quantify the relative proportion of viable and non-viable cells. This
667 method works through the combined application of two fluorescent dyes, one that freely
668 penetrates all cells and binds to nucleic acids and a second nucleic acid fluorescent dye that
669 normally only penetrates cells with damaged membranes, indicative of dead or dying cells
670 (Figure 4a). The fluorescent profile then determines the ratio of cells that are viable (stain
671 only with the first dye) or dead cells, stained by both dyes. The permeability of these dyes are
672 however dependent on the types of cellular membranes present in bacteria and hence, are not
673 necessarily applicable to mixed communities. Alternatively, redox active fluorophores (*e.g.*
674 5-cyano2,3-ditolyl tetrazolium chloride or CTC),[224, 225] that fluoresces in the presence of an
675 active electron transport chain can be used to visualise and quantify active *vs* non-active cells
676 in the microbial community. This may have limited function where cells are fermentative, for
677 example.

678

679



680

681 **Figure 4.** (a) Epifluorescence images of biofilms on copper pipes with (1) aggregating
682 bacteria and (2) homogeneously distributed bacteria stained with the BacLight viability
683 reagents.[216] Green: bacteria with intact membranes, red: bacteria with damaged membranes.
684 Scale bars = 10 µm. (b) Environmental scanning electron micrographs of biofilms on copper
685 surfaces.[216] Image (1) to (4) show the presence of multi-layered bacterial aggregates with
686 different morphologies on Cu surfaces. Note the multi-species microbial communities in (3).

Phylogenetic analyses of microbial communities in biofilms

Although viable, many of the microbial members of sampled biofilms are often uncultivable as they do not grow on commonly used cell culture media.[226] Culture-based techniques therefore most of the time underestimate the diversity and relative abundances of microorganisms in biofilms.[71] Cultivation-independent molecular techniques applicable to drinking water biofilms have been developed, and as herein discussed, these can be classified into two approaches, those that are based on DNA fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP) or terminal restriction fragment length polymorphism (T-RFLP) and the 16S rRNA gene sequencing method.

DGGE examines microbial diversity in mixed-culture biofilms, as well as population shift in response to altered environmental conditions or stress.[65, 227-230] Relevant to the DWDS, the method has been used to investigate dominant bacterial members in microbial communities on different plumbing materials, to compare the effect of material choice on biofilm formation,[216, 231, 232] and to determine any population shift during water treatment steps and subsequent distribution.[217, 228] DGGE separates polymerase chain reaction (PCR) amplified gene fragments of the same length but with different base pair sequences based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants.[233] The number of bands observed in DGGE profiles provides an estimate of species richness in biofilms while the relative intensity of each band is thought to reflect the relative abundance of each species. DNA bands from DGGE gels can be further processed for sequencing to identify the corresponding microbial species. Gradually being abandoned, the method is limited by the risk of bias introduced during PCR amplification,[234, 235] co-migration of DNA from different

711 species forming the same band,[236] as well as formation of multiple bands in the amplification
712 of genes from single genomes.[237, 238]

713 Other DNA fingerprinting methods, like SSCP[8] or T-RFLP[239] have also been used for
714 microbial community analyses of biofilms in drinking water systems. In PCR-SSCP analysis,
715 target sequences in genomic DNA are simultaneously amplified, then denatured to a single-
716 stranded form and subjected to non-denaturing gel electrophoresis. SSCP separates PCR
717 amplicons of the same fragment length with different nucleotide sequences on the basis of the
718 conformation of single-stranded DNA. Using PCR-SSCP analysis, Henne *et al.*[8] reported
719 unique microbial composition in drinking water biofilms across the distribution network, with
720 only little similarities to those of the bulk water. This is despite the highly similar bulk water
721 microbial composition observed across the network. In T-RFLP analysis, PCR is performed
722 on DNA extracted from mixed microbial communities with fluorescently labeled primer(s).
723 The PCR products are then digested using specific restriction endonucleases to generate DNA
724 fragments of different sizes. When subjected to capillary electrophoresis, only the fragments
725 that contain the labeled primer are detected. Microbial diversity in drinking water biofilms
726 can be estimated based on the number of peaks of the terminal restriction fragment patterns
727 and their heights.[66, 240] T-RFLP has also been used to assess shifts in the microbial population
728 as a result of variation in environmental conditions or disinfection practices.[241] Similar to
729 DGGE however, the SSCP and T-RFLP methods detect only the most dominant members of
730 microbial communities.

731 In recent years, biomolecular approaches based on the sequencing of 16S rRNA genes
732 amplified from microbial biomass – using the high-throughput Next-generation sequencing
733 (NGS) method, have been used to characterize microbial communities in biofilms. For
734 example, by using 16S rRNA gene analysis, Schmeisser *et al.*[242] found that the majority of
735 microbes in drinking water biofilms were closely related to *Proteobacteria*. Also using the

736 16S gene analysis, Lin *et al.*[63] reported that *Proteobacteria* were the dominant organisms in
737 biofilms formed on PVC, stainless steel and cast iron surfaces. Importantly, the technique
738 could detect microorganisms present at low abundances. Analysing biofilms in a model
739 DWDS to simulate regions with low assimilable organic carbon content (10 μg/L) and no
740 disinfection, Martiny *et al.* detected bacteria from 12 phyla in the growth using the 16S gene
741 analysis, including members from *Nitrospirae*, *Acidobacteria* and *Planctomycetes*, in
742 comparison to detection of only bacteria from the *Proteobacteria* and *Bacteriodetes* phyla
743 using cultivation-based method.[243] Apart from showing that the dominant bacterial
744 population was related to *Nevskia* spp. (γ-*Proteobacteria*), Keinanen-Toivola *et al.*[244] also
745 described the presence of novel bacteria lineages in drinking water biofilms that have not
746 been listed in the current databases. The 16S gene analysis offers many advantages over
747 DNA fingerprinting method as it can more thoroughly characterize biofilm communities, and
748 owing to a drop in gene sequencing costs, is likely to become more attractive to the water
749 industry. Comprehensive identification of DWDS microbial members will allow for spot-on
750 treatments for biomass growth in the distribution system. Chlorination for example, while
751 intended to kill fecal pathogens, may lead to outbreak of resistant bacteria.[16] It is important to
752 note however, that the technique does not differentiate inactive bacteria, *e.g.* persisters
753 (dormant forms of cells) or VBNCs (viable by non-culturable cells) from the active ones.
754 Further, presence of DNA does not mean presence of viable biomass as extracellular DNA
755 amplifies just as well as intracellular DNA.

756 In addition to microbial community sequencing, which gives the relative proportion of
757 microorganisms present, it is also possible to quantify the numbers of organisms present with
758 techniques such as quantitative PCR or qPCR. In this approach, DNA are extracted from the
759 DWDS biofilms and amplified using specific primers in combination with a fluorescent DNA
760 marker, which allows for simultaneous detection and quantification of the target

761 species.[218] The primers can either target microbes at the kingdom or phylum level or can be
762 designed to quantify specific bacteria based on the presence of genes of interest. For the latter,
763 the genes associated with ammonia oxidation for example, can be quantified,[245, 246] which
764 may indicate the extent to which the microbial community are able to metabolise and
765 inactivate chloramine added to the distribution system as disinfectant. Similarly, qPCR
766 primers can be designed to quantify specific pathogens in the DWDS[246] and this information
767 can be integrated into the risk management strategy of the operator. More specifically, the
768 technique could detect presence in DWDS of the only few pathogenic strains of *E. coli*, as
769 opposed to the non-specific detection of the bacteria by coliform-based test. This is to refrain
770 from any unnecessary treatment response that may adversely impact the public health.[16]

771 With today's technological advances in biofilm sampling and characterization, more
772 thorough and frequent monitoring of biofilm development has become feasible for DWDS.
773 From an array of biofilm sampling devices for *ex situ* analysis to *in situ* biofilm
774 characterization techniques, these technologies could form an integral part in the efforts to
775 control microbial growth in water distribution systems.

776

777 **Current practices to limit biofilm growth in water distribution systems**

778 In 2004, the World Health Organization (WHO) published its first set of guidelines to
779 ensure drinking water safety, called the Water Safety Plans (WSPs).[247] Unique to each water
780 supply system, WSPs are a comprehensive 'source to tap' risk assessment and preventative
781 management approach, guided by health-based targets and supervised by (preferably
782 independent) auditor. The Plans include multi-barrier practices to prevent growth of
783 pathogens in the DWDS, including controls on biofilm growth as potential reservoirs for
784 pathogens, as herein described.[15, 54, 248]

785 Control on biofilm formation in DWDS has been mainly achieved by means of chemical
786 disinfection. Chlorine, a cheap, efficient and most widely used disinfectant, affects biofilm
787 formation at every stage of development. Chlorine is a highly reactive oxidizing agent, and its
788 bactericidal activity has been linked to the generation of reactive oxygen species, which
789 induce broad damage to bacterial cells affecting DNA, proteins and lipids.[249] Activity of
790 chlorine on biofilms has been shown to localize around the periphery of cell clusters.
791 Chlorine slows the kinetics of microbial deposition onto the pipe wall by degrading cell
792 membrane functional groups and associated polymers and in turn, inhibiting the reversible-to-
793 irreversible transition of cell attachment to surfaces.[22] Chlorine reduces microbial growth
794 rate,[250, 251] and yet is incapable of complete inhibition of biofilm growth.[252] For the latter,
795 several studies have reported slow penetration of chlorine into biofilms, with chlorine
796 neutralization by the organic matter in the surface layers of biofilms occurring faster than its
797 diffusion into the biofilm interior.[253-256] Further, chlorine is able to promote detachment of
798 cells from biofilms.[257-259] It is noteworthy to mention however, that the use of chlorination
799 while effective in killing faecal pathogens for example, may lead to selection of resistant
800 bacteria, such as the opportunistic pathogen *Mycobacterium avium* due to their relative
801 chlorine resistance.[16] In many cases, bacteria could still form biofilms at high chlorine
802 concentrations (0.8 to 1.5 mg/L, relevant to those in DWDS),[250, 260] and this is a reflection of
803 the antimicrobial tolerance of biofilms. Increased resistance to chlorine has been observed
804 with mature biofilms (at the highest thickness), compared to those at early stages[261] and is
805 further enhanced within multi-species biofilms, compared to those of single-species, whereby
806 the mixed species communities may share multiple mechanisms of chlorine resistance.[165, 262]
807 Chlorination was also found to enrich the prevalence of antibiotic resistant bacteria (ARB) in
808 drinking water.[263] Shi et al.[264] found higher proportion of surviving bacteria that exhibit
809 resistance to chloramphenicol, trimethoprim and cephalothin following chlorination.

Chlorine is typically dosed in excess, while at levels below the safety and aesthetic, taste and odour standard limits, to provide effective residual concentrations preventing bacterial re-growth during water distribution. Free chlorine degrades due to reactions with organic and inorganic compounds (ammonia, Br^-, I^-, SO_3^{2-}, NO_2^-, Fe(II)) in bulk water,[265] corrosion products,[266] pipe materials[267] and even through interactions with microorganisms and their EPS.[268] In most cases however, the maintenance of an effective disinfectant concentration is challenging. Chlorine degradation is most likely to occur at the dead ends of large networks and in low velocity regions. To solve the problem, at least partly, supplementary chlorine are added at strategic points through booster stations installed along the distribution lines. Such loss in disinfection residual also typically leads to additional application of chemical disinfectants, which in turn increases operating costs and the likelihood of generation of hazardous disinfection by-products. The increasing stringency of guidelines and regulations on disinfection by-products mandates better control of disinfectant application.

More stable compounds, such as chloramines, formed from a reaction between chlorine and ammonia, maintain disinfection residual for a longer period throughout the distribution system and generates fewer harmful regulated disinfection by-products.[16] Chloramines are often used in distribution systems where free chlorine residuals are difficult to maintain or chlorine use leads to excessive by-product formation. Though less reactive compared to chlorine, chloramines may penetrate biofilms more effectively[269] because unlike chlorine, they less readily react with the presence of organic matters in the biofilm surface layers. Caution must be taken however, as in some cases the use of chloramine has been associated with the growth of certain nitrifying bacteria (due to the release of ammonia from chloramine decay) within biofilm that in turn degrade disinfectant residual.[16]

The water distribution system is also subjected to cleaning *via* flushing, pigging or air/water scouring, which are considered to be the best routine management practices for

biofilm control,[6] also removing any biomass killed or inactivated by disinfections. Flushing involves forcing high-speed water through the pipes to flush out particulates. Strong shear forces from flushing could enhance mass transport of disinfectants and cause areas of biofilms to slough and in turn, not only altering the microbial composition in biofilms but also those of the bulk water.[16, 61] Recent enquiries have observed changes in microbial richness and diversity between pre- and post-flushing samples. The relative abundance of γ-*Proteobacteria* for example, decreased following either low or highly varied flushing regimes, while the opposite occurred for β-*Proteobacteria*.[61] Flushing in most cases is incapable of thorough removal of biofilms from pipe walls.[61] Pigging involves forcing an object fitted to the pipe diameter, such as a hard sponge bullet, ball or ice, through the pipe to physically scrub biofilms from the pipelines. A build-up in back-pressure causes the bullet to rotate while in motion.

The current practices for biofilm control also include strategies to reduce the levels of biodegradable organic matters (BOMs) or assimilable organic carbon (AOC) as well as the concentration of suspended microbial in drinking water prior to entering the distribution system (typically through membrane biofiltration). It has been shown in various laboratory scale systems that nutrient limitation can inhibit biofilm formation and/or induce biofilm dispersal, ultimately, reducing or delaying the impact of biofilms on engineered systems. Granular activated carbon filters have been used to reduce AOC levels in water, discouraging bacterial growth.[270] Following adsorption of AOCs on the activated carbon, the technology facilitates degradation of the organic matters through the metabolic activity of artificially inoculated microorganisms along with, to a certain extent, the activity of naturally-occurring aquatic microorganisms adsorbing on the activated carbon.[271] Such nutrient control practice has been reported to effectively reduce biofilm accumulation in membrane based water purification systems.[272, 273] In place of the use of disinfectants in the DWDS, large treatment

860 plants in Europe (Germany, Netherlands, Denmark, Luxembourg, Switzerland) have used
861 both biofiltration and nutrient limitation as final treatment steps to minimise biofilm growth
862 and therefore, avoiding the distribution of water with residual disinfectants.[221, 274-276] There
863 are valid pros and cons in regard to the latter and various factors are to be taken into account
864 when deciding on whether or not to apply disinfection residual in DWDS; including the types
865 of treatment process and quality of water entering the network as well as the network's age,
866 materials, hydraulic and structural integrity.

867 Other technologies to reduce suspended microorganisms in drinking water prior to entering
868 the distribution system, which includes UV disinfection, oxidative treatments, such as
869 ozonation and a combination of UV/H_2O_2 treatment, could also reduce chlorine demand and
870 corrosion potential.[277] It is important to note that UV disinfection is not effective on UV
871 resistant microorganisms[278-280] and that the presence of UV absorbing organic and inorganic
872 compounds or suspended particles in water will reduce the UV fluence, and therefore higher
873 UV doses are required to inactivate microorganisms.

874 Further, corrosion control in distribution systems is also key to limit biofilm growth in
875 distribution systems.[111] As described earlier, corroded pipe surfaces are favourable over
876 'smooth' surfaces for microbial attachment and colonization while corrosion products have
877 been known to promote growth of unique biofilm-forming bacteria. In real practice, it is not
878 always achievable to extract old corroded pipes from distribution networks, in particular in
879 larger systems whereby pipes are only being replaced every 100 years.[16] Instead, although the
880 tendency to promote biofilm growth, the earlier mentioned addition of or coating of pipes
881 with phosphates is still in practice to control corrosion, along with pH adjustment of the water
882 entering the distribution system[16, 281, 282] For the latter, abatement of corrosion is generally
883 accomplished by increasing the pH.

884 Positive results from the implementation of these biofilm control practices as part of WSPs
885 in water utilities have been reported not only in industrialized but also in developing
886 countries.[283-286] For example, an improved drinking water quality and better public health was
887 reported in Iceland, which saw a substantial reduction in the concentration of HPC
888 (heterotrophic plate count) bacteria in both the source and distributed water and
889 correspondingly, the incidence of diarrhea.[287] WSPs are now legally required in a number of
890 countries, including Iceland.[284] In fact, many well-managed water utilities have implemented
891 the WSPs' principles for years,[283, 284, 288] such as those in Germany with their DVGW's TSM
892 (The German Technical and Scientific Association for Gas and Water's technical security
893 management) approach.[289] Further, international networks (e.g. the IWA Bonn Network, the
894 Latin America and Caribbean WSP network, the African WSP network and the Asia-Pacific
895 WSP network) have been established to provide a platform for water professionals to share
896 knowledge and experiences in implementing the WSPs. These networks will help to address
897 challenges in the implementation of biofilm control strategy and ultimately, safeguarding the
898 existing and future investments in water supply. For the latter, emerging biofilm control
899 technologies could be considered for better management of the biofilm growth in DWDS, as
900 discussed in the following.
901
902 **Future outlook for biofilm control in water distribution systems**
903 Effective control of biofilm growth in DWDS requires suitable antimicrobial agents and
904 design of treatment processes to limit the initial presence of colonizing microbial community
905 at the treatment plant. A wide range of engineered nanomaterials have been increasingly
906 demonstrated to exhibit potent and versatile antimicrobial properties through diverse toxicity
907 mechanisms, from compromising the integrity of microbial cell wall (*e.g.* nanosilver,[290-292]
908 copper oxide,[293] zinc oxide,[294, 295] carbon nanotubes,[296] chitosan[297]), stimulation of cellular

909 reactive oxygen species (ROS) generation that can damage proteins, lipids and even DNA
910 (*e.g.* silver,[292] ZnO,[298, 299] TiO_2[300] and fullerol[301]), to inhibition of enzyme activity and DNA
911 synthesis (*e.g.* chitosan[302]). Incorporation of these antimicrobial nanomaterials in membranes
912 or filters could potentially decrease the microbial population, prior to entering the water
913 distribution system. It is necessary to confine the nanomaterials within the treatment plant to
914 minimize any unintended impacts on human health and the environment. In most cases, the
915 treatment system will require downstream processing to capture trace amounts of the
916 nanoparticle-derived leached soluble species in water as well as the residual solid-form
917 (undissolved) nanomaterials (membranes or filters materials are designed to secure the
918 packed in nanoparticles and therefore, the minimal leaching or release of the particulates).[303,
919 304] As with the use of any antimicrobial agents in contact with drinking water, the potential
920 applications of nanomaterials will require to comply with existing safety regulations (still
921 regulated based on existing regulations for the corresponding regular or non-nano-scale
922 materials)[305, 306]

923 Alternative non-toxic, environmentally-friendly biofilm control and prevention strategies
924 currently exploit the application of chemical signals used by bacteria to regulate biofilm
925 developmental processes. One potential target is the quorum sensing bacterial signaling
926 system[307, 308] with a diverse class of natural and synthetic compounds being developed to
927 inhibit the QS mediated cell-to-cell communication, including halogenated furanones,[309]
928 dihydropyrrolones[310] or natural products such as ajoene.[311] The technology offers a potential
929 strategy for disrupting and preventing development of biofilms on water pipes, with the
930 successful coating of numerous QS inhibitor, such as dihydropyrrolones onto surfaces
931 whereby they limit biofilm formation.[310] Another promising approach is to use low levels of
932 nitric oxide (NO) to induce biofilm dispersal. NO, an ubiquitous biological signaling free
933 radical, was recently discovered to induce biofilm-to-planktonic phenotype transition in many

934 bacteria.[312] NO triggers a decrease in the intracellular concentration of c-di-GMP, which
935 leads to not only biofilm dispersal, but also rendering the biofilm more susceptible to
936 biocides. Compounds that spontaneously release NO, called the NO donors and include
937 compounds such as sodium nitroprusside, Proli-NONOate and DETA-NONOate, have been
938 shown to disperse drinking water biofilms[312, 313] as well as biofilms formed on membranes for
939 water treatment.[314, 315] While NO is a highly reactive molecule and thus its delivery over long
940 distances in water pipe networks may prove difficult, novel coatings have been developed
941 that are capable of catalytically generating NO *via* conversion of nitrite ions commonly found
942 in water.[316] Such solution could prove effective for applications in DWDS. Further, given that
943 c-di-GMP is a key regulator of biofilm formation in a broad range of organisms, an ideal
944 treatment could be one that also targets the enzymes responsible for the production of this
945 compound.[317]

946 Apart from the earlier described practice to limit nutrient content in the distribution system,
947 biofilm control could be achieved by adding inhibitors of key metabolic enzymes. Target
948 metabolic pathways for biofilm control could be identified through better understanding of
949 microbial metabolism in biofilms. In the case of ammonia-oxidizing biofilms, adding
950 inhibitors of the key metabolic enzyme ammonia oxidase has been shown to suppress biofilm
951 growth.[318] Finally, a quite recent bacteriophage-based technology has been developed for
952 control of biofilms on hard surfaces,[319-321] a potentially attractive application for drinking
953 water biofilms. Bacteriophages are natural predators of bacteria and enzymes produced
954 during phage infection have been shown to degrade polysaccharide components of the EPS
955 matrix in biofilms, leading to destruction of the biofilms.[322, 323] Bacteriophages are self-
956 generating as long as the appropriate host bacterium is present. This feature could enable
957 phage distribution throughout the drinking water network without the need for re-dosing.
958 Further, bacteriophages are inherently self-limiting in that once the host or target bacterium is

present below a threshold sufficient for phage replication, they become inert particles. The potential application of the technology in distribution systems requires a detailed understanding of the key biofilm-forming bacteria in the system, as targets for the appropriate bacteriophages, as well as the suitable phage removal technique following treatment. For the latter, various filter- and surface-based phage capturing technologies have been developed.[324-326] For all of these novel approaches, research will be required to find the optimum compounds, concentrations and dosing strategies to demonstrate efficacy. Additional considerations faced by all new technologies, such as cost for implementation relative to benefit, as well as the environmental impact and fate of such compounds, would also need to be addressed here. Finally, some testing of these novel approaches under realistic conditions, *e.g.* high flow rates, and benchmarking them against existing technologies would also enable decisions about their large scale utility as biofilm control strategies in the field.

SUMMARY AND PERSPECTIVES

Growth of biofilms in drinking water distribution networks although unavoidable, is potentially controllable. An in-depth understanding of biofilm characteristics and how the conditions of the distribution systems affect their development, will allow for potential tuning of the operational parameters to limit the growth. The choice of pipe materials, flow rates, temperature and pH of distribution system affect all stages of biofilm development, from the initial attachment of microorganisms onto pipe surfaces, to the extent and characteristics of biofilm growth. Whenever applicable, the 'biofilm-limiting' operating conditions of distribution systems, together with the already implemented biofilm control practice, such as removal of organic and inorganic nutrients and treatment with disinfectants, feature a potential for improved management of biofilm growth throughout the network. Equally important is the implementation of suitable biofilm monitoring practice to probe the likely

changes in biofilm characteristics as a result of fluctuations in operating conditions and disinfection treatments. The quite recent development of *in-situ* biomass sensors and the increasingly cost effective biomolecular analysis of microbial communities will enable more frequent and thorough assessments of biofilm characteristics and their distribution profile across the drinking water networks. This will allow for timely administration of control measures, particularly in response to unforeseen changes in operating conditions that could promote biofilm growth in distribution systems, including an abrupt temperature increase or a pH drop due to growth of nitrifiers. Management of the persistent biofilm growth requires an integrated approach of water pretreatment, biofilm monitoring and control, as no single practice thus far appears to be sufficiently effective. In closing, a systematic survey of DWDS microbial ecosystems and their correlation to water characteristics and the systems' operational conditions, is key for effective monitoring and treatment strategy and importantly, for anticipation of potential shifts in the microbial profile in response to treatment change. The survey is indispensable, in particular with the now known prevalence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in drinking water, with no regulation currently in place for such presence of resistance entities. In the current reality, *"water utilities, in a sense, are forced to 'fly blind' when making treatment decisions without a detailed inventory of the microorganisms growing within distribution systems"* (a quote from the Microbes in Pipes[16] report by the American Academy of Microbiology, 2012).

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1010

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