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

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1 **ABSTRACT**: In drinking water distribution systems (DWDS), biofilms are the predominant 2 mode of microbial growth with the presence of extracellular polymeric substance (EPS) 3 protecting the biomass from environmental and shear stresses. Biofilm formation poses a 4 significant problem to the drinking water industry as a potential source of bacterial 5 contamination, including pathogens and in many cases also affecting the taste and odor of 6 drinking water and promotes corrosion of pipes. This article critically reviews important 7 research findings on biofilm growth in DWDS, examining the factors affecting their 8 formation and characteristics, as well as the various technologies to characterize, monitor and 9 ultimately, to control their growth. Research indicates that temperature fluctuations 10 potentially affect not only the initial bacteria-to-surface attachment but also the growth rates 11 of biofilms. For the latter, the effect is unique for each type of biofilm-forming bacteria -12 ammonia oxidizing bacteria for example, grow more developed biofilms at typical summer 13 temperature of 22°C compared to 12°C in fall, while the opposite occurs for the pathogenic 14 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 15 16 loosely attached biofilms at low flow rates. Further, in addition to the rather well-known 17 tendency of significant biofilm growth on corrosion-prone metal pipes, research efforts also 18 found leaching of growth-promoting organic compounds from the increasingly popular use of 19 polymer-based pipes. Knowledge of the unique microbial members of drinking water 20 biofilms and importantly, the influence of water characteristics and operational conditions on 21 their growth, can be applied to optimize various operational parameters to minimize biofilm 22 accumulation. More detailed characterizations of the biofilm population size and structure are 23 now feasible with fluorescence microscopy (epifluorescence and CLSM imaging with DNA, 24 RNA, EPS, protein and lipid stains) and electron microscopy imaging (ESEM). Importantly, 25 thorough identification of microbial fingerprints in drinking water biofilms is achievable with

26 DNA sequencing techniques (the 16S rRNA gene-based identification), which have revealed 27 prevalence of previously undetected bacterial members. Technologies are now moving toward in situ monitoring of biomass growth in distribution networks, including the 28 29 development of optical fibres capable of differentiating biomass from chemical deposits. 30 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 31 32 potential implementation of 'biofilm-limiting' operational conditions and finally, to the 33 careful selection of available technologies for biofilm monitoring and control. For the latter, conventional practices, including chlorine – chloramine disinfection, flushing of DWDS as 34 35 well as nutrient removal, and emerging technologies are discussed with their associated 36 challenges.

TOC



#### 51 INTRODUCTION

Safe drinking water is a basic need and its provision has been a top priority issue world-52 wide. The main challenge to the drinking water industry is to deliver a product that is 53 54 microbiologically and chemically safe, as well as aesthetically pleasing. While disinfection 55 practices remove the majority of microorganisms found in raw water, the treated water is not 56 sterile and low levels of microorganisms persist in the water when entering the distribution 57 networks. Studies on drinking water distribution systems (DWDS) have indicated that more 58 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.<sup>1</sup> Biofilms are 59 60 ubiquitous and persistent microbial communities growing on surfaces, capable of continuous 61 shedding of cells that promotes the spread of microorganisms. Biofilms in DWDS range from a few tens of micrometers to a few mm.<sup>2, 3</sup> The biofilms consist of complex and functionally 62 organized microbial communities composed of cells embedded in a gelatinous matrix of 63 biological origin comprised of extracellular polymeric substances (EPS).<sup>4</sup> The EPS matrix is 64 65 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 66 from adverse environmental conditions. Recent studies revealed that microorganisms can also 67 68 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.<sup>5-7</sup> 69

DWDS harbor biofilms even in the presence of disinfectants,<sup>8</sup> potentially affecting the turbidity, taste, odor and color of the water,<sup>9</sup> and in many cases, promoting the decay of residual disinfectants.<sup>10</sup> 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,<sup>3</sup> affecting the network's hydraulic efficiency in the long run. Moreover, biofilms in many cases secrete acid metabolites that corrode concrete and
 metallic pipes.<sup>11-13</sup>

Posing a major health threat, biofilms have been known to harbor pathogenic 78 microorganisms.<sup>14-16</sup> potentially releasing them into the water flow through the natural 79 shedding cycle of biofilm.<sup>17</sup> The consumption of contaminated water has been known to 80 cause a wide range of diseases and health problems, particularly affecting infants, young 81 children, the elderly and the immune-compromised population.<sup>18</sup> Examples of pathogens 82 83 found in DWDS include Vibrio cholerae (causes cholera), Salmonella typhimurium (typhoid fever), Escherichia coli, Giardia lamblia and Cryptosporidium parvum (gastroenteritis), 84 85 Naegleria fowleri (amoebic meningoencephalitis), Mycobacterium avium (pulmonary infections) and hepatitis viruses.<sup>18-20</sup> In the USA alone, waterborne infections are responsible 86 for over 40,000 hospitalizations, costing the economy \$970 million per year.<sup>21</sup> 87

88 A core yet still debated issue is the development and impact of biofilms in DWDS, that is, 89 how the characteristics of water and operating conditions of DWDS determine the traits of 90 the growth and subsequently, their roles on corrosion, degradation of disinfectants as well as in facilitating proliferation of pathogens.<sup>16</sup> The knowledge is key for appropriate monitoring 91 92 and control strategy of biofilm growth in the distribution system. In this Review, we describe 93 the occurrence and characteristics of biofilms in DWDS and how the growth is potentially 94 affected by the various operational conditions, including their fluctuations. The choice of pipe 95 materials, flow rate variations, and guite often, changes in temperature and pH, affect biofilm 96 formation, including the initial stage of microorganism attachment onto pipe surfaces. The 97 *Review* further describes a range of technologies that have been available or that are currently 98 being developed for potential use in the monitoring and characterization of drinking water 99 biofilms. Finally, practices to control biofilm development are discussed, including the 100 emerging catalysis and biometabolic based technologies. The *Review* is expected to provide 101 insights into the susceptibility of water distribution systems to biofilm growth, featuring 102 potential for manipulation of operational parameters as well as the selection of the right 103 technologies for the challenging issue of biofilm monitoring and control.

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# 105 Occurrence and characteristics of biofilms in water distribution systems

106 In comparison to their planktonic, 'free-living' counterparts, biofilm microbes in general 107 show increased protection against a range of stressors. In the case of drinking water biofilms, these include resistance against disinfectants,<sup>22, 23</sup> shear stress conditions,<sup>24</sup> thermal stresses 108 and predators.<sup>25, 26</sup> The increased resistance of bacteria within biofilms is in part due to the 109 110 EPS matrix that they produce. EPS can retain and store compounds including nutrients to provide food reserves for microbial members during starvation period,<sup>27</sup> as well as bind and 111 inactivate disinfectants such as chlorine and chloramines.<sup>28</sup> Further, specific EPS 112 components, such as the Psl exopolysaccharides formed by Pseudomonas aeruginosa 113 biofilms have been known to increase the elasticity and cross-linking within the matrix, 114 115 which in addition to increase protection against shear stress, is thought to facilitate formation of microcolonies.<sup>29</sup> 116

117 Biofilms form through a series of developmental stages (Figure 1), which are generally 118 controlled genetically in response to environmental cues and signals. Of the genetic factors 119 controlling biofilms, one of the better studied systems include the intra- and inter-species 120 cell-to-cell communication systems called quorum-sensing (QS), which are responsive to 121 changes in cell population density or local diffusion parameters. Quorum sensing bacteria 122 produce and detect diffusible signal molecules, called autoinducers, enabling cells to sense, 123 communicate with other cells and subsequently adjust to changing physiological needs under 124 different growth conditions and to do so in a coordinated, population level response. Two of 125 the best described quorum-sensing systems in bacteria are the acylated-homoserine lactone

(AHLs)<sup>30</sup> system present in many Gram-negative species and the peptide-based signaling 126 system present in many Gram-positive species.<sup>31</sup> Quorum sensing has been shown to 127 influence biofilm formation by controlling EPS synthesis in V. cholerae<sup>32</sup> and by controlling 128 cell aggregation in *Serratia marcescens* (*liquefaciens*).<sup>33</sup> Referred to as diffusion sensing, QS 129 130 is thought to play a role on biofilm development in specific geometric configurations of the 131 DWDS, such as small diameter pipes or dead-end pipes, whereby QS molecules bounce off 132 neighbouring surfaces, thus triggering QS-mediated gene expression, even with only presence of low cell density.<sup>34</sup> In high velocity regions on the other hand, it is still unclear as to 133 134 whether QS molecules could accumulate to the required threshold concentration to play a role in biofilm formation.<sup>35</sup> Nonetheless, bacterial isolates from drinking water have been shown 135 136 to produce QS signals as well as QS quenching molecules, suggesting that these signalling systems are active in DWDS.<sup>36</sup> 137

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In addition to QS, biofilm formation is genetically controlled by bis-(3',5')-cyclic 139 140 dimericguanosine monophosphate (c-di-GMP) signaling. C-di-GMP is a highly conserved 141 secondary messenger molecule that controls the transition from a free-living, motile lifestyle to a biofilm mode in many bacteria.<sup>37</sup> Cells adjust their c-di-GMP levels in response to 142 143 environmental cues and intracellular signals. High concentrations of c-di-GMP tend to 144 promote cell attachment to surfaces, biofilm formation, EPS production, and attenuation of 145 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' 146 and 'low' c-di-GMP thresholds are unique for different bacterial strains).<sup>38-40</sup> For example, 147 148 upon sensing nutrient limitation, e.g. depletion of carbon, nitrogen or oxygen sources, 149 intracellular c-di-GMP level decreases in *P. aeruginosa*, resulting in rapid dispersal of the

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



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- 153 **Figure 1.** (a) Biofilm growth on different pipe materials. Reprinted with permission from
- 154 Ren *et al.*<sup>43</sup> Copyright (2015) Springer. (b) Biofilm life cycle in DWDS.
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156 Biofilms in water distribution systems are mainly comprised of water from the gelatinous 157 matrix, which can occupy up to 99% of the total volume, while microorganisms in fact represent only 2–5% of the volume.<sup>44-46</sup> EPS accounts for 50–90% of the total organic carbon 158 in biofilms<sup>47, 48</sup> and is generally comprised of polysaccharides and proteins as the major 159 components (75-89%),<sup>49</sup> with varying amounts of nucleic acids, lipids, phospholipids and 160 humic substances.<sup>50</sup> Inorganic particles such as corrosion products, suspended solids and 161 162 sand, may also be incorporated in biofilms, increasing its mechanical strength and biomass accumulation.<sup>51</sup> According to Characklis and Marshall,<sup>52</sup> bacteria are generally the dominant 163 members of biofilm microbial communities in DWDS due to their high growth rates, 164 165 relatively small size, adaptation capabilities and ability to produce EPS. Viruses, filamentous fungi, algae and protozoa may also be present in drinking water biofilms.<sup>15, 53, 54</sup> These 166 'secondary' microorganisms, in particular, viruses and protozoa could rapidly attach and 167 persist in existing drinking water biofilms,<sup>17</sup> while the involvement of filamentous fungi in 168 biofilms has not yet been satisfactorily established.<sup>55</sup> Protozoan opportunistic pathogens 169 170 including Acanthamoeba (causing keratitis mostly from contact lenses stored or washed in 171 tap water) and Naegleria (encephalitis via nasal washes) are regularly found in DWDS, probably mainly in reservoirs. Cryptosporidium (protozoa responsible for gastrointestinal 172 173 diseases) was reported to be present in some DWDS, potentially introduced via faecal 174 contamination.<sup>16</sup>

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 cellto-cell communication, facilitating their initial attachment, subsequent colonization and biofilm formation on pipe surfaces, even in chlorinated drinking water.<sup>56</sup> 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.<sup>56</sup> In many cases, the co-presence of microorganisms has been shown to enhance 181 biofilm formation. For example, Min and Rickard<sup>57</sup> reported that co-aggregation of bacteria 182 promotes biofilm development by facilitating attachment to the partner species. Further, 183 Simoes *et al.*<sup>58</sup> investigated the role of species-to-species interactions in the formation of 184 185 mixed-species drinking water biofilms, and observed a range of synergistic interactions. 186 Acinetobacter calcoaceticus for example, was found to co-aggregate with bacteria commonly found in drinking water, such as Burkholderia cepacia and Mycobacterium mucogenicum to 187 form biofilms and interestingly, no bacterial co-aggregation was observed in its absence. The 188 results suggest the 'bridging' function of A. calcoaceticus in drinking water biofilm 189 formation.<sup>58</sup> Similarly, despite its inability to attach to solid surfaces, *Escherichia coli* 190 191 PHL565 was able to form mixed biofilms with 'adhesive' bacteria, such as Pseudomonas putida MT2.<sup>59</sup> 192

193 Research efforts have revealed wide variation in the identity and composition of microbial communities in drinking water biofilms,<sup>8, 60</sup> which are also noticeably different when 194 compared to the corresponding planktonic population in bulk water.<sup>8, 61, 62</sup> The latter suggests 195 that only specific members of the free-living bulk water microbes are capable of attaching to 196 pipe surfaces and form biofilms. Proteobacteria, particularly those belonging to the  $\alpha$ ,  $\beta$ ,  $\gamma$ 197 and  $\delta$  subclasses, have been found to dominate biofilms in DWDS (Figure 1b),<sup>8, 62, 63</sup> 198 199 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 200 material.<sup>64, 65</sup> biofilm age.<sup>66</sup> phosphate treatment<sup>67</sup> as well as disinfection practices.<sup>67, 68</sup> Two 201 202 separate studies revealed that  $\alpha$ -Proteobacteria such as *Sphingomonas* and *Hyphomicrobium* predominate in water with low chlorine residuals (<0.02 mg/L), and in chloraminated water,<sup>68</sup> 203 whereas  $\beta$ - and  $\gamma$ -Proteobacteria flourish with increased chlorination.<sup>69</sup> Within the class of  $\beta$ -204 205 Proteobacteria, examples of predominant bacterial genera include Janthinobacterium,

Methylophilus, Burkholderia, Nitrosomonas and Alcaligenes.<sup>2, 26, 30</sup> A number of pathogens 206 207 and opportunistic pathogens belonging to  $\gamma$ -Proteobacteria subclass have been particularly found in water distribution systems, which are thought to also exist as members of drinking 208 209 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 Mycobacterium avium and M. kansasii can cause serious pulmonary and lymphatic disease, 211 212 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 USA alone in 2008.<sup>16, 70</sup> Some pathogens, including *M. avium* and *L. pneumophilla* can even 216 proliferate within various amoebas in biofilm.<sup>16, 71</sup> Other  $\gamma$ -Proteobacteria pathogens and 217 218 opportunistic pathogens found in DWDS include Enterobacter, Acinetobacter, Klebsiella, Aeromonas.<sup>67, 72, 73</sup> Note that the occurrence of such hygienically-relevant microorganisms in 219 220 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 buildings.<sup>74</sup> Additional bacteria found in biofilms in DWDS include members of 223 224 Actinobacteria, Chloroflexi, Bacteroidetes, Nitrospirae, Firmicutes, Verrucomicrobia and Acidobacteria.<sup>62</sup> Mixed community biofilms display enhanced protection against 225 226 environmental stresses, that renders them significantly more stable than the monospecies systems.75,76 227

Advances in molecular biology technique, such as the 16S rRNA gene-based identification (discussed in later section) have allowed detection of water relevant 'viable but non culturable' (VBNC) bacteria,<sup>77, 78</sup> 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 physiologically viable but non-proliferating state as a response to adverse environmental 233 234 conditions such as unfavourable temperatures, chlorination, pH fluctuations, nutrient depletion, and oxygen stress.<sup>79, 80</sup> The potential presence of pathogenic 'viable but non 235 236 culturable' (VBNC) bacteria in drinking water biofilms is a threat to public health due to their ability to regain virulence under favourable growth conditions.<sup>80, 81</sup> Undetectable by 237 conventional culturing methods, the population density of VBNC bacteria are often 238 239 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 within bacterial populations. Engemann et al.<sup>82</sup> found that tetracycline resistance genes readily 243 244 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 and surface water to naturally-present drinking water bacteria.<sup>83</sup> It is also possible that the 251 genes were part of the genome of VBNC bacteria.<sup>83</sup> Several studies have detected ARB in 252 drinking water systems. Faria et al.<sup>84</sup> detected *Staphylococcus* with resistance to multiple 253 antibiotics in drinking water samples. Xi et al.<sup>85</sup> detected ARGs and heterotrophic ARB in all 254 255 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.

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

262 Growth of biofilms in DWDS is a complex phenomenon, consisting of a number of 263 interconnected growth stages (Figure 1b). Biofilm development typically starts with the 264 formation of conditioning films composed of macromolecules (such as polysaccharides, 265 lipids, proteins and humic substances found in drinking water and/or secreted by 266 microorganisms) on surfaces, and the subsequent initial attachment/adhesion of microorganisms on the films.<sup>86, 87</sup> Note that the formation of a conditioning film is particularly 267 268 important in nutrient-depleted environment, such as drinking water, where the accumulation 269 of organic molecules at surfaces create a relatively nutrient-rich local environment. This is 270 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 271 272 single cells into the bulk water to form new colonies elsewhere, thus completing the biofilm 273 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.<sup>16</sup> Herein, 274 275 comprehensively established from numerous drinking water biofilm studies, we found that the 276 development of biofilms in the distribution system is likely to be affected by a number of 277 inter-related factors (Figure 2).

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Organic and inorganic matter as nutrients for biofilm growth in water distribution systems 281 282 Organic matter content in water distribution systems is fuel for biofilm formation, with 283 most aquatic microorganisms metabolizing biodegradable organic matter (BOM) for energy 284 sources (dissimilation) and for the production of cellular materials (assimilation). Studies 285 have correlated the structural and physicochemical characteristics of organic matters to their 286 biodegradability or in other words, their bioavailability. Unsaturated aliphatic compounds, 287 such as simple carbohydrates, low molecular weight proteins and organic acids are in general 288 more accessible for microbial degradation compared to the more hydrophobic aromatic 289 compounds, including aromatic carboxylic acids, phenolic compounds and humic substances.<sup>88-91</sup> Sun *et al.*<sup>90</sup> reported a positive correlation between the percentage of aliphatic 290 291 carbon, indicated by H-to-C ratio of organic matters with their bioavailability. Significantly 292 influencing the species of microbial growth, the levels of BOM are unique to each 293 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.<sup>16</sup> Regardless, oxidative 294 drinking water treatments, such as ozonation<sup>92, 93</sup>, UV radiation<sup>94-96</sup> and a combination of 295 UV/H<sub>2</sub>O<sub>2</sub> treatment<sup>97</sup>, have been suggested to increase the bioavailability of organic matters 296 297 in distribution systems due to alteration of their chemical structures. Reduction of BOM 298 contents, typically by biological filters, is therefore necessary prior to the water entering the 299 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.<sup>98-100</sup> Growth of bacteria in drinking water needs phosphorus,<sup>99, 101</sup> 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.<sup>102</sup> 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 metals,<sup>103</sup> which limits further corrosion. Changes in the biofilm structure and microbial 308 community have been reported following phosphate (or phosphoric acid) addition.<sup>104, 105</sup> A 309 clear example is given by Fang et al.<sup>106</sup> who observed the formation of thicker, more 310 heterogeneous biofilms with higher number of micro-colonies upon phosphate treatment. 311 Batté et al.<sup>67</sup> reported a significant increase in  $\gamma$ -Proteobacteria within biofilms, which 312 potentially includes common pathogens. The work however, further reported no change in the 313 bacterial counts following addition of a relatively high concentration of phosphate (500  $\mu$ g/L) 314 to systems with already established biofilms.<sup>67</sup> This suggests that the phosphorus was not 315 316 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 planktonic and biofilm growth have been reported to increase with 1 to 300 µg/L phosphate 319 addition.<sup>107-109</sup> Biofilms have also been shown to elevate their EPS production in response to 320 phosphorus limitation,<sup>110</sup> which appears to serve as protective mechanisms against the growth 321 322 inhibiting effect of phosphorus limitation.

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

Another key inorganic nutrient affecting biofilm development is nitrogen, a building block 330 for proteins and genetic materials (DNA and RNA). A major class of microorganisms that 331 form biofilms in DWDS are the autotrophic nitrifying bacteria or nitrifiers, which utilize 332 nitrogen-based compounds such as ammonia, nitrate, nitrite and in some species, urea, as an 333 energy source,<sup>113</sup> with ammonia as the preferential compound for biomass production.<sup>114</sup> 334 Ammonia is often present in untreated water and is also released during chloramine decay.<sup>115</sup> 335 Ammonia also forms from reactions of nitrate with metal surfaces in distribution systems.<sup>116</sup>, 336 <sup>117</sup> As with phosphorus, it was reported that the water's nitrogen content could modulate the 337 composition of microbial communities in biofilms. Biofilms with predominantly autotrophic 338 339 bacteria tend to form at high nitrogen-to-carbon ratios, whereas low nitrogen-to-carbon ratios promote growth of heterotrophic bacteria.<sup>118, 119</sup> A modelling work by Zhang et al.<sup>120</sup> (based 340 on the work of Verhagen and Laanbroek<sup>119</sup>) predicted that autotrophic bacteria will flourish 341 342 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 heterotrophic and autotrophic bacterial population at between 0.1 to 10 nitrogen-to-carbon 344 345 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.

Finally, trace metals such as iron and copper are also known to affect biofilm development in DWDS. Iron is essential for almost all bacterial growth and development, but at high concentrations can be toxic to the cells.<sup>121</sup> Growth in biofilms is often associated with expression of iron acquisition genes, suggesting that iron is a limited resource in biofilms.<sup>122,</sup> <sup>123</sup> Further, several studies reported that iron sequestration can inhibit biofilm formation,<sup>124</sup> while others have reported that addition of iron can induce dispersal from biofilms.<sup>125</sup> Copper on the other hand, is reported to enhance bacterial aggregation at toxic levels, which is

thought to act as protective responses to stress.<sup>126, 127</sup> Presence of copper in drinking water 355 has also been shown to induce VBNC state on the opportunistic pathogen P. aeruginosa.<sup>81</sup> 356 357 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 358 359 nutrients, including nitrogen, in the bulk water prior to entering the distribution system. In 360 some countries and even parts of the US, such nutrient limitation has been a common practice 361 for decades. Bulk water pretreatment could be performed in the case of high nitrate-362 containing ground water through ion-exchange processes, reverse osmosis and even 363 biological denitrification. In regard to the routine practice of phosphate addition to 364 distribution systems, an appropriate monitoring strategy (as later discussed) is necessary to 365 anticipate potential biofilm growth, particularly in systems with initially low phosphate bulk 366 water content.

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# Initial microbial attachment onto pipe surfaces



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- 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 in fall and 6°C in winter,<sup>128</sup> while temperatures ranging from 6°C to 35°C<sup>129</sup> are not 377 378 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 bacteria, such as Listeria monocytogenes,<sup>130-132</sup> P. aeruginosa<sup>133</sup> as well as other bacteria,<sup>134</sup> 383 that have been found to form biofilms. L. monocytogenes for example, is reported to produce 384 EPS at 21°C and therefore adhering on surfaces and forming biofilm, but not at 10°C or 385  $35^{\circ}C$ ,<sup>130</sup> as also observed by other studies.<sup>131</sup> 386

387 Relevant biofilm-forming bacteria, including Acinetobacter, Agrobacterium radiobacter, Alcaligenes, Arthrobacter sp., Corynebacterium sp., E. coli, P. aeruginosa, P. fluorescens 388 and *P. putida*,  $^{135}$  have been known to become more hydrophobic during the exponential 389 growth phase,<sup>135</sup> and temperature variations are reported to modulate cell surface 390 391 hydrophobicity in a growth phase-dependent manner. With decreasing temperature from 37°C to 8°C, Chavant et al.<sup>136</sup> observed a more prominent decrease in cell hydrophobicity 392 with stationary phase *L. monocytogenes* compared to exponentially growing cells.<sup>136</sup> It has 393 394 been suggested that bacteria modify their cellular membrane lipid composition as a function 395 of temperature, leading to changes in hydrophobicity,<sup>137</sup> 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

398 attachment onto hydrophilic surfaces (stainless steel) compared to hydrophobic surfaces 399 (polytetrafluoroethylene, PTFE).<sup>136</sup> In contrast, at 8°C, the cell surface becomes hydrophilic 400 and cell attachment was observed not only on the hydrophilic surfaces, but comparably also 401 on the hydrophobic surfaces.<sup>136</sup> Despite potential differences in the initial bacteria-to-surface 402 attachment, it has been frequently observed that over prolonged periods (days or months), 403 there is generally no observable difference in the extent of biofilm accumulation on 404 hydrophilic surfaces compared to those on hydrophobic surfaces.<sup>138</sup>

Microorganisms tend to form biofilms at a lesser extent at lower temperatures.<sup>128, 136</sup> This 405 406 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.<sup>139, 140</sup> For example, L. 407 408 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 409 8°C.<sup>136</sup> In other drinking water relevant cases, temperature fluctuation does not appear to 410 411 affect the presence of ammonia oxidizing bacteria (AOB) in chloraminated systems - the bacteria are capable to deplete monochloramine and generate nitrate.<sup>128</sup> However, less 412 developed AOB biofilms are formed at 12°C compared to those formed at 22°C.<sup>128</sup> 413

414 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 415 c-di-GMP level in the pathogenic V. cholerae, in turn, enhancing biofilm growth.<sup>141</sup> The 416 cellular physiological responses was linked to 6 DGC genes, which encode for the synthesis 417 418 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.<sup>141</sup> In other studies, a 419 temperature increase by 5°C or more was found to induce dispersal of a pre-established P. 420 421 aeruginosa (an opportunistic pathogen) biofilm, and the effects are also linked to changes in cellular c-di-GMP level.<sup>142</sup> 422

423 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 424 425 biofilms. An understanding of the temperature-dependent susceptibility of water distribution 426 systems to biofilm formation will allow for prompt implementation of appropriate biofilm 427 monitoring and control strategies. It is noteworthy to mention however, that in general an increase in temperature leads to higher rates of disinfectant degradation,<sup>143</sup> which in turn, 428 429 increases disinfectant demand. Applications of higher doses of disinfectant are therefore 430 necessary, in particular during warmer temperatures, to maintain the microbiological quality 431 of the water.

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# 433 Effect of pipe materials on biofilm formation

434 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), 435 436 copper or cement based materials, while polymer based materials such as, polyvinyl chloride 437 (PVC) and polyethylene (PE) are becoming increasingly popular as they are easier to handle 438 and install. The choice of pipe materials could affect development of biofilms in distribution 439 systems. Polymeric pipes could be a source of biodegradable volatile organic compounds (VOCs) in drinking water,<sup>144</sup> due to leaching of polymer additives, polymer degradation as 440 441 well as by-products of polymer oxidation. It has been shown that microorganisms could 442 proliferate by metabolizing small molecular weight plasticizer, residual monomers as well as anti-oxidants, potentially promoting biofilm growth on pipe surfaces.<sup>145, 146</sup> Many studies 443 444 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).<sup>147-150</sup> In 445 446 contrast to the 'smooth' surfaces of polymeric pipes, it is thought that the pitted surfaces of 447 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 greater surface area.<sup>16, 138, 148, 151</sup> Further, dissolved and solid iron corrosion products in 450 DWDS could support the growth of specific biofilm-forming bacteria. Iron-oxidizing 451 bacteria, such as *Gallionella* spp. oxidize ferrous iron to ferric iron,<sup>152, 153</sup> while iron-reducing 452 bacteria, such as P. aeruginosa, P. fluorescens and some members of Bacillus spp.<sup>13, 154</sup> 453 reduce soluble<sup>155</sup> or solid iron (III) species<sup>156, 157</sup> to iron (II) species.<sup>158</sup> Corroded pipe 454 material could retain nutrients, including carbon, nitrogen, phosphorus,<sup>159, 160</sup> for subsequent 455 utilization by biofilm bacteria. Corrosion products could also react with disinfectants, 456 depleting residuals particularly near pipe surfaces.<sup>161</sup> Indeed, an increase in microbial 457 458 concentration and diversity has been observed on biofilms formed on severely corroded pipes.162 459

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

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#### 473 Flow rate variation in distribution systems affects biofilm growth

The hydrodynamic conditions in DWDS may dramatically vary between different 474 475 locations, alternating from laminar to turbulent flow and vice versa. Flowing water affects 476 biofilm development, giving rise to structurally-unique biofilm growth depending on the flow 477 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 478 with surfaces as a result of convective diffusion.<sup>163</sup> Further, high shear force has been shown 479 to boost EPS production in established biofilms and enhance cell-to-substratum adhesion.<sup>164</sup> 480 481 Such enhanced EPS production (in particular polysaccharides) may further contribute to the 482 mechanical stability of the growing biofilms and aid certain types of bacteria to remain attached to the surface.<sup>165</sup> Moreover, the nutrient transport rate from bulk water into the 483 biofilm increases at high flow rates and in turn, stimulates further growth.<sup>166, 167</sup> Such 484 485 enhanced growth was observed to be more pronounced on polymeric-based (polyethylene) pipes compared to those on copper pipes.<sup>167</sup> There is considerable evidence indicating that 486 487 turbulent flow and high shear stress conditions promote the growth of thinner, denser, and less porous biofilms.<sup>164, 168, 169</sup> High flow rates however, also promote detachment of mature 488 biofilms due to increased shear stress on the outer layers of the microbial communities.<sup>166, 170,</sup> 489 <sup>171</sup> Dispersed biofilms can compromise the microbiological quality of the drinking water. In 490 contrast, at low flow rates, both the nutrient transport and shear effects are dampened,<sup>166, 172</sup> 491 492 and this appears to result in the formation of more loosely attached and more porous biofilms.<sup>164</sup> Knowledge of the formation of distinct biofilm structures under different flow 493 494 characteristics can be included as a factor when selecting the appropriate strategy for biofilm 495 control, including the physical and chemical removal of biofilms, as later described.

496 Effective management of the distribution system hydraulics to avoid slow moving or even 497 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.<sup>16</sup> The sedimentation and low disinfectant levels are likely 502 to promote extensive biofilm growth.<sup>173, 174</sup>

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#### *pH adjustment and fluctuations in distribution systems affects biofilm development*

505 Drinking water pH is often adjusted to facilitate optimum water treatment processes, to minimize the decay of disinfectants or for corrosion control.<sup>175</sup> The growth of nitrifying 506 507 bacterial biofilms in distribution systems is most favourable at pH 7 - 8 with the actual optimum pHs vary among different bacteria.<sup>176</sup> For example, *Nitrobacter* spp. grow optimally 508 at pH 7.2 – 7.6,<sup>177</sup> while *Nitrosomonas* spp. at pH 7.9 – 8.2.<sup>177</sup> When it occurs, nitrification 509 510 will most likely decrease the pH of the distribution system to 6 or less, particularly in poorly buffered systems.<sup>175</sup> This pH fluctuation in distribution systems could in fact further promote 511 or inhibit nitrification through a number of known mechanisms<sup>175</sup>: (1) binding of  $H^+$  at low 512 pH or OH<sup>-</sup> at high pH to weak basic or acid groups of enzyme active sites,<sup>178, 179</sup> (2) pH 513 affects nutrient availability by governing the chemical equilibrium of the mineral carbon 514 source  $(CO_3^2)^2$  to  $HCO_3^2$  to  $CO_2$ ).<sup>178</sup> At high pH, the mineral carbon will predominantly exist 515 as the insoluble and hard-to-metabolize carbonates,<sup>178</sup> (3) pH affects the concentrations of the 516 non-ionic ammonia and nitrous acid, which could inhibit nitrification.<sup>178, 180</sup> Free ammonia 517 dominates at high pH while nitrous acid dominates at low pH.<sup>181</sup> 518

The pH in distribution systems could also affect bacteria-to-surface interactions and in turn, their initial attachment on pipes. At around pH 7, many biofilm-forming bacteria will have a net negative surface charge due to presence of anionic groups (*e.g.* carboxyl and phosphate) on cell surfaces.<sup>182-184</sup> Electrostatic repulsion could take place upon their 523 interaction with negatively-charged pipe surfaces<sup>185, 186</sup>, for example, PVC pipes at around 524 pH 7 (isoelectric point = pH 5.4).<sup>187</sup> A pH drop in distribution systems close to the isoelectric 525 pH, due to growth of nitrifiers for instance, could reduce the bacteria-to-surface electrostatic 526 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.

531 Up to this stage, the current article has reviewed important research efforts to reveal how 532 operational conditions of distribution systems affect biofilm growth, from the affinity and 533 initial attachment of microorganisms onto surfaces, to the extent and characteristics of 534 growth. The knowledge provides insights into the susceptibility of water distribution systems 535 to biofilm formation, which signifies the need for water pretreatment and biofilm monitoring 536 strategies. The knowledge will also allow potential tuning of operational parameters 537 whenever applicable, to better manage the growth.

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# 539 Characterization and monitoring of biofilms in water distribution systems

540 Continuous monitoring of biofilm growth in water distribution systems is essential to limit 541 their potential adverse impact on the drinking water quality and safety. Conventionally, 542 biofilms are extracted by scraping pipe surfaces followed by *ex situ* analyses of samples in 543 laboratories. Biofilms are not uniformly distributed throughout the water distribution system 544 and therefore, obtaining a representative sample is difficult. There are several devices that 545 can be placed either directly into the flow or in a by-pass line, and used to assess biofilm 546 growth in DWDS. Examples of these devices include (as shown in Figure 3): Corporation Sampling Device,<sup>188</sup> modified Robbins device,<sup>189, 190</sup> biofilm sampler,<sup>191</sup> Pennine Water 547

Group coupon,<sup>192</sup> and a column filled with glass cylinders.<sup>193, 194</sup> All of these devices contain 548 549 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 biofilm sampling devices have been tested in the DWDS,<sup>190, 191</sup> they are still not widely used 552 553 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 water distribution system.<sup>195</sup> The small and flexible optical fibers are non-conducting and 560 561 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 biofilms with more than  $10^{10}$  cells cm<sup>-2</sup> due to saturation of optical signals. Fischer *et al.*<sup>196</sup> 564 565 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 technology allows *in situ* discrimination of biological deposits from chemical fouling as well 567 as capability to evaluate the viability of the biomass.<sup>197</sup> The device is capable of measuring 568 569 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 bacteria).<sup>198</sup> ALVIM operate in two modes: (a) potentiostatic technique provides information 578 579 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 therefore,<sup>198</sup> providing meaningful information to optimize treatment, *i.e.* the concentrations, 584 585 timing and duration of chemical additions.

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Figure 3. Schematic diagrams of sampling devices for biofilm monitoring: (a) Robbins
 device,<sup>190</sup> (b) Pennine water group coupon,<sup>192</sup> (c) Corporation sampling device<sup>188</sup>, Reprinted

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

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#### 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 process, which is responsible for ammonia metabolism, involves multiple organisms to 603 effectively convert ammonia to N2.199, 200 This is in part achieved by close spatial 604 605 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 more detail).<sup>201</sup> More mature biofilms of more than 3 to 4  $\mu$ m thick can be non-destructively 613 614 visualized using confocal laser scanning microscopy (CLSM), which allows optical

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

632 For mature biofilms of up to 2 mm thick, optical coherence tomography (OCT) offers high resolution and relatively large imaging area without cell staining.<sup>215</sup> The current OCT 633 634 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 to evaluate biofilm coverage and thickness,<sup>216, 217</sup> ESEM does not require dehydration of 638 639 samples, therefore enabling visualization of biofilm structure in their natural wet or partially

hydrated states without dehydration artifacts (Figure 4b).<sup>218</sup> Despite these advantages, ESEM 640 has inherent limitations, such as reduced resolution and increased beam damage at high 641 magnification due to the absence of metal coating. Obscured surface topography is also 642 common with presence of alternate dark and light areas as a result of differences in local 643 electric charge.<sup>218, 219</sup> Further, elemental composition mapping of macromolecules within 644 biofilm matrices (e.g. polysaccharides, proteins, lipids and nucleic acids) is feasible with 645 scanning transmission X-ray microscopy (STXM),<sup>220</sup> which can be used to generate a 646 detailed correlative map of biofilm structure and composition in the water distribution 647 648 system.

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## 650 Measurements of active biomass

651 It may be of interest to determine what fraction of the microbial community is active in the distribution system, that is, distinguishing dead or recalcitrant biomass that persists from 652 biochemically active biomass. The latter may represent either a health risk – as reservoir for 653 654 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 metabolism. Example of such biochemical tests that are applicable to the drinking water 659 660 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 detection limits of 0.05 ng ATP  $L^{-1}$ .<sup>221, 222</sup> The analysis of ATP is based on the luciferase 662 catalyzed reaction of ATP with luciferin to produce a luminescent signal.<sup>223</sup> This signal is 663 proportional to the amount of ATP present, which correlates well to the number of viable 664

665 cells. Other measures of active biomass include the use of the earlier mentioned Live/Dead Baclight staining to quantify the relative proportion of viable and non-viable cells. This 666 method works through the combined application of two fluorescent dyes, one that freely 667 668 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 necessarily applicable to mixed communities. Alternatively, redox active fluorophores (e.g. 673 5-cyano2,3-ditolyl tetrazolium chloride or CTC),<sup>224, 225</sup> that fluoresces in the presence of an 674 675 active electron transport chain can be used to visualise and quantify active vs non-active cells in the microbial community. This may have limited function where cells are fermentative, for 676 677 example.

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**Figure 4.** (a) Epifluorescence images of biofilms on copper pipes with (1) aggregating bacteria and (2) homogeneously distributed bacteria stained with the BacLight viability reagents.<sup>216</sup> Green: bacteria with intact membranes, red: bacteria with damaged membranes. Scale bars = 10  $\mu$ m. (b) Environmental scanning electron micrographs of biofilms on copper surfaces.<sup>216</sup> Image (1) to (4) show the presence of multi-layered bacterial aggregates with different morphologies on Cu surfaces. Note the multi-species microbial communities in (3).

#### 687 *Phylogenetic analyses of microbial communities in biofilms*

688 Although viable, many of the microbial members of sampled biofilms are often uncultivable as they do not grow on commonly used cell culture media.<sup>226</sup> Culture-based 689 690 techniques therefore most of the time underestimate the diversity and relative abundances of microorganisms in biofilms.<sup>71</sup> Cultivation-independent molecular techniques applicable to 691 692 drinking water biofilms have been developed, and as herein discussed, these can be classified 693 into two approaches, those that are based on DNA fingerprinting methods, such as denaturing 694 gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP) or terminal restriction fragment length polymorphism (T-RFLP) and the 16S rRNA gene 695 696 sequencing method.

697 DGGE examines microbial diversity in mixed-culture biofilms, as well as population shift in response to altered environmental conditions or stress.<sup>65, 227-230</sup> Relevant to the DWDS, the 698 699 method has been used to investigate dominant bacterial members in microbial communities 700 on different plumbing materials, to compare the effect of material choice on biofilm formation,<sup>216, 231, 232</sup> and to determine any population shift during water treatment steps and 701 subsequent distribution.<sup>217, 228</sup> DGGE separates polymerase chain reaction (PCR) amplified 702 703 gene fragments of the same length but with different base pair sequences based on the 704 decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants.<sup>233</sup> The number of 705 706 bands observed in DGGE profiles provides an estimate of species richness in biofilms while 707 the relative intensity of each band is thought to reflect the relative abundance of each species. 708 DNA bands from DGGE gels can be further processed for sequencing to identify the 709 corresponding microbial species. Gradually being abandoned, the method is limited by the risk of bias introduced during PCR amplification,<sup>234, 235</sup> co-migration of DNA from different 710

species forming the same band,<sup>236</sup> as well as formation of multiple bands in the amplification
of genes from single genomes.<sup>237, 238</sup>

Other DNA fingerprinting methods, like SSCP<sup>8</sup> or T-RFLP<sup>239</sup> have also been used for 713 microbial community analyses of biofilms in drinking water systems. In PCR-SSCP analysis, 714 715 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 conformation of single-stranded DNA. Using PCR-SSCP analysis, Henne et al.<sup>8</sup> reported 718 719 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 and their heights.<sup>66, 240</sup> T-RFLP has also been used to assess shifts in the microbial population 727 as a result of variation in environmental conditions or disinfection practices.<sup>241</sup> Similar to 728 729 DGGE however, the SSCP and T-RFLP methods detect only the most dominant members of 730 microbial communities.

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

16S gene analysis, Lin et al.<sup>63</sup> reported that Proteobacteria were the dominant organisms in 736 737 biofilms formed on PVC, stainless steel and cast iron surfaces. Importantly, the technique could detect microorganisms present at low abundances. Analysing biofilms in a model 738 739 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 analysis, including members from Nitrospirae, Acidobacteria and Planctomycetes, in 741 742 comparison to detection of only bacteria from the Proteobacteria and Bacteriodetes phyla using cultivation-based method.<sup>243</sup> Apart from showing that the dominant bacterial 743 population was related to Nevskia spp. (y-Proteobacteria), Keinanen-Toivola et al.<sup>244</sup> also 744 745 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 intended to kill fecal pathogens, may lead to outbreak of resistant bacteria.<sup>16</sup> It is important to 751 752 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.

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

species.<sup>218</sup> The primers can either target microbes at the kingdom or phylum level or can be 761 762 designed to quantify specific bacteria based on the presence of genes of interest. For the latter, the genes associated with ammonia oxidation for example, can be quantified,<sup>245, 246</sup> which 763 may indicate the extent to which the microbial community are able to metabolise and 764 765 inactivate chloramine added to the distribution system as disinfectant. Similarly, qPCR primers can be designed to quantify specific pathogens in the DWDS<sup>246</sup> and this information 766 767 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 from any unnecessary treatment response that may adversely impact the public health.<sup>16</sup> 770

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

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

In 2004, the World Health Organization (WHO) published its first set of guidelines to ensure drinking water safety, called the Water Safety Plans (WSPs).<sup>247</sup> Unique to each water supply system, WSPs are a comprehensive 'source to tap' risk assessment and preventative management approach, guided by health-based targets and supervised by (preferably independent) auditor. The Plans include multi-barrier practices to prevent growth of pathogens in the DWDS, including controls on biofilm growth as potential reservoirs for pathogens, as herein described.<sup>15, 54, 248</sup> 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 bactericidal activity has been linked to the generation of reactive oxygen species, which 788 induce broad damage to bacterial cells affecting DNA, proteins and lipids.<sup>249</sup> Activity of 789 790 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-toirreversible transition of cell attachment to surfaces.<sup>22</sup> Chlorine reduces microbial growth 793 rate,<sup>250, 251</sup> and yet is incapable of complete inhibition of biofilm growth.<sup>252</sup> For the latter, 794 795 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 diffusion into the biofilm interior.<sup>253-256</sup> Further, chlorine is able to promote detachment of 797 cells from biofilms.<sup>257-259</sup> It is noteworthy to mention however, that the use of chlorination 798 799 while effective in killing faecal pathogens for example, may lead to selection of resistant bacteria, such as the opportunistic pathogen Mycobacterium avium due to their relative 800 chlorine resistance.<sup>16</sup> In many cases, bacteria could still form biofilms at high chlorine 801 concentrations (0.8 to 1.5 mg/L, relevant to those in DWDS),<sup>250, 260</sup> and this is a reflection of 802 the antimicrobial tolerance of biofilms. Increased resistance to chlorine has been observed 803 with mature biofilms (at the highest thickness), compared to those at early stages<sup>261</sup> and is 804 further enhanced within multi-species biofilms, compared to those of single-species, whereby 805 the mixed species communities may share multiple mechanisms of chlorine resistance.<sup>165, 262</sup> 806 807 Chlorination was also found to enrich the prevalence of antibiotic resistant bacteria (ARB) in drinking water.<sup>263</sup> Shi et al.<sup>264</sup> found higher proportion of surviving bacteria that exhibit 808 809 resistance to chloramphenicol, trimethoprim and cephalothin following chlorination.

810 Chlorine is typically dosed in excess, while at levels below the safety and aesthetic, taste 811 and odour standard limits, to provide effective residual concentrations preventing bacterial regrowth during water distribution. Free chlorine degrades due to reactions with organic and 812 inorganic compounds (ammonia, Br<sup>-</sup>, I<sup>-</sup>, SO<sub>3</sub><sup>2-</sup>, NO<sub>2</sub><sup>-</sup>, Fe(II)) in bulk water,<sup>265</sup> corrosion 813 products,<sup>266</sup> pipe materials<sup>267</sup> and even through interactions with microorganisms and their 814 EPS.<sup>268</sup> In most cases however, the maintenance of an effective disinfectant concentration is 815 816 challenging. Chlorine degradation is most likely to occur at the dead ends of large networks 817 and in low velocity regions. To solve the problem, at least partly, supplementary chlorine are 818 added at strategic points through booster stations installed along the distribution lines. Such 819 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 820 821 hazardous disinfection by-products. The increasing stringency of guidelines and regulations 822 on disinfection by-products mandates better control of disinfectant application.

823 More stable compounds, such as chloramines, formed from a reaction between chlorine and 824 ammonia, maintain disinfection residual for a longer period throughout the distribution system and generates fewer harmful regulated disinfection by-products.<sup>16</sup> Chloramines are 825 often used in distribution systems where free chlorine residuals are difficult to maintain or 826 chlorine use leads to excessive by-product formation. Though less reactive compared to 827 chlorine, chloramines may penetrate biofilms more effectively<sup>269</sup> because unlike chlorine, 828 829 they less readily react with the presence of organic matters in the biofilm surface layers. 830 Caution must be taken however, as in some cases the use of chloramine has been associated 831 with the growth of certain nitrifying bacteria (due to the release of ammonia from chloramine decay) within biofilm that in turn degrade disinfectant residual.<sup>16</sup> 832

833 The water distribution system is also subjected to cleaning *via* flushing, pigging or 834 air/water scouring, which are considered to be the best routine management practices for

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biofilm control,<sup>6</sup> also removing any biomass killed or inactivated by disinfections. Flushing 835 836 involves forcing high-speed water through the pipes to flush out particulates. Strong shear 837 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 838 also those of the bulk water.<sup>16, 61</sup> Recent enquiries have observed changes in microbial 839 richness and diversity between pre- and post-flushing samples. The relative abundance of  $\gamma$ -840 841 Proteobacteria for example, decreased following either low or highly varied flushing regimes, while the opposite occurred for  $\beta$ -Proteobacteria.<sup>61</sup> Flushing in most cases is 842 incapable of thorough removal of biofilms from pipe walls.<sup>61</sup> Pigging involves forcing an 843 844 object fitted to the pipe diameter, such as a hard sponge bullet, ball or ice, through the pipe to 845 physically scrub biofilms from the pipelines. A build-up in back-pressure causes the bullet to 846 rotate while in motion.

847 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 848 849 concentration of suspended microbial in drinking water prior to entering the distribution 850 system (typically through membrane biofiltration). It has been shown in various laboratory 851 scale systems that nutrient limitation can inhibit biofilm formation and/or induce biofilm 852 dispersal, ultimately, reducing or delaying the impact of biofilms on engineered systems. 853 Granular activated carbon filters have been used to reduce AOC levels in water, discouraging bacterial growth.<sup>270</sup> Following adsorption of AOCs on the activated carbon, the technology 854 855 facilitates degradation of the organic matters through the metabolic activity of artificially 856 inoculated microorganisms along with, to a certain extent, the activity of naturally-occurring aquatic microorganisms adsorbing on the activated carbon.<sup>271</sup> Such nutrient control practice 857 has been reported to effectively reduce biofilm accumulation in membrane based water 858 purification systems.<sup>272, 273</sup> In place of the use of disinfectants in the DWDS, large treatment 859

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

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

Further, corrosion control in distribution systems is also key to limit biofilm growth in 874 distribution systems.<sup>111</sup> As described earlier, corroded pipe surfaces are favourable over 875 876 '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 larger systems whereby pipes are only being replaced every 100 years.<sup>16</sup> Instead, although the 879 880 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 entering the distribution system<sup>16, 281, 282</sup> For the latter, abatement of corrosion is generally 882 883 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 countries.<sup>283-286</sup> For example, an improved drinking water quality and better public health was 886 reported in Iceland, which saw a substantial reduction in the concentration of HPC 887 888 (heterotrophic plate count) bacteria in both the source and distributed water and correspondingly, the incidence of diarrhea.<sup>287</sup> WSPs are now legally required in a number of 889 countries, including Iceland.<sup>284</sup> In fact, many well-managed water utilities have implemented 890 the WSPs' principles for years, 283, 284, 288 such as those in Germany with their DVGW's TSM 891 (The German Technical and Scientific Association for Gas and Water's technical security 892 management) approach.<sup>289</sup> Further, international networks (e.g. the IWA Bonn Network, the 893 894 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.

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# 902 Future outlook for biofilm control in water distribution systems

Effective control of biofilm growth in DWDS requires suitable antimicrobial agents and design of treatment processes to limit the initial presence of colonizing microbial community at the treatment plant. A wide range of engineered nanomaterials have been increasingly demonstrated to exhibit potent and versatile antimicrobial properties through diverse toxicity mechanisms, from compromising the integrity of microbial cell wall (*e.g.* nanosilver,<sup>290-292</sup> copper oxide,<sup>293</sup> zinc oxide,<sup>294, 295</sup> carbon nanotubes,<sup>296</sup> chitosan<sup>297</sup>), stimulation of cellular 909 reactive oxygen species (ROS) generation that can damage proteins, lipids and even DNA (e.g. silver,  $^{292}$  ZnO,  $^{298, 299}$  TiO<sub>2</sub><sup>300</sup> and fullerol<sup>301</sup>), to inhibition of enzyme activity and DNA 910 synthesis (*e.g.* chitosan<sup>302</sup>). Incorporation of these antimicrobial nanomaterials in membranes 911 912 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 minimize any unintended impacts on human health and the environment. In most cases, the 914 915 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 packed in nanoparticles and therefore, the minimal leaching or release of the particulates).<sup>303,</sup> 918 <sup>304</sup> As with the use of any antimicrobial agents in contact with drinking water, the potential 919 920 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 materials)<sup>305, 306</sup> 922

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 system<sup>307, 308</sup> with a diverse class of natural and synthetic compounds being developed to 926 inhibit the QS mediated cell-to-cell communication, including halogenated furanones,<sup>309</sup> 927 dihydropyrrolones<sup>310</sup> or natural products such as ajoene.<sup>311</sup> The technology offers a potential 928 929 strategy for disrupting and preventing development of biofilms on water pipes, with the successful coating of numerous QS inhibitor, such as dihydropyrrolones onto surfaces 930 whereby they limit biofilm formation.<sup>310</sup> Another promising approach is to use low levels of 931 932 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

bacteria.<sup>312</sup> NO triggers a decrease in the intracellular concentration of c-di-GMP, which 934 935 leads to not only biofilm dispersal, but also rendering the biofilm more susceptible to biocides. Compounds that spontaneously release NO, called the NO donors and include 936 compounds such as sodium nitroprusside, Proli-NONOate and DETA-NONOate, have been 937 shown to disperse drinking water biofilms<sup>312, 313</sup> as well as biofilms formed on membranes for 938 water treatment.<sup>314, 315</sup> While NO is a highly reactive molecule and thus its delivery over long 939 940 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 in water.<sup>316</sup> Such solution could prove effective for applications in DWDS. Further, given that 942 943 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 compound.<sup>317</sup> 945

946 Apart from the earlier described practice to limit nutrient content in the distribution system, biofilm control could be achieved by adding inhibitors of key metabolic enzymes. Target 947 948 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 growth.<sup>318</sup> Finally, a quite recent bacteriophage-based technology has been developed for 951 control of biofilms on hard surfaces,<sup>319-321</sup> a potentially attractive application for drinking 952 953 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 matrix in biofilms, leading to destruction of the biofilms.<sup>322, 323</sup> Bacteriophages are self-955 956 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

959 present below a threshold sufficient for phage replication, they become inert particles. The 960 potential application of the technology in distribution systems requires a detailed 961 understanding of the key biofilm-forming bacteria in the system, as targets for the appropriate 962 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.<sup>324-</sup> 963 <sup>326</sup> For all of these novel approaches, research will be required to find the optimum 964 965 compounds, concentrations and dosing strategies to demonstrate efficacy. Additional 966 considerations faced by all new technologies, such as cost for implementation relative to 967 benefit, as well as the environmental impact and fate of such compounds, would also need to 968 be addressed here. Finally, some testing of these novel approaches under realistic conditions, 969 e.g. high flow rates, and benchmarking them against existing technologies would also enable 970 decisions about their large scale utility as biofilm control strategies in the field.

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#### 972 SUMMARY AND PERSPECTIVES

973 Growth of biofilms in drinking water distribution networks although unavoidable, is 974 potentially controllable. An in-depth understanding of biofilm characteristics and how the 975 conditions of the distribution systems affect their development, will allow for potential tuning 976 of the operational parameters to limit the growth. The choice of pipe materials, flow rates, 977 temperature and pH of distribution system affect all stages of biofilm development, from the 978 initial attachment of microorganisms onto pipe surfaces, to the extent and characteristics of 979 biofilm growth. Whenever applicable, the 'biofilm-limiting' operating conditions of 980 distribution systems, together with the already implemented biofilm control practice, such as 981 removal of organic and inorganic nutrients and treatment with disinfectants, feature a 982 potential for improved management of biofilm growth throughout the network. Equally 983 important is the implementation of suitable biofilm monitoring practice to probe the likely

984 changes in biofilm characteristics as a result of fluctuations in operating conditions and 985 disinfection treatments. The quite recent development of *in-situ* biomass sensors and the 986 increasingly cost effective biomolecular analysis of microbial communities will enable more 987 frequent and thorough assessments of biofilm characteristics and their distribution profile 988 across the drinking water networks. This will allow for timely administration of control 989 measures, particularly in response to unforeseen changes in operating conditions that could 990 promote biofilm growth in distribution systems, including an abrupt temperature increase or a 991 pH drop due to growth of nitrifiers. Management of the persistent biofilm growth requires an 992 integrated approach of water pretreatment, biofilm monitoring and control, as no single 993 practice thus far appears to be sufficiently effective. In closing, a systematic survey of DWDS 994 microbial ecosystems and their correlation to water characteristics and the systems' 995 operational conditions, is key for effective monitoring and treatment strategy and 996 importantly, for anticipation of potential shifts in the microbial profile in response to 997 treatment change. The survey is indispensable, in particular with the now known prevalence 998 of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in drinking 999 water, with no regulation currently in place for such presence of resistance entities. In the 1000 current reality, "water utilities, in a sense, are forced to 'fly blind' when making treatment 1001 decisions without a detailed inventory of the microorganisms growing within distribution systems" (a quote from the Microbes in Pipes<sup>16</sup> report by the American Academy of 1002 1003 Microbiology, 2012).

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