Unwanted coliforms can hide in mature drinking water biofilms, grown in full-scale distribution networks

Fien Waegenaar[1,2], Cristina García-Timermans[1,2], Bart De Gusseme[1,2,3], Nico Boon[1,2,*]

[1]Center for Microbial Ecology and Technology (CMET), Department of Biotechnology, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium.

[2]Center for Advanced Process Technology for Urban Resource Recovery (CAPTURE), Frieda Saeysstraat 1, B-9000 Ghent, Belgium.

[3]Farys, Department R&D – Innovation Water, Stropstraat 1, B-9000 Ghent, Belgium.

Running title:

Coliform invasion in drinking water biofilms.

***Correspondence:** nico.boon@ugent.be

Abstract

Biofilms within drinking water distribution systems are an important habitat for drinking water microorganisms. However, they can play a role in microbial regrowth, water discoloration and can be a reservoir for unwanted microorganisms. In this study, we investigated whether indicator organisms for drinking water quality, such as coliforms, can settle in mature drinking water biofilms. Therefore, a biofilm monitor consisting of glass rings was used to grow and sample drinking water biofilms. Two mature drinking water biofilms were characterized by flow cytometry, ATP measurements, confocal laser scanning microscopy and 16S rRNA sequencing. Overall, biofilms developed under treated chlorinated surface water supply exhibited lower cell densities in comparison with biofilms resulting from treated groundwater. We observed that water sources shaped the biofilm community composition while drinking water disinfection determined the biofilm density. In addition, the response of the biofilm microbiome and possible biofilm detachment after minor water quality changes were

investigated. Limited changes in pH and free chlorine addition, to simulate operational changes that are relevant for practice, were evaluated. It was shown that both biofilms remained resilient. Finally, mature biofilms were prone to invasion of the coliform, *Serratia fonticola*. After spiking low concentrations (i.e. ± 100 cells/100 mL) of the coliform to the corresponding bulk water samples, the coliforms were able to attach and get established within the mature biofilms. These outcomes are emphasizing the need for continued research on biofilm detachment and its implications for water contamination in distribution networks.

1 Introduction

Microbial communities are ubiquitously present in drinking water distribution systems (DWDS). More than 98% of these microorganisms form biofilms on pipe materials or are associated with loose deposits (1–3). Biofilms can play a role in drinking water discoloration, the transformation of organic compounds, the decay of free chlorine, microbial regrowth, the formation of disinfection by-products and unwanted odor compounds, etc. (4–7). Furthermore, it is known that they can be a potential source of opportunistic pathogens (3, 8–12). Previous researchers detected pathogens such as *Mycobacteria* spp. and *Legionella* spp. as well as fecal indicator bacteria such as *Escherichia coli* in biofilm samples from a full-scale distribution network (9, 11). Furthermore, *Pseudomonas aeruginosa*, *Mycobacterium avium* and *Legionella pneumophila* can survive in young biofilms after spiking 10^5-10^6 cells/mL (12, 13). As a result, biofilms are a potential risk to human health as biofilm cells can be released to the planktonic water phase in some conditions. Nowadays, the measurement of coliform bacteria is used as an indicator for fecal contamination and to control the effectiveness of disinfection processes and the cleanliness and integrity of distribution systems (14, 15). In Flanders, 3.30% norm exceedances of the coliform indicator parameter were measured in 2021, however, the source of microbial contaminations is often not retrievable (14, 16, 17). Though, Kilb et al. (17) detected coliforms on rubber-coated valves from distribution networks, which could be possible sources of contaminations. However, more insights about pathogen invasion and detachment are needed to estimate corresponding unwanted consequences on the final water quality and safety.

Characterizing biofilm microbial compositions and physical characteristics, and therefore investigating the drinking water biofilm, is challenging as sampling is difficult in practice. Previous researchers used lab set-ups to determine biofilm microbial development and community compositions (18–25). Biofilm formation rates in terms of ATP activity and

deposition of iron and manganese is previously studied using biofilm monitors consisting of glass rings (26–28). Relatively new sampling techniques consist of a coupon holder to implement in a part of the drinking water distribution systems (DWDS) or in pilot-scale networks (29, 30). Ginige et al. (27) showed that the microbial ATP content on glass rings was 80% less than on plastic coupons for young biofilms (i.e. 6 months). However, inert glass allows the bulk water characteristics to be the only variable determining biofilm formation and composition (26).

A typical drinking water biofilm consists of a diverse microbial community attached to distribution pipes and immersed in a self-produced matrix of extracellular polymeric substances (EPS), which mainly contain polysaccharides and proteins (3, 31, 32). The bacterial biofilm community is dominated by *Proteobacteria*, more specifically *Alpha*- and *Gammaproteobacteria*. The most prominent biofilm genera on distribution pipes are *Pseudomonas*, *Sphingomonas* and *Acinetobacter* (1, 6, 30, 33–40). The drinking water biofilm environment offers protection against antibiotics, metals, disinfectants such as free chlorine and changing operational conditions such as shear stress, because of the EPS matrix and the interconnected processes among the biofilm bacteria (41–43). Additionally, EPS can serve as precursor for the formation of disinfection by-products (7). However, more research is needed to determine the role and influences of EPS on the final drinking water quality. Biofilm formation, its corresponding physical structure and the present microbial community are mainly influenced by the raw water source and treatment (e.g. microbial and nutrient composition) (3, 20, 44–47). For example, previous studies reported a shift in the microbial biofilm community and concentrations on distribution pipes after switching from drinking water with more carbon and a higher conductivity to less turbid water with a lower nutrient content (45, 46). In addition, the drinking water biofilm composition, and therefore biofilm detachment, is affected by the water residence time, pipe materials, free chlorine concentrations and temperature (5, 22, 24,

48–54). Previous researchers mainly investigated the bacterial removal efficacy of hydrodynamic stressors such as flushing, increasing chlorination concentrations or interrupted water flow (23, 49, 51, 54). For example, shock chlorination (10 mg Cl_2/L, 60 minutes contact time) removes 75% of the biofilm bacteria (23). However, the influence of minor operational changes, which often happens in practice, on biofilm dispersal to the water phase is lacking in literature.

Here, two biofilm monitors consisting of glass rings were set up to investigate whether unwanted coliforms can intrude into mature drinking water biofilms. First, the biofilms were characterized regarding bacterial cell density and community compositions as well as physical parameters such as biofilm biomass and thickness. Secondly, the response of the biofilm microbiome and possible biofilm detachment after minor water quality changes were investigated. These quality changes (pH, free chlorine concentration) were specifically chosen to simulate operational changes that are relevant for the full-scale DWDS in operation. Finally, survival of the coliforms in the bulk water phase was evaluated and attachment of these coliforms on the biofilm was investigated using confocal laser scanning microscopy and qPCR. Again, we explicitly chose to spike coliforms in low concentrations, in order to simulate real-life contaminations and thus conditions relevant for practice.

2 Materials and Methods

Biofilm sampling device, conditions and experimental design. A KIWA biofilm monitor was used (KWR, The Netherlands) consisting of 40 glass rings to collect drinking water biofilm (Figure A1). This monitor was placed at two different locations (Table A1). Monitor 1 was placed at the outlet of a drinking water reservoir receiving treated groundwater, before the UV post-disinfection step. Monitor 2 was placed at the outlet of a drinking water tower receiving treated surface water with residual free chlorine. The monitors were operating for 17 months at the respective locations and there was a continuous flow of 270 L/h, according to the manufacturer's instructions. Water quality parameters were measured by the respective drinking water providers (55). The total organic carbon (TOC) concentration was measured at the end of the experiment (16).

After 11 months and 17 months, 3 rings were analyzed with confocal laser scanning microscopy (CLSM). 4 rings were put in 10 mL autoclaved tap water and sonicated in a water bath (37 kHz, Elma – Ultrasonic, Belgium) 3 times for 2 minutes with a vortex step in between according to the manufacturer's instructions, to detach the biofilm. The biomass was quantified in terms of heterotrophic plate counts (HPC), ATP and total cell concentration (TCC) and the microbial community was characterized with 16S rRNA amplicon sequencing as described further. In the second part of the study, the KIWA monitors were transported to laboratory conditions to be able to manipulate the water conditions and to perform invasion experiments. During these experiments, the corresponding water types were used (Table A1). The monitor was connected to a 10 L plastic vessel and the water was pumped (WM 323 peristaltic pump, Watson Marlow, Belgium) and recirculated over the biofilm monitor. The pump was operating on 150 rpm and the flow was 42 L/h. The experiments were performed at room temperature (i.e. 22 °C).

Changing operational conditions in terms of the Langulier Saturation Index and HOCl addition. The LSI was calculated according to WAC/III/A/011 (56) (Table 1). The pH, conductivity and calcium concentration were measured using a Multi-parameter analyser C1010 (Consort, Belgium), a Hanna Edge Conductivity Meter (HANNA Instruments, Belgium) and the Total Hardness Test (Merck, Belgium), respectively. The alkalinity of the water samples was determined based on WAC/III/A/006 (57). Using 1M NaOH and 1M HCl (Chem-lab, Belgium), the pH was adapted to the a higher and lower Langelier Saturation Index (LSI) (Table 1). In addition, HOCl solution was added to have a free chlorine concentration of 0.20 mg/L and 0.28 mg/L, for the groundwater and surface water samples, respectively. The chlorine concentrate solution was prepared by addition of a NaOCl tablet (B-Care Chemicals, Belgium) to 1 L ultrapure water (Milli-Q, Merck Millipore, Germany). The amount of free chlorine was quantified with the Pocket Colorimeter II (Hach,Belgium).

Invasion experiments with GFP-labelled *Serratia fonticola.* The strain was isolated from the Flemish drinking water distribution network (Antwerp, Pidpa). The strain was made ampicillin resistant by serial selections on Luria Broth (LB) agar (Carl Roth, Belgium) with increasing concentrations ampicillin (5 µL/mL to 100 µL/mL) (Merck, Belgium). Each time, the cultures were incubated for 24 hours at 28°C. GFP-labelling was performed using triparental mating. Briefly, the donor strain *Escherichia coli* DH5α pME6012 containing the plasmid ptac-gfp and the helper strain *Escherichia coli* HB101 containing the plasmid pRK2013 needed for conjugation were grown in LB medium at 37°C for 24 hours with 8 µg/mL tetracycline and 50 µg/mL kanamycin, respectively. The acceptor, *Serratia fonticola*, was grown in LB medium with 50 µg/mL ampicillin at 28°C for 24 hours. Next, the cultures were washed twice. They were centrifuged for 5 minutes at 3000 g, the supernatant was removed and 0.22 µm filtered sterile PBS (PBS tablet, Merck, Belgium) was added. Triparental mating was performed by adding10 µL of each culture on LB agar. Plates were incubated at 28°C for 24 hours. GFP-

labelled coliforms were selected for resistance to tetracycline (8 µg/mL) and ampicillin (50 µg/mL) and screened for GFP fluorescence using a dark reader (Clare Chemical Research, USA). The final identification of the pure culture was performed with 16S rRNA gene Sanger sequencing as described in (58) and NCBI BLAST (59).

Two invasion experiments were performed by spiking GFP-labelled *Serratia fonticola* to the corresponding water samples (V = 10 L) in plastic vessels which were connected to the KIWA monitor. Therefore, a few colonies were picked from the agar plate and resuspended in 25 mL of 3 g/L sterile R2A broth medium (Oxoid, England) with tetracycline (8 µg/mL) and ampicillin (50 µg/mL) and the tubes were incubated at 28°C and 100 rpm for 24 hours. Subsequently, the culture was washed using sterile 8.5% NaCl as explained before. Afterwards, the culture was transferred to a diluted liquid medium of 50 mg/L R2A broth medium (Oxoid, England) with tetracycline (8 µg/mL) and ampicillin (50 µg/mL) and incubated at 28°C and 100 rpm for 24 hours. The culture was washed before measuring the TCC with flow cytometry. The culture was diluted using sterile 8.5% NaCl and final spike concentrations were ranging from 50 – 500 cells/100 mL. The concentration of *Serratia fonticola* in the bulk water was determined by filtering (3x 100 mL) on S-Pack filters 0.45 µm (Merck, Belgium) using a filtration unit consisting of six filtration funnels and a Microsart e.jet vacuum pump (Sartorius, Germany) and incubation (18 – 23 h, 37°C) on coliforms chromogenic agar (Carl Roth, Belgium), according to the ISO 9308-1:2014 method for drinking water (55). Water was refreshed after 4 days of recirculation.

Confocal laser scanning microscopy. Visual characterization of the biofilms was done with a Nikon A1R confocal microscope (Nikon Instruments Inc., USA), which consists of 4 lasers with in total 7 laser lines (405, 457, 476, 488, 514, 561, 639 nm). Prior to analysis, samples were fixed in 4% paraformaldehyde (Merck, Belgium) for 24 hours. Fixated biofilm rings were stored at -20°C in a PBS:EtOH 1:1 solution prior to analysis. All samples were

analyzed within 14 days. First, the EPS protein content was stained for 30 minutes with FilmTracer Sypro Ruby Biofilm Matrix Stain (Thermofisher Scientific, Belgium) and the carbohydrates with 25 µL/mL Concanavalin A CF640R (Biotium, USA) for 30 minutes. Nucleic acids were labelled with 3 µM DAPI (Merck, Belgium) for 20 minutes. Staining was performed at room temperature (i.e. ± 22°C) and in the dark, after each staining step a washing step with cold (i.e. ± 6°C), filtered through 0.2 µm, PBS. Finally, the rings were air-dried and Fluoroshield mounting medium (Merck, Belgium) was added to prevent bleaching. A construction was made and Nunc™ Glass Bottom Dishes (Thermofisher Scientific, Belgium) were used to fit the biofilm ring under the CLSM. For each location, 3 biofilm rings (n = 3) were analyzed with confocal microscopy and technical replicates (n = 4) were taken. Image stacks were processed in ImageJ and the plugin Comstat2 was used to determine biomass, roughness and thickness of the biofilms (61). An automatic threshold (Otsu's method) was used for all image processing.

Biomass analysis. HPC, ATP and TCC were determined as described in (16). In brief, The ATP concentration was measured using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Belgium) and luminescence was measured with the Infinite M Plex, multimode microplate reader (Tecan, Switzerland). TCC was measured using an Attune NxT BRXX flow cytometer (ThermoFisher Scientific, USA) and staining was performed with 1 v% of SYBR Green I solution. Biofilm samples were 10 times diluted in 0.2 µm filtered Evian and all samples were measured in technical triplicate.

Molecular analysis of microbial communities. 16S rRNA gene amplicon sequencing was performed on biofilm and bulk samples. Biofilm samples (volume = 15 mL) were filtered using Millipore Express PLUS Membranes (Merck, Belgium) and Polycarbonate syringe filter holder (Sartorius, Germany). MF-Millipore Membrane Filters (Merck, Belgium) and a filtration unit consisting of six filtration funnels and a Microsart e.jet vacuum pump (Sartorius, Germany)

were used to filter bulk water samples. DNA extraction was performed using the DNeasy PowerSoilPro kit (Qiagen, Germany), following the manufacturer's protocol. PCR amplification was performed according to Van Landuyt et al. (62). 10µL genomic DNA extract was send out to LGC genomics GmbH (Berlin, Germany) for library preparation and sequencing on an Illumina Miseq platform with v3 chemistry (Illumina, USA).

Quantitative polymerase chain reaction (qPCR) to detect Serratia fonticola. QPCR assays were performed using a StepOnePlus real-time PCR system (Bio-Rad, Belgium). Specific primers were used to detect the gfp-gen: Forward (5'-AGTGGAGAGGGTGAAGGTGA-3') and Reverse (5'-ACGGGAAAAGCATTGAACAC-3'). Reactions were performed in a volume of 20 µL consisting of 10 µL of 2x iTAQ universial SYBR Green supermix (Bio-Rad Laboratories, USA), 2.0 µL DNA template, 0.8 µL (10 µM) of each primer, and 6.4 µL nuclease-free water. Amplification conditions were outlined according to the manufacturer's instructions. Quantification was done a standard curve based on known concentrations of DNA standard dilutions from 10^7 copies/µL to 10 copies/µL. Reactions were performed in technical triplicates, a negative and positive control was included.

Data analysis and Statistics. Data analysis was done in R (63) in RStudio version 4.2.1 (64). The Flow Cytometry Standard (.fcs) files were imported using the flowCore package (v2.12.2) (65). The background data was removed by manually drawing a gate on the FL1-H (green) and FL3-H (red) fluorescence channels as described in Props et al. (2016). The xlsx package (v4.2.5.2) was used to analyze the data from the confocal microscopy (67). Illumina data was processed using the DADA2 pipeline (v1.28.0) as outlined by https://benjjneb.github.io/dada2/tutorial_1_2.html (68). Taxonomy was assigned using the Silva database v138 (69). Further data analysis was performed using statistical packages such as the phyloseq package (v1.44.0) and the vegan package (v2.6-4) (70, 71). Data visualization

was done using the ggplot2 (v3.4.3) and ggpubr (v0.6.0) packages (72, 73). Statistical analysis was done with the dplyr package (v1.1.2) and the vegan package (v2.6-4) (71, 74).

3 Results

Mature biofilm characterization. A KIWA biofilm monitor was used to grow and sample a drinking water biofilm (Figure A1). The monitor was set up at two different places receiving two different water types: treated groundwater without a disinfection step and treated surface water with a chlorination step. Treated surface water contained more minerals such as aluminum, calcium, nitrate, resulting in a higher hardness, whereas treated groundwater had a higher TOC content and more total colony counts (Table A1).

After 11 and 17 months of biofilm development, the biofilms were characterized by flow cytometry, ATP analysis, confocal laser scanning microscopy (CLSM) and 16S rRNA sequencing (Table 2, Figure 1). The cell densities and ATP concentrations of the groundwater biofilms were 10 times higher than the surface water biofilms. Similar observations were seen using CLSM, where DAPI staining was used to determine the biofilm biomass, roughness and average thickness. Significant statistical differences were observed between the two biofilms (Table 2). In addition, using 16S rRNA sequencing, a significant difference between the two biofilm community compositions was observed (ANOSIM, $p < 0.05$, Figure 1). *Chloroflexi* and *Proteobacteria* were identified as the most dominant phyla in the groundwater biofilm samples, with an abundance of 37% and 35% respectively. *Chloroflexi* were mainly represented by uncultured and unclassified JG30-KF-CM66 (~30%) and S085 (~7%) bacteria, however, only 1.5% of the groundwater bulk bacteria belonged to this phyla. The bulk community was dominated by *Cyanobacteria* (~26%) and *Alphaproteobacteria* (~22%), for example, *Hyphomicrobiaceae* (~3%) and *Hyphomonodaceae* (~3%) (Figure A2). These families were also detected in the surface water biofilm samples (i.e. ~3% and ~11%, respectively) and to a lower extent in the corresponding bulk samples (i.e. ~2% and ~7%, respectively). More than 65% of the bacteria in the biofilms developed under surface water supply were *Alphaproteobacteria*, more specifically *Acetobacteraceae* (~12%), *Beijerinckiaceae* (~8.5%)

and *Sphingomonadaceae* (~ 7%). Furthermore, these bacteria were predominant in the surface water bulk samples (~70%, Figure A2). These results indicate that the raw water sources are mainly influencing the bacterial community traits whereas treatment (e.g. disinfection) is determining the biofilm cell densities.

As extracellular polymeric substances (EPS) are an important part of a biofilm structure, the protein and sugar content were determined as well (Table 2, Figure 2). In accordance with the other measurements, 10 times more EPS biovolume was noticed for the groundwater biofilm over the surface water biofilm. However, the ratio sugars:biomass and proteins:biomass were similar for both mature biofilms (Figure A3). Higher bacterial and EPS content in the groundwater biofilm could be related to the higher carbon content in the raw water and the fact that no disinfectant was used (Table A1). To illustrate that the biofilms used in further experiments were mature, statistical analyses were performed between the two time points, more precisely after 11 and 17 months. Biomass and community composition were not significantly different over time for both biofilms (Table A2).

Effect of operational water quality changes on biofilm detachment. After 17 months of growing a drinking water biofilm, the KIWA monitors were transferred from the water reservoirs to laboratory conditions and the original water quality was adapted to examine the response of the biofilm microbiome towards limited changes that are also observed in practice. More concretely, the effects of minor pH variations and the addition of small concentrations of HOCl on biofilm detachment were investigated. These small pH changes were leading to an increase or decrease in the Langelier Saturation Index (LSI) (Table 1). This qualitative index is used to predict the scale forming potential of water and is based on the measurement of pH, conductivity, alkalinity, calcium ions and temperature (75, 76). Cell counts in the bulk were measured with flow cytometry for 8 hours. In general, little or no detachment of the biofilm was observed compared to the blank (Figure 3). Linear regression with a confidence interval of

95% was performed to quantify cell release rates to the bulk. If the cell release rate is higher than zero, there is an indication that biofilm cells are dispersed into the bulk water phase. Briefly, a detachment was always observed except for two blank conditions. Furthermore, the dispersal of biofilm cells to the bulk water was faster (i.e. higher release rates) when the LSI was changed or when HOCl was added. Therefore, statistics on the residuals and the slopes of the linear regression models were done and no significant difference was observed between the blanks and the applied operational changes (Table A3).

Invasion of coliforms onto drinking water biofilms. *Serratia fonticola*, isolated from the Flemish distribution network, was labelled with a green fluorescent protein (GFP) to distinguish this specific strain from other drinking water biofilm bacteria and spiked (15 – 500 cells/100 mL) to the water fed to the biofilm monitors. Two spike experiments were performed for each water type, and the start concentration and survival of the coliform in the bulk water were followed using selective media. Additionally, qPCR and CLSM were used to determine whether *Serratia fonticola* was attached to the biofilm (Table 3, Figure 4). For treated surface water, the coliforms were still present in the bulk water phase after three hours. After 24 hours, the coliforms were below detection limit (< 0 cells/100 mL) for both water types. Biofilms on the glass rings were analyzed after 4 or 7 days using CLSM and qPCR. For the first spike experiment, the gene copies were below the limit of quantification (LOQ = 28.57 gene copies/cm²), whereas for the second spike experiment, 578.59 ± 38.45 and 358.36 ± 24.03 gene copies/cm² of the invader were measured in the groundwater and surface water biofilms, respectively. Detection of the GFP-labelled *Serratia fonticola* in the biofilm was also done with CLSM and coliforms were detected 7 or 8 days after the spike for each water type. Even though mature biofilms seem strong microbial ecosystems and minor water quality changes do not lead to major detachment into the bulk phase, they are susceptible towards unwanted invasion of coliforms.

4. Discussion

Water sources shape the biofilm community composition while drinking water disinfection determines the biofilm density. Two biofilm monitors consisting of glass rings were used to investigate drinking water biofilms. They were placed at two different drinking water reservoirs, one received treated groundwater without disinfection step whereas the other received treated surface water, with residual free chlorine. Using glass as a carrier material is a reproducible way of sampling biofilms and it only considers the biofilm formation potential. However, as glass does not leach nutrients, the biofilm density is lower compared to for example on plastic coupons (27). In general, bacterial cell densities of biofilms vary from 10^4 – 10^8 cells/cm² (2, 3, 21). We observed similar concentrations, although a lower cell density was observed for biofilms developed under surface water supply probably due to the use of a free chlorine disinfectant (Table 2). Furthermore, our results were in line with previous studies where ATP concentrations of 100 – 1100 pg/cm² and 10 – 100 pg ATP/cm² were measured for unchlorinated and chlorinated water respectively (37, 77). Regarding the results of the CLSM analysis, lower biomass content was observed for treated surface water biofilms but still around 1 µm³/µm² which was early reported in literature (5). In a study from Shen et al. (78) it was shown that biofilm roughness is positively correlated with adhesion and negatively with detachment. Here, a significantly higher biofilm roughness was measured for the surface water biofilm, but this did not result in a higher biomass content probably due to the chlorine treatment. Overall, the biofilm resulting from treated groundwater without chlorination had a 10 times higher FCM cell concentration, biofilm thickness, ATP and biomass content. Higher cell and TOC concentrations in the bulk water and the fact that no disinfection was applied are plausible explanations. Moreover, chlorination does not only lower biofilm density, but also EPS contact points resulting in less EPS production, which was also observed in our study (Figure 2, Table 2) (22).

16S rRNA sequencing showed a significant difference between treated groundwater and surface water biofilms (Figure 1). The groundwater biofilm community was dominated by *Alphaproteobacteria*, *Chloroflexi* and *Actinobacteriota*, whereas the surface water biofilm mainly consists of *Alphaproteobacteria* and *Gemmatimonadota*. Within the *Chloroflexi* phylum, the class JG30-KF-CM66 was most abundant, even though only small concentrations were found in the bulk. The latter is similar to raw groundwater measurements reported in literature (Figure 1, Figure A2) (79). Additionally, the members of the *Chloroflexi* and *Actinobacteriota* clusters are known to degrade complex organic matter structures that could be present in biofilms (80, 81). On the other hand, previous studies confirmed that the biofilm and bulk core microbiome of treated chlorinated surface water mainly consists of *Alphaproteobacteria* such as *Hyphomicrobiaceae* and *Sphingomonadaceae* (18, 25, 52, 82–84). These taxa are both known to easily colonize surfaces, produce EPS and can degrade a wide range of organic carbon compounds (18, 83, 85).

To conclude, treated groundwater and chlorinated treated surface water resulted in a significantly different biofilm regarding cell density as well as community composition. Previous studies already showed that source water is shaping the biofilm community composition, which was also observed in this study (35, 40). Although, other researchers showed that there is no difference in biofilm community regarding the drinking water source, and that treatment (e.g. disinfection) is more important for the biofilm core community in distribution pipes (86).

Mature biofilms react minimally towards operational water quality changes. During drinking water distribution water quality remains mainly constant, however, minor operational changes can happen which can in turn influence water microbiology (87). For example, additional chlorination because of water works, changing quality of raw water sources, mixing different water types in the DWDS, etc. (88, 89). Where previous studies are focused on the

influence of harsh operational changes such as flushing and shock chlorination, the goal of this work was to investigate the influences of these minor variations, that often happen in practice, on biofilm detachment.

Drinking water providers maintain fixed pH values and free chlorine dosage during treatment to control biofilm formation and corrosion of pipe materials, valves, pumps, etc. (15). To evaluate water corrosivity, the LSI, a qualitative index that predict the scale forming potential of water, is determined. The European as well as Belgian drinking water directives report to measure an LSI above -0.5 (14, 90). A slightly negative LSI means more aggressive water and a positive LSI indicates $CaCO_3$ supersaturated water that has the potential to form scale (75). In this study, the LSI of the tested waters was adapted by changing the pH (Table 1). Also, HOCl solution was added to have a free chlorine concentration of 0.20 mg/L and 0.28 mg/L, for the groundwater and surface water samples, respectively. In general, there was detachment towards the bulk water phase as the slopes of the regression models were higher than zero, but no significant biofilm detachment was observed between the blank waters and the adapted waters (Figure 3, Table A3). Besides, our drinking water setup, and distribution systems in general, were quite resilient (35). The pH was recovered after 4 hours and the free chlorine concentration was under the detection limit (< 0.05 mg Cl_2/L) after 2 hours of recirculation (data not shown). Similarly, the research of Trihn et al. (5) observed the role of biofilms in free chlorine decay.

However, the implementation of the results should be handled with care as only the short effect (i.e. 8 hours) of the operational changes was investigated. Continuous pH changes could alter the electrostatic interactions between materials and microorganisms as well as between microorganisms (8, 78). Former studies examined the long-term effect of chlorine on biofilms, and mentioned that small increases in chlorine concentrations could lead to a decrease in culturable biofilm bacteria and EPS production (42, 51). Furthermore, in our study, biofilms

were grown on glass rings, whereas in a full-scale distribution network aged biofilms on iron or plastic piping materials are used, which are more susceptible towards corrosive water. For example, biofilms attached to stainless steel compound pipes are more sensitive towards flushing than those attached to ductile cast iron pipes (54).

Mature biofilms are susceptible towards the invasion of *Serratia fonticola*. Even though mature biofilms seemed resilient ecosystems and were not susceptible to minor operational quality changes, such as pH and chlorine, they were prone to invasion. In this study, we spiked a GFP-labelled coliform, *Serratia fonticola* (i.e. ± 100 cells/100 mL) to the treated groundwater and surface water samples. Water was recirculated over the biofilm monitor for 4 days and *Serratia fonticola* was followed using selective media. Our results showed that the coliform was not able to survive in the bulk waters possibly because of the oligotrophic drinking water environment or competition with the resident drinking water community (87, 91–93). However, after 7 days, the coliform was detected in the biofilm samples (Figure 4, Table 3). Since confocal microscopy is limited (e.g. operator dependent) and bleaching of the fluorescent protein was observed, the results were confirmed using qPCR based on the detection of the fluorescent gene, which was incorporated into the genome of the coliform. During the first spike experiment, the gene copies were below the limit of quantification, but the second spike experiment resulted in detection with qPCR (Table 3). This could be related to the sampling day (after 7 days instead of 4 days), more favorable invasion circumstances or variability over the glass rings in the biofilm monitor. Overall, adhesion and invasion onto a drinking water biofilm depend on nutrient availability, surrounding microorganisms, local hydrodynamics and biofilm roughness (78, 91).

Previous researchers already showed that biofilms could be a reservoir for pathogens (8, 10). Pathogens such as *Pseudomonas aeruginosa* were able to persist in drinking water biofilms after spiking to bulk water samples (12). However, in comparison with the microbial drinking

water legislation, too high spike concentrations were used (i.e. 10^5-10^7 cells/mL) in former studies, as the drinking water guidelines report a limit for *E. coli* and total coliform bacteria of 0/100 mL (12, 13, 15, 91). Here, in this study, we demonstrated that even low concentrations of coliforms (± 100 cells/100 mL) can attach and get established in mature drinking water biofilms. We explicitly chose to use low concentrations as they are relevant for the full-scale practice, where the bigger volumes and flow rates in the DWDS might get contaminated by for example groundwater intrusion after pipe burst, or rain-, river- or wastewater contamination due to wrong connections (94, 95). Furthermore, it is important to notice that *Serratia fonticola* was able to settle in two different biofilms regarding cell density and community composition (Figure 1, Table 2). As biofilms can be disrupted by water stagnation, changes in water flow rates and flushing after water works, coliforms could be released to the water phase, and this could result in water contamination (49, 54, 89). Moreover, detached coliforms could settle elsewhere in the DWDS. Despite, further research about biofilm detachment is needed to understand the occurrence of contaminations in distribution networks.

4 Conclusion

In conclusion, we have used a biofilm monitor consisting of glass rings to study drinking water biofilms. More specifically, we investigated the response of the biofilm microbiome towards limited operational variations in pH and free chlorine concentration, and the ability of the coliform, *Serratia fonticola*, to settle onto these biofilms. Two mature drinking water biofilms were characterized using several techniques and it was shown that they were significantly different from each other regarding cell density and community composition. To summarize, the biofilm resulting from treated groundwater had a 10 times higher cell density, biofilm thickness, ATP and biomass content than biofilm resulting from treated chlorinated surface water. Next, it was observed that the biofilms remained resilient when applying limited changes that are seen in the full-scale drinking water network. Finally, *Serratia fonticola*, spiked at low concentrations to the bulk water samples, demonstrated the ability to attach and get established within the mature biofilms, highlighting the potential for biofilms to act as reservoirs for unwanted microorganisms in drinking water distribution systems.

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Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://github.com/waegenaarfien/2023_Unwanted-coliforms-can-hide-in-mature-drinking-water-biofilms.git, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1015597.

Supplemental material

Supplemental material for this article may be found at ...

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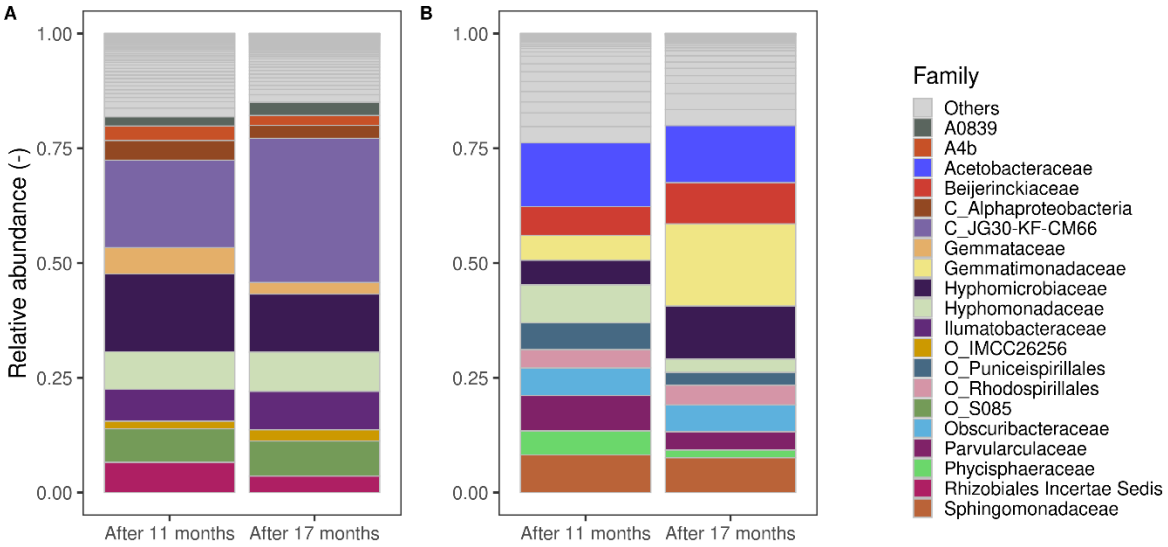

Figure 1: Relative abundances of the 20 most abundant families from the treated groundwater (not disinfected) **(A)** and treated surface water (chlorinated) **(B)** biofilm. Biofilm samples (n = 2) were taken after 11 and 17 months. Significant differences were observed between both biofilms at each timepoint (ANOSIM, $p < 0.05$).

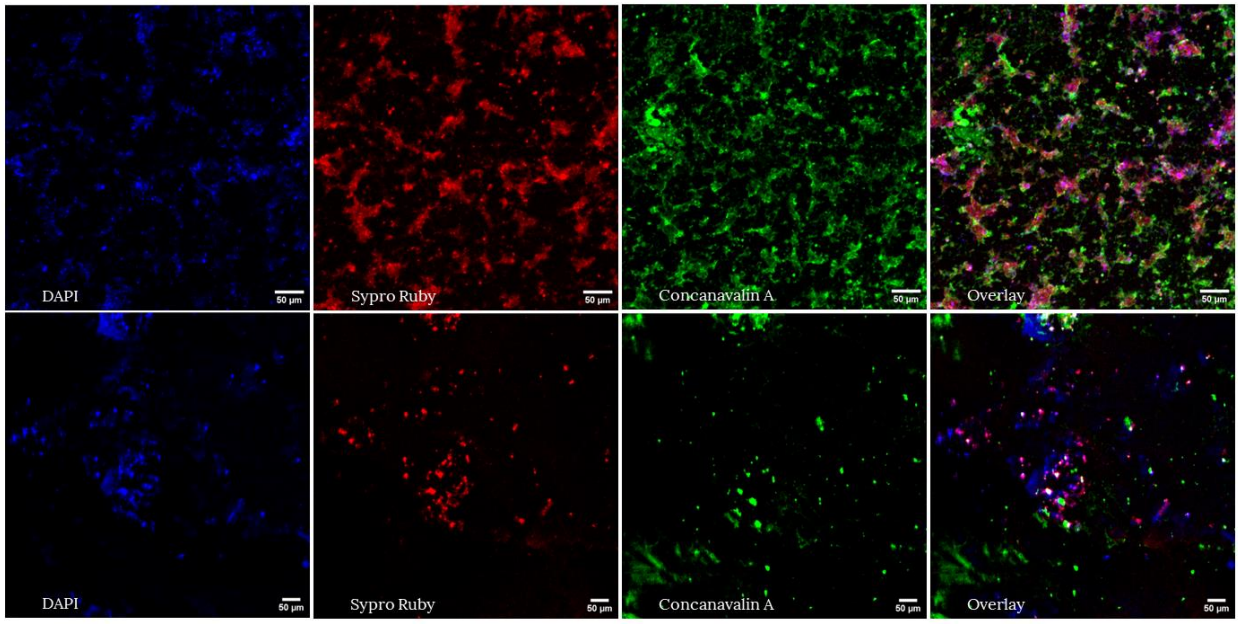

Figure 2: Confocal microscopy images of the treated groundwater (top images) and treated surface water (bottom images) biofilms. Biofilm cells were stained using DAPI and the EPS content, more specifically

the protein and sugar content were stained with Sypro Ruby and Concanavalin A, respectively. Samples were analyzed after 17 months and the images were processed using ImageJ.

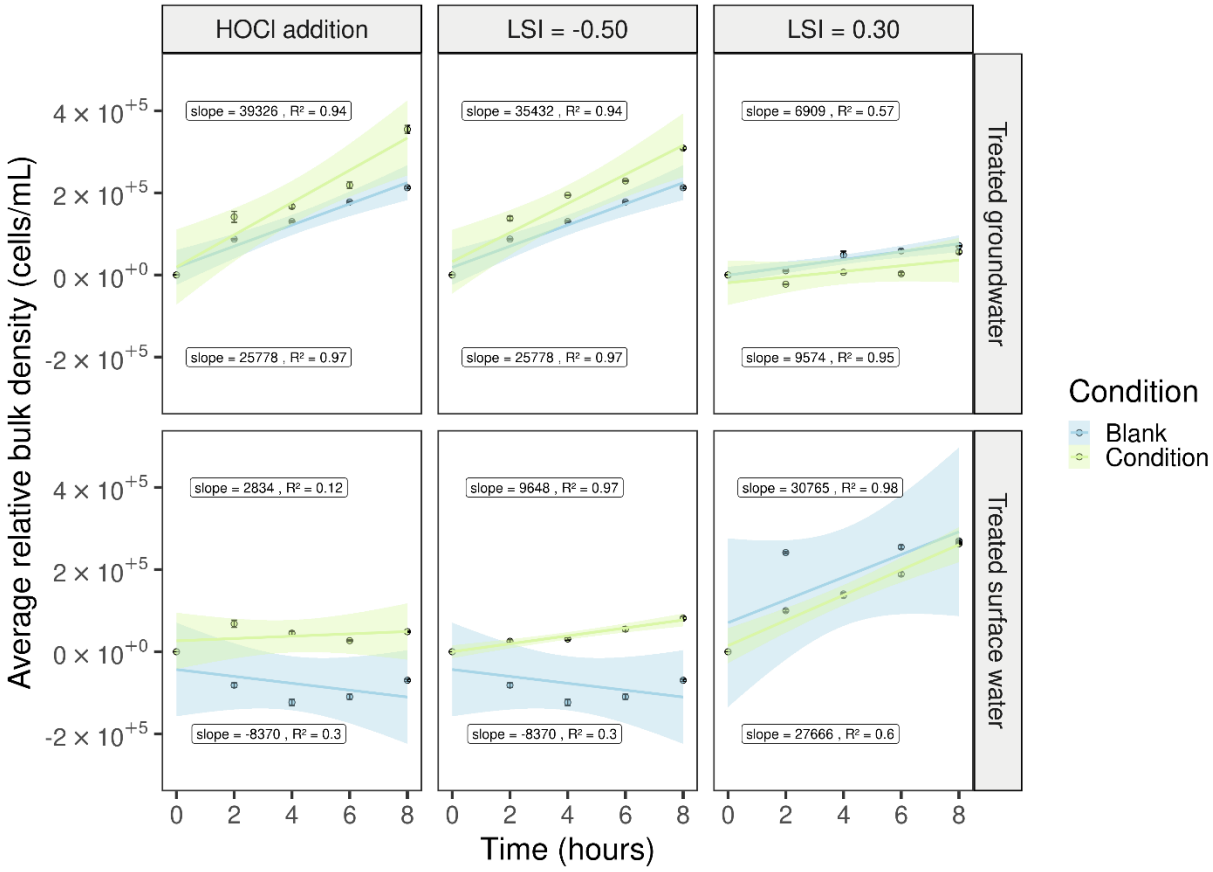

Figure 3: Average cell density of the bulk water phase (n = 3) in function of time (hours) for each water type. The reference detachment profile is indicated with blue, the water cell density after applying different water quality changes (free chlorine, pH) is indicated with green. A linear regression model was calculated (95% confidence interval) and the corresponding slopes and R^2 is shown in a black box.

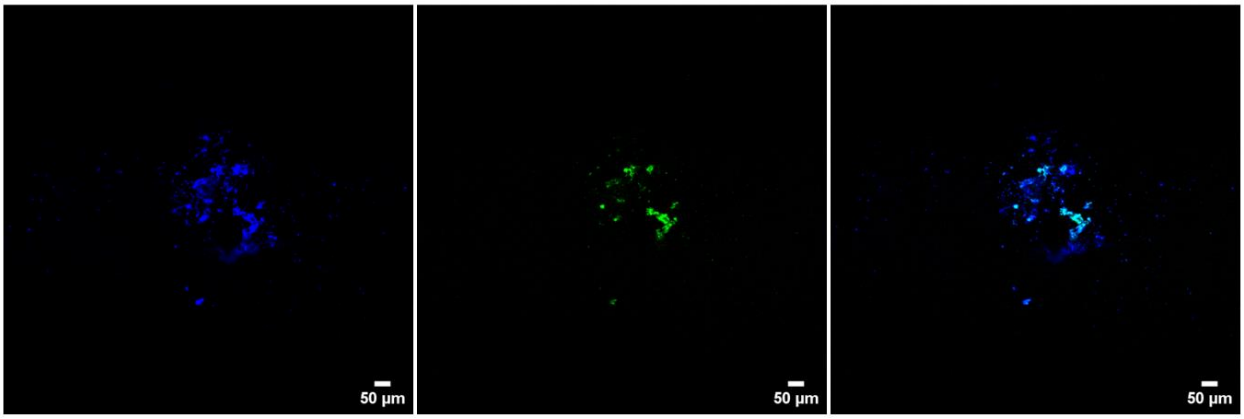

Figure 4: Confocal microscopy images of the invasion experiment with *Serratia fonticola* for the treated surface water biofilm. From left to right: biofilms stained with DAPI, the GFP-labelled coliforms, an overlay image.

Table 1: Original pH and corresponding Langelier Saturation Index (LSI) for each water type. To evaluate biofilm detachment, the LSI was changed by decreasing or increasing the pH using HCl and NaOH, respectively.

Water type	Original pH	Original LSI	pH adapted	LSI	pH adapted	LSI
Treated groundwater (no disinfection)	7.10	-0.04	6.84	-0.52	7.58	0.25
Treated surface water (chlorination)	7.75	0.10	7.30	-0.55	7.90	0.31

Table 2: Characterization of the biofilms using different techniques after 11 and 17 months. Statistics are done using the Wilcoxon rank sum test or the t test indicated with a ''. Biofilms resulting from treated groundwater were more dens and active than biofilms resulting treated chlorinated surface water.*

Technique	**Parameter**	**After 11 months**		**P value**	**After 17 months**		**P value**
	Water type	**Treated groundwater (no disinfection)**	**Treated surface water (chlorinated)**		**Treated groundwater (no disinfection)**	**Treated surface water (chlorinated)**	
Flow cytometry	**Total cell density (cells/cm²)**	$(1{,}01 \pm 0{,}04) \times 10^{7}$	$(5{,}96 \pm 1{,}01) \times 10^{5}$	4.11×10^{-5}	$(7{,}46 \pm 0{,}58) \times 10^{6}$	$(5{,}23 \pm 3{,}68) \times 10^{5}$	4.11×10^{-5}
ATP analysis	**ATP content (pg/cm²)**	411,34 ± 52,58	51,86 ± 3,22	8.23×10^{-5}	896,54 ± 72,11	77,26 ± 41,48	4.04×10^{-4}
CLSM	**Roughness (Ra)**	1,25 ± 0,39	1,97 ± 0,03	0.06	0,78 ± 0,14	1,68 ± 0,04	3.97×10^{-5}
CLSM	**Average thickness (µm)**	15,78 ± 5,89	0,94 ± 0,57	0.06	4,95 ± 2,62	1,31 ± 0,33	7.18×10^{-3}
CLSM	**Biomass (µm³/µm²)**	5,65 ± 2,23	0,57 ± 0,45	0.08*	4,10 ± 3,07	0,25 ± 0,10	1.59×10^{-4}
CLSM EPS	**Protein biovolume (µm³/µm²)**	Not determined	Not determined	/	1.23 ± 1.09	0.13 ± 0.07	3.97×10^{-5}
CLSM EPS	**Sugar biovolume (µm³/µm²)**	Not determined	Not determined	/	2.73 ± 2.54	0.29 ± 0.25	7.94×10^{-5}

Table 3: Serratia fonticola was spiked in the bulk water phase and followed with selective plating (CCA). Invasion of the coliform in the biofilm was determined using CLSM and qPCR. Positive detection on the respective day after the spike (Dx) with CLSM is indicated with "check". It was not possible to perform CLSM for the groundwater biofilms of the second spike experiment because of calcium precipitation on the rings. Using qPCR, the GFP-labelled coliform was found in the biofilm after 7 days (D7).

	Bulk			**Biofilm**	
Technique	**Coliform Chromogenic Agar (CCA)**			**CLSM**	**qPCR**
Water type	Start concentration (cells/100 mL)	After 3 hours (cells/100 mL)	After 24 hours (cells/100 mL)	/	(Gene copies/cm²)
Treated groundwater (no disinfection)	104.0 ± 10.6	0	0	D8: check	D4: under LOQ
Treated groundwater (no disinfection)	15.3 ± 3.0	0	0	D7: not possible	D7: 578.59 ± 38.45
Treated surface water (chlorinated)	650.0 ± 14.1	420.0 ± 17.0	0	D8: check*	D4: under LOQ
Treated surface water (chlorinated)	39.5 ± 5.0	1.0 ± 1.4	0	D7: check	D7: 358.36 ± 24.03