



# Quantification of the bacterial community of drinking water-associated biofilms under different flow velocities and changing chlorination regimes

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## Abstract

Microbial growth in drinking water distribution systems (DWDS) depends on a great number of factors, and its control represents a great challenge for management of these engineering systems. The present case study assessed the influence that a pair of factors—water chlorination and flow velocity—had on the biofilms formed in a model DWDS in 626 days. The culturable bacteria number and biomass of the biofilms developed under the flow velocities of 0.3 m/s, 0.5 m/s, 0.7 m/s and 1 m/s were determined during three consecutively applied regimes of water chlorination to 0.05 mg/l (in 380 days), 0.42 mg/l (in 46 days) and 0.14 mg/l free chlorine (in 200 days). The results demonstrated that biofilm formation was a prolonged process directly depended on flow velocity at drinking water chlorination to 0.05 mg/l. The increase in the water chlorination to 0.42 mg/l chlorine resulted in both the reduction in culturable bacteria number and biomass removal, but the bacteria killing and the biofilm removal were distinct processes. The biocide action of chlorine was faster and more effective than its biomass removal effect. The chlorine decreasing from 0.42 to 0.14 mg/l resulted in increasing the biofilm HPC densities, although the biomass removal process was still continuing. The study carried out contributes for better understanding the biofilm behavior in DWDS and demonstrates that biofilm formation could be managed within a DWDS through operational decisions on parameters that can be changed and controlled as flow velocity and chlorination to safeguard drinking water quality.

**Keywords** Biofilm · Biocide effect · Biomass removal · Chlorination · Drinking water distribution system · Flow velocity · Hydraulics

## Introduction

Drinking water is an oligotrophic environment where most of the microorganisms predominantly live attached to the surfaces in a complex, dynamic microbial assemblage called biofilm (Flemming 1991; LeChevallier et al. 1987). In drinking water distribution systems (DWDS), microbial biofilms comprise about 95% of overall active biomass (Flemming et al. 2002). Biofilms are formed by microbial communities that are embedded in a self-produced matrix of extracellular polymer substances, such as polysaccharides, proteins and extracellular DNA that provides architecture and stability of

the biofilm (Flemming et al. 2016; Lemos et al. 2015). Most of their characteristic features such as social interactions, resource capture and enhanced survival of exposure to antimicrobials rely on the structural and functional properties of the biofilm matrix (Flemming et al. 2016).

The biofilms in the individual DWDSs represent diverse environments for microbial growth. The biofilm communities differed in their species composition depending on a combination of factors such as chemical and biological characteristics of the source water, hydrodynamics and pipe materials (LeChevallier et al. 1987; Hallam et al. 2001; Liu et al. 2017; Revetta et al. 2013; Yu et al. 2010). Drinking water biofilms are mainly formed by autochthonous aquatic microflora, but have potential to harbor opportunistic pathogens that are able to persist or even multiply there (Berry et al. 2006; Lethola et al. 2007; Storey et al. 2004; Torvinen et al. 2007). Hygienically relevant bacteria and parasitic protozoa could attach to preexisting biofilms (Flemming et al. 2002; Torvinen et al. 2007), and soil-inhabiting filamentous

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fungi also do appear as colonizers (Dogget 2000; Douterelo et al. 2016). The recent use of molecular tools and metagenomics approach allow for determining the entire microbial communities and a much more sensitive detection of pathogens in the biofilm. These studies indicated a high diversity within the biofilm communities of DWDSs with the majority of bacteria closely related to *Proteobacteria*, but also *Actinobacteria* and the *Cytophaga–Flavobacterium–Bacteroidetes* group (Martiny et al. 2003; Schmeisser et al. 2003; Revetta et al. 2013; Yu et al. 2010). The biofilm communities in DWDS demonstrated temporal and spatial variations in bacterial richness and diversity, bacterial species succession over time and domination of a few taxa (Douterelo et al. 2014; Schmeisser et al. 2003; Kelly et al. 2014; Martiny et al. 2003). The differences in dominant taxa are driven by the different environmental conditions (Kelly et al. 2014). The microbiome of the DWDSs varies across the phases (planktonic, biofilms and loose deposits) and changes through the stages of water treatment and distribution (Liu et al. 2017; Proctor and Hammes 2015). Biofilm species richness and diversity depended on pipe material (Yu et al. 2010), biofilm age (Martiny et al. 2003), water treatment and disinfection practice (Douterelo et al. 2013, 2014; Revetta et al. 2013; Roeder et al. 2010). In drinking water networks, biofilms may cause a number of hygienic and operational problems at water transport and distribution due to the biofilm destabilization and mobilization (Douterelo et al. 2014; Flemming et al. 2002; Liu et al. 2017). Detachment of single cells and small portions of the biofilm or sloughing of larger pieces may increase bacteria numbers in drinking water (Daly et al. 1998; Moreira et al. 2015; Paul et al. 2012; Stoodley et al. 2001; Wricke et al. 2002). Single cells and small clusters detached more frequently than larger aggregates (Stoodley et al. 2001). In this way, the biofilms can act as a continuous microbial source of water contamination and dissemination of pathogens as well (Lethola et al. 2007; Revetta et al. 2013; Storey et al. 2004).

Many factors have influence on the processes of biofilm accumulation and mobilization, and the water flow velocity is considered as a determinative physical factor by nutrients supply to the biofilm and shear stress exerted on its surface (Flemming 1991; Ohl et al. 2004; Ollos et al. 1998; Paul et al. 2012; Stoodley et al. 1999). The hydrodynamics in pipelines define the transport of microorganisms, oxygen and nutrients from bulk water to the biofilm (Lemos et al. 2015; Manuel et al. 2007; Melo and Vieira 1999; Moreira et al. 2015; Simoes et al. 2006; Stoodley et al. 1999). Besides, the external mass transfer is not uniform throughout the complex biofilm structure: The water channels can carry solutes into and out of the biofilm depths, but do not guarantee penetration to the interior of the cell clusters (Stoodley et al. 1998; Stewart 2003). Hydrodynamics greatly influence the mass transfer mechanisms, but also create stresses

that have a direct action on biofilm structure, deformation and detachment. Several biofilm studies showed that the hydraulic regimes did affect the mechanical stability of biofilms (Liu et al. 2017; Manuel et al. 2007; Melo and Vieira 1999; Moreira et al. 2015). However, their viscoelasticity allows structural deformation when biofilms are exposed to varying shear stress (Stoodley et al. 1999; Paul et al. 2012). Microorganisms respond to higher hydrodynamic shear forces by producing more compact and stronger biofilms, while biofilms become more porous and weaker under low shear forces (Moreira et al. 2015). Melo and Vieira (1999) found that the higher fluid velocities resulted in a smaller amount of attached biomass and lower biofilm thickness, as the thinner biofilms had more compact and stable structure and higher resistance to detachment. In stagnant water conditions, biofilms grew to a less extent than under continuous flow (Manuel et al. 2007). Some studies demonstrated that biofilm accumulation increases with flow velocity (Torvinen et al. 2007; Percival et al. 1999; Simoes et al. 2006), while others found an opposite effect (Melo and Vieira 1999; Tsai 2005).

Several studies assessed the effect of hydrodynamics on bacteriological composition and biological activity of the biofilms (Percival et al. 1999; Simoes et al. 2006; Tsai, 2006; Torvinen et al. 2007). According to Simoes et al. (2006), the higher percentage of culturable cells determined in the turbulent flow-generated biofilms is related to a thinner layer of cells and a more uniform distribution in comparison with the laminar flow-generated biofilms. The hydraulic regimes had insignificant effect on the bacteriological composition of the biofilms at the early stages of their formation (Douterelo et al. 2014), but they had influence on their physical structure and distribution on pipes. It was also observed that water flushing (flow at high shear stresses inside the pipes) might shift bacterial community profile (Douterelo et al. 2013).

Drinking water disinfection and maintaining a disinfectant residual is a conventional approach to control of bacterial growth in DWDSs (LeChevallier et al. 1988; Chen and Stewart 2000; Hallam et al. 2001; Tsai 2006; Wricke et al. 2002). However, it is well known that the attached bacterial cells exhibit greater tolerance to antimicrobial agents compared to the planktonic counterparts (Bridier et al. 2011). Among the oxidative biocides used for drinking water disinfection, chlorine has from 150 to 3000 times greater biocide effect on planktonic bacteria than on sessile cells, while chloramine—from 2 to 150 times higher (LeChevallier et al. 1988). The antimicrobial resistance is intimately related to the three-dimensional structure of biofilms and their heterogeneity. It has a multifactorial nature as a result of accumulation of a number of different acting mechanisms: a limited penetration of biocide into the biofilm matrix; a low bacterial growth rate; acquisition of resistant phenotypes; a higher

bacterial density of the biofilm (Bridier et al. 2011; Flemming et al. 2016; Lemos et al. 2015). The disinfectants may promote persisters and could cause a considerable shift of populations in biofilm community (Roeder et al. 2010). It was found that the diversity of bacterial species and their association in the biofilm community enhance their resistance and functional adaptability (Simões et al. 2009). The multi-species biofilms of bacterial strains isolated from drinking water were much more resistant to inactivation with sodium hypochlorite in comparison with their single-species biofilm species (Simões et al. 2010).

Most of the reported studies on the effect of biocides on drinking water-associated biofilms covered short periods of time, with a few exceptions (Codony et al. 2005; Martiny et al. 2003; Revetta et al. 2013; Roeder et al. 2010), and did not consider the combined effect of hydrodynamics and biocide treatment. Because of that, the main goal of the present study was to assess the influence of hydrodynamics (as different mean flow velocities) and drinking water chlorination (as different chlorine doses) on the culturable bacteria density and biomass of the biofilms developed in a long period of time. The study was carried out in a physical model of DWDS which recreates hydraulics and water environment of real DWDSs to determine the biofilm behavior under the realistic conditions of water distribution network. The study aims to contribute for a better understanding of the dynamic response of drinking water-associated biofilms to two factors that can be intentionally changed in DWDSs—the water flow velocities and disinfection regime, and provide reliable and

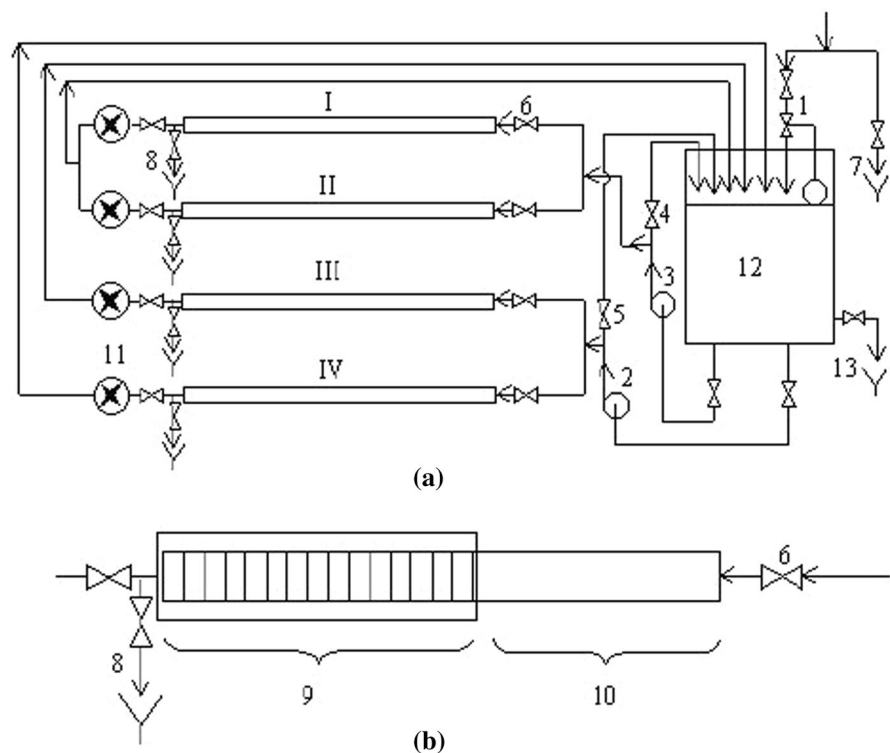
useful information for effective operational management of DWDSs.

## Materials and methods

The study was carried out in a model drinking water distribution system made from polypropylene, a pipe material usually used for construction of domestic installations. The model (Fig. 1) consists of four pipe loops (each 12 m long) working in parallel under the defined mean water flow velocities of 0.3 m/s, 0.5 m/s, 0.7 m/s or 1 m/s maintained by recirculating pumps and valves. The total water volume of the model was 35 L; the feed water flow was 145 ml/min ensuring hydraulic retention time of 4 h.

Each pipe loop included an end part, referred to as a biofilm ‘tube-in-tube’ reactor, designed as an outer pipe tube (outer  $d$  of 32 mm; inner  $d$  of 21.2 mm) containing 30 removable pipe pieces (each one with inner surface of  $13 \text{ cm}^2$ ) that formed an inner tube (outer  $d$  of 20 mm; inner  $d$  of 13.2 mm) with the same diameter as the main pipeline. The model was connected to the laboratory tap and supplied directly with drinking water from the municipal distribution network. The drinking water originated from a surface water source, purified in drinking water treatment plant.

**Fig. 1** Scheme of the model DWDS: **a** the whole model and **b** a pipe loop with biofilm ‘tube-in-tube’ reactor (9). I, II, III and IV—pipe loops; 1—automatic valve for water level; 2, 3—recirculating pumps; 4, 5, 6, 8—valves; 7—sample point for inlet water; 9—biofilm reactor with replaceable pipe pieces; 10—main pipe part of a loop; 11—flowmeter; 12—buffer vessel; 13—valve for regulation of water residence time



## Experiments performed

The experiments carried out were designed to study simultaneously the effects of the selected flow velocities and chlorination doses on the biofilm formation and characteristics. The biofilms were studied under turbulent water flows with the velocities of 0.3 m/s, 0.5 m/s, 0.7 m/s or 1 m/s (and, respectively, the Reynolds number,  $Re$ , of 4320, 7200, 10,080 or 14,400). The Reynolds number used to predict flow pattern is calculated based on hydraulic diameter and characteristics of water fluid (flow velocity; fluid viscosity; and density at the operation temperature).

First, the model distribution system worked for a period of 380 days supplied with drinking water containing  $0.05 \pm 0.01$  mg/l free chlorine. Next, it worked for 46 days at  $0.42 \pm 0.02$  mg/l residual-free chlorine, produced through continuous addition of fresh daily prepared stock solution of sodium hypochlorite in the buffer vessel by peristaltic pump. At last, it worked for 200 days in conditions of  $0.14 \pm 0.001$  mg/l free chlorine. Water temperature was daily measured, and residual chlorine content of the recirculating water was daily analyzed by *o*-tolidine spectrophotometric method.

## Biofilm analyses

Prior to each sampling event, the pipe loops were dewatered and two (or rarely, one) pipe test pieces from each pipe loop were taken for biofilm analysis. During the sampling events, each test coupon was replaced with new sterile one. The outer surface of the test coupon was cleaned and disinfected. Biofilm sample was removed from the inner surface of the test piece through sterile cotton swab into 100 ml physiological solution, and sample was vortex. The biofilm suspension was analyzed for heterotrophic plate count (HPC) bacteria by culture technique in R2A agar (Merck) at 22 °C for 7 d incubation. The test piece weight was measured before and after biofilm removal procedure, and the total wet biomass accumulated on a unit of surface area was calculated.

## Water analyses

Samples from the recirculating water were taken from the buffer vessel prior to each biofilm sampling event. The samples were analyzed for HPC bacteria by pour technique (R2A agar; 7 d; 22 °C). The feeding water samples were analyzed by R2A agar (7 d; 22 °C) and yeast extract agar (Merck)/24 h/36 °C.

## Statistical analyses

The HPC of the biofilm samples was presented as mean values with 95% confidence intervals. The significance *t* test

for comparison between a pair of samples was used. The difference between logarithms of two compared values was considered significant when the experimental *l*<sub>t</sub> value was greater than the *t*-value from *t*-distribution at probability of 0.05 and degree of freedom *n* (Miller and Miller, 1988). The data were analyzed by one-way ANOVA test based on confidence level equal or higher than 95%. The test procedure was used to compare the equivalence between the biofilms behavior at different flow velocities during each particular chlorination regime.

## Results and discussion

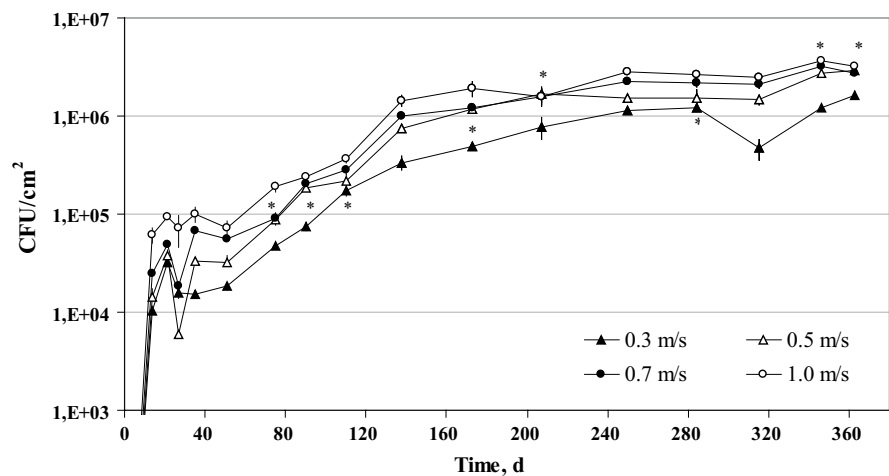
The tap water used to feed the model met all requirements for drinking water quality, including oxidizability of  $1.9 \pm 0.2$  mg O<sub>2</sub>/l, nitrates of  $3.5 \pm 1.8$  mg/l and phosphates of  $0.01 \pm 0.01$  mg/l. The residual chlorine content was  $0.05 \pm 0.01$  mg/l. The HPC value of the feeding water was  $6 \pm 4$  CFU/ml (YEA/24 h/36 °C) or  $580 \pm 70$  CFU/ml (R2A/7 days/22 °C), and no *E. coli* and coliforms were detected.

The temperature of the water recirculating in parallel in the pipe loops of the model was  $21.6 \pm 0.5$  °C. The HPC of the bulk recirculating water was  $14,100 \pm 4470$  CFU/ml during the first chlorination period (at 0.05 mg/l free chlorine),  $1270 \pm 370$  CFU/ml during the second period (at 0.42 mg/l) and  $2480 \pm 80$  CFU/ml during the third period (at 0.14 mg/l).

The biofilm analyses (Fig. 2) found a fast colonization of the pipe test surfaces—the initial HPC density of the biofilms varied from  $10^4$  to  $10^5$  CFU/cm<sup>2</sup> up to the 50th day. This significant difference ( $P < 0.05$ ) between the biofilm samples depending on the flow velocity demonstrated that the water flow could have crucial effect on the initial bacteria attachment to the pipe surfaces. Then, such a difference between the HPC densities of all biofilms was maintained, but the growth rates of the biofilms between the 50th and 175th day were quite similar ( $0.0014$  h<sup>-1</sup>; unpublished data).

In the present study, a steady state of biofilm was assumed when only relatively small changes in the biofilm HPC numbers were measured in consecutive samples. As pointed out by Boe-Hansen et al. (2002a, b), true biofilm stationary state is never achieved, since selection is continually occurring and slight changes in environmental conditions may favor growth of different microorganisms. The obtained data show that formation of steady-state biofilms in drinking water was a prolonged process. The biofilms reached a steady state after 250 days (but 207 d at 0.5 m/s), and these results corresponded with the data of Boe-Hansen et al. (2002a) for biofilm quasi-stationary state in low substrate-loaded drinking water observed after approximately 200 days.

**Fig. 2** Dynamics of the biofilm formation (measured as HPC density) under mean flow velocities 0.3 m/s, 0.5 m/s, 0.7 m/s or 1 m/s in drinking water containing  $0.05 \pm 0.01$  mg/l residual chlorine. The culturable bacteria number with 95% confidence intervals are plotted, number of analyses,  $n = 3 \div 6$ ; the asterisk (\*) indicates the insignificant difference between a pair of values compared by *t*-test ( $|t| < t_{0.05,n}$ )



The mean HPC densities of the steady state biofilms were  $3.0 \pm 0.4 \times 10^6$  CFU/cm<sup>2</sup> at 1 m/s,  $2.6 \pm 0.5 \times 10^6$  CFU/cm<sup>2</sup> at 0.7 m/s,  $2.1 \pm 0.7 \times 10^6$  CFU/cm<sup>2</sup> at 0.5 m/s and  $1.2 \pm 0.4 \times 10^6$  CFU/cm<sup>2</sup> at 0.3 m/s. The detected biofilm HPC densities were in the range of values usually reported for drinking water-associated biofilms (Boe-Hansen et al. 2002a, b; Simoes et al. 2006; Ndiongue et al. 2005). The HPC densities of the biofilms were similar to the data obtained by Ndiongue et al. (2005) for the biofilms in non-chlorinated drinking water or to the value of  $2.6 \times 10^6$  CFU/cm<sup>2</sup> reported by Boe-Hansen et al. (2002a) for drinking water biofilm reached quasi-stationary state.

During the entire biofilm formation period, the biofilm HPC density was proportional to the flow velocity: The highest HPC values were detected in the biofilm at the highest velocity of 1 m/s and the lowest ones—at 0.3 m/s. The differences between the biofilm samples under the studied flow velocities were statistically significant at each particular sampling event, with only a few exceptions (marked by \* at Fig. 2). In general, the CFU density of the biofilms was significantly different when comparing between the biofilms developing at each of the tested flow velocities ( $P < 0.05$ ). Our results corresponded with the finding of Percival et al. (1999) for higher viable (and also total) cell counts at the higher flow rates of 0.96 m/s and 1.75 m/s compared to a flow rate of 0.32 m/s. Torvinen et al. (2007) detected 1.5 times higher HPCs in the biofilms at 0.24 m/s than those at 0.1 m/s. A higher percentage of culturable cells in the turbulent flow-generated biofilms than in the laminar ones was detected by Simoes et al. (2006), while the experimental results of Tsai (2005) showed that the increase in the flow velocity from 0.2 to 0.4 m/s did not change the biofilm biomass, but it was significantly affected by further increase to 0.6 m/s.

Despite the different HPC densities of all steady-state biofilms, the observations of their biomass showed a quite similar voluminous and slimy appearance and a uniformly

coverage and distribution on the pipe test surfaces, excepting the smaller biofilm amount at 0.3 m/s. From the 364th to the 380th day, the differences between the biofilm HPC densities at all flow velocities decreased, except at 0.3 m/s. That could be related to an increased impact of the flow shear force for removal of the external biofilm layers and an achieving balance between the shear forces and external nutrient transport effects (Stoodley et al. 2001; Moreira et al. 2015). As it is known, the flow shear starts acting barely after the biofilm thickness exceeded the laminar boundary layer (Flemming 1991). The influence of flow velocity on the biofilm HPC density was altering during the individual stages of the biofilm formation process because of the hydraulic action complexity related to both the diffusion/mass transfer processes potentiating the biofilm growth and the shear stress promoting the biofilm erosion/sloughing. Here, presented data from the 380-day biofilm study in the model DWDS fed with real drinking water support the general understanding that the higher water flow velocities promote biofilm formation and higher biofilm HPC density, and that the biofilm characteristics (such as bacteria viability and biofilm amount) are impacted by the hydrodynamic conditions.

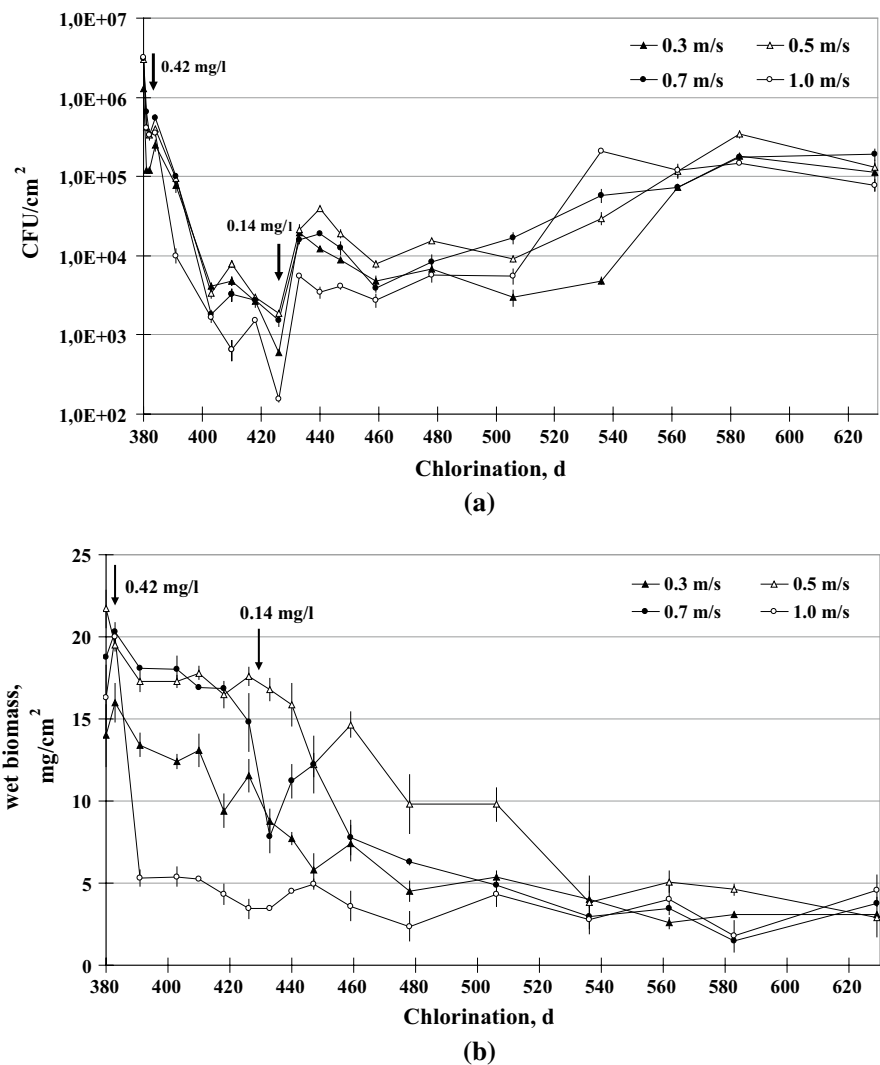
### Effect of the chlorination regimes on the biofilms

The start of drinking water treatment to 0.42 mg/l free chlorine on the 380th day of the biofilm growth caused significant reduction in the biofilm HPC density (Fig. 3a).

At the end of the disinfection mode (on the 426th day), the culturable bacteria number in the biofilms decreased to  $1.5 \pm 0.1 \times 10^2$  CFU/cm<sup>2</sup> (at 1 m/s),  $1.5 \pm 0.3 \times 10^3$  CFU/cm<sup>2</sup> (at 0.7 m/s),  $1.9 \pm 0.1 \times 10^3$  CFU/cm<sup>2</sup> (0.5 m/s) or  $6.0 \pm 0.2 \times 10^2$  CFU/cm<sup>2</sup> (0.3 m/s). The differences between the biofilm samples under the four studied flow velocities were statistically significant at each particular sampling event (with a few exceptions), these demonstrating the impact of hydrodynamics during that water chlorination



**Fig. 3** Dynamics of the biofilms under the flow velocities of 0.3 m/s, 0.5 m/s, 0.7 m/s or 1 m/s during both successive water chlorination regimes to 0.42 mg/l or 0.14 mg/l free chlorine: **a** bacterial densities of the biofilms (number of analyses,  $n = 3 \div 6$ ) and **b** wet biofilm biomass ( $n = 2$ ). Chlorination, d—chlorine exposure time (days) as a part of the total operation of the model; the start of chlorination modes are indicated by arrow



period. However, an opposite hydrodynamic effect was observed in comparison with the effect previously found in the conditions of low chlorine-containing water, due to the chlorine dose. Initially, the biofilm HPC density decreased rapidly and the highest velocity of 1 m/s resulted in the largest HPC reduction. The biofilms at 1 m/s were found to have one order of magnitude lower HPC density in comparison with the biofilms at 0.5 m/s and 0.7 m/s, and the biofilms' response to chlorine treatment at the different flow velocities was significantly different ( $P < 0.05$ ). At the end of that chlorination regime, the bacteria viability in the biofilms at 0.3 m/s decreased at a larger extent than in the biofilms at 0.5 m/s and 0.7 m/s. The lower initial HPC density and biomass amount of the biofilm samples at 0.3 m/s could account for this biofilm behavior.

Despite the significant effect of chlorine on the bacterial viability in the biofilms, no major changes in their biomass amount and surface coverage were visually observed. Only at 1 m/s, the biofilm showed an apparent slough off

behavior and a partly denudation of the pipe surface, a phenomenon probably related to the most intensive chlorine mass transfer within the biofilm. Like biofilm appearance, the accumulated biomass underwent weak changes during this chlorination period (Fig. 3b). Again, only at 1 m/s the biofilm biomass showed a stronger decrease (up to 79% of biomass was removed), while under the rest flow velocities the biomass was removed up to 20% (Table 1). The biofilm biomass reduction at flow velocity of 1 m/s was significant ( $P < 0.05$ ) in comparison with the biofilm behavior at the rest velocities.

Applying the next water chlorination regime to decreased content of 0.14 mg/l free chlorine resulted in recovery of biofilm growth and a fast increase in the biofilm HPC density by one order of magnitude compared to the preceding chlorination mode to 0.42 mg/l (Fig. 3a). Then, excepting at 1 m/s, the HPC density of the biofilms weakly decreased up to 506th day at 0.5 m/s and 0.7 m/s or to 540th day at 0.3 m/s. This phenomenon of decreasing the biofilm HPC

**Table 1** Effects of drinking water chlorination to 0.42 mg/l residual chlorine experienced on the HPC density of the biofilm samples and the biofilm removal under the studied flow conditions

Total operation time, days	Chlorine exposure time, days	Biocide effect (%)				Wet biomass removal (%)			
		0.3 m/s	0.5 m/s	0.7 m/s	1 m/s	0.3 m/s	0.5 m/s	0.7 m/s	1 m/s
380	0	0	0	0	0	0	0	0	0
380.2	0.2	79.13	78.41	76.26	78.67				
382	2	90.75	88.66	89.19	89.64	0	0	0	0
391	11	94.02	96.84	96.71	99.69	4	11	3	68
403	23	99.68	99.89	99.94	99.95	11	11	4	68
410	30	99.63	99.73	99.90	99.98	6	9	10	68
418	38	99.79	99.90	99.91	99.95	33	16	10	74
426	46	99.95	99.94	99.95	99.98	17	10	20	79

density probably was related to the biomass removal process that had started during the preceding water chlorination regime to 0.42 mg/l chlorine and occurred with delay over time compared to the biocidal action (Fig. 3b). After a prolonged time, the biofilms reached a new pseudo-steady state, when their HPC densities were one order of magnitude lower compared to the biofilms formed at the conditions of water chlorination to 0.05 mg/l. These data for a long growth of the biofilms prior to reaching steady state were in contrary to findings for fast biofilm recovery after stopping the chlorination process (Ollos et al. 1998).

The biofilm growth in water containing 0.14 mg/l residual chlorine did not show a clear dependence on the flow velocity, in spite of the significant differences found between the biofilm samples formed under all tested flow velocities at each particular sampling event. The dynamics of the biofilms composition at all flow velocities was insignificantly different ( $P > 0.05$ ), unlike the trend found for the biofilms developed in 0.05 mg/l chlorine-containing water. This outcome could be related to the different HPC density of the biofilms at the start of the third chlorination regime, but mainly to the further action of the preceding chlorination to 0.42 mg/l for continuing of the biomass removal. It is known the oxidizing biocides destabilize the biofilm matrix (Douterelo et al. 2014; Flemming et al. 2016; Simoes et al. 2006) and potentiate biomass removal. However, the applied dose of 0.42 mg/l free chlorine had been low to provide fast and total biofilm removal and for that reason, the processes of biofilm detachment/sloughing were continuing during the next water chlorination mode to 0.14 mg/l free chlorine.

The chemical agents and mechanical forces are often involved simultaneously in the sanitation and removal of biofilms in engineered water systems, since biocides tend to leave biofilm intact when no mechanical treatment is implemented (Simoes et al. 2005; Simões et al. 2009). There are data that the combined effect of chemical and mechanical treatment (with increased shear force) provided increased biofilm removal (Simoes et al. 2005), while the diversity of bacterial species and their association in the biofilm

community enhanced their resistance and fitness (Simões et al. 2009). The higher antimicrobial resistance of the mixed biofilms is associated with an alteration in the biofilm consortium and individual role of the participating bacterial species and may be due to the resistance of one or two key strains; for example, the partnership with *Acinetobacter calcoaceticus* could contribute for increased resistance of the mixed biofilms against chlorine (Simões et al. 2010).

The obtained data of the combined effect of chemical treatment (by different chlorine doses) and hydrodynamics of water flow (at different velocities) showed that the flow regimes had a weaker impact on the biofilm HPC density under the selected test conditions than the chlorine dose: At one and the same chlorination mode, the biofilms formed under all studied flow velocities differed only several times, while between the applied three chlorination regimes they differed from 1 to 3 orders of magnitude. For example, the triple increase in chlorine dose in water (from 0.05 to 0.14 mg/l) at 1.0 m/s resulted in difference between the HPC densities of all steady-state biofilms of one order of magnitude, or the increase in the chlorine dose about 8 times (from 0.05 to 0.42 mg/l) reduced the culturable bacteria number in the biofilms at 1 m/s by three orders of magnitude, while the biofilms at the four flow velocities (that roughly triple vary from 0.3 to 1 m/s) differed up to 2.5 times at the chlorination regime of 0.05 mg/l or up to 13 times at one at 0.42 mg/l.

The present case study quantified the influence that pair of factors—flow velocity and water chlorination, had on culturable bacteria number in drinking water-associated biofilms and demonstrated their combined action as well. The combination of chemical and hydrodynamic stress made the biofilms more vulnerable to chlorine action, and the co-effect experienced on the biofilms increased with an increase in the flow velocity. However, the drinking water chlorination at the range of 0.05–0.42 mg/l free chlorine had stronger influence on the biofilm HPC density than the hydrodynamic conditions. Although the culturable bacteria represent a very small part of the total bacteria number in the drinking water-associated biofilms, the destabilization of biofilm matrix

by the chlorine action and consequent emission of viable bacteria can deteriorate bacteriological quality of drinking water, and as well, cause organoleptic complaints of consumers (Flemming et al. 2016; Douterelo et al. 2014; Wricke et al. 2002). Entering into the drinking water, the culturable bacteria may create serious problems for water consumers' health, especially when some pathogens could survive in the biofilm. Together with the data for increasing viability of the biofilm bacteria during discontinuous water chlorination in a model DWDS and increased effect on the bacteriological water quality (Codony et al. 2005), the present data demonstrate the importance of HPC analyses of the biofilms for the health risk assessment in water consumption.

### Biocide effect versus biofilm removal effect

The water chlorination to 0.42 mg/l free chlorine had low and quite similar initial biocide effect on all biofilms (Table 1), but it was changing over the treatment period. The biofilm at 1 m/s most quickly reached bacterial reduction by 99% (on the 11th day of chlorination), while the same biocide effect was registered quite latter for the rest biofilms. The HPCs of the biofilms at 0.7 m/s and 1.0 m/s reduced by 99.9% for 23 days, but for 38 days at 0.5 m/s or for 46 days at 0.3 m/s. Hence, the time needed for achieving maximum biocide effect on the sessile bacteria was dependent on the flow velocity and relied on the potentially different chlorine transfer in the biofilms. At the same time, this chlorine dose caused rising biomass removal at velocity of 1 m/s—from 68% (on the 11th day) to 79% (at the end of the chlorination), while at the rest flow velocities the biomass removal remained quite weak.

After the shift of water chlorination to 0.14 mg/l free chlorine, the biofilm mass continued decreasing and a significant biomass removal was registered at all velocities (Fig. 3b). As a result, the biofilms still had a patchy appearance on the 506th day of the model operation, as opposed to the thin uniform coverage of the biofilm at 1 m/s (See 'Appendix'). On the 536th day (110 days after starting this chlorination regime), about 80% of total biomass of all biofilms was removed, and afterward no further changes in biomass were registered. The biofilms became fine, smooth and uniformly distributed. All visible changes of the biofilm morphology (surface coverage, distribution, etc.) at each flow velocity coincided with the biofilm detachment and reduction in the attached biomass.

The obtained biomass data confirmed the limited ability of chemical agents for complete removal of biofilm layers (Simoes et al. 2005; Chen and Stewart 2000). In spite of the fact that at least 99.9% inactivation of the biofilm-associated bacteria was detected, the part of the biofilm matrix remained attached and probably could act as an additional source of nutrients and a surface for further cell attachment.

Destroying the biofilm structure through destabilization of biofilm matrix chlorine made biofilms more vulnerable to hydrodynamic stress that was best demonstrated at the highest flow velocity. These results oppose the findings of Simoes et al. (2005) that the biofilms formed under laminar flow were more easily inactivated than the ones formed under turbulent flow.

Comparison between running of the curves of the biofilm HPC density (Fig. 3a) and the wet biofilm mass (Fig. 3b) showed that the biofilm removal effect at water chlorination to 0.42 mg/l free chlorine was manifested later compared to the HPCs reduction. The biocide effect was faster and greater than the biomass removal effect, although the both processes were incomplete. Next, the biomass removal was continuing over the succeeding water chlorination to 0.14 mg/l chlorine, in spite that the biofilm HPC densities were already started increasing.

Our results corresponded with the findings of Chen and Stewart (2000) that the biofilm removal by chemical treatment did not correlate with the reduction in viable cell number in the biofilm. These researchers reported that some chemical treatments of the biofilms could cause significant killing but not much biomass removal, while others caused biomass removal with little killing. However, the effect of the tested chemical agents have been studied on 7–9-day-old biofilms after short 1-hour treatment periods, while the present case study investigated the biofilms behavior in model DWDS under continuous chlorine treatment during a 626-day period. The experimental range of the chlorine doses mimics the conditions in real DWDSs, where the residual chlorine levels of 0.3–0.4 mg/l are considered acceptable to consumers. Our results underscore the fact that biofilm removal and reduction in viable bacteria number in mature drinking water biofilms are distinct processes. Both parameters are important as long as the viable biofilm represents an acute safety problem, and the devitalized but non-removed biofilm may be a source of nutrients and/or cause increase in bacterial adhesion and subsequent formation of a new biofilm (Roeder et al. 2010).

On the base of the discussed results, it could be summarized:

- Formation of steady-state biofilms in drinking water with low-free chlorine content was a prolonged process required 200–250 days. The HPC density of the biofilms grown was directly dependent on the flow velocity.
- Changing the water chlorination regime through increasing the residual chlorine content to 0.42 mg/l resulted in both the reduction in culturable bacteria number and biomass removal, but the bacteria killing and the biofilm removal were distinct processes. The biocide action of chlorine was faster and more effective than its biomass removal effect. The biomass removal occurred with delay



and was incomplete (up to 20% reduction) compared to the bactericidal effect of 99.9%.

- Decreasing the water chlorination from 0.42 to 0.14 mg/l free chlorine resulted in increasing the biofilm HPC densities, although the biomass removal process was still continuing.
- Changing the water chlorination dose in the range of 0.05–0.42 mg/l free chlorine had more significant effect on the HPC density of the biofilms than the water flow velocity in the range of 0.3 m/s–1.0 m/s.

The outcomes evidence that the local conditions in a DWDS, such as water chlorination and hydrodynamics, were shaping the biofilm bacterial composition and amount, and therefore, their managing is of practical importance for the better operation of DWDS to safeguard water quality.

## Conclusions

The complexity of DWDSs themselves makes it difficult to control the microbial growth in water and attached on the surfaces in contact with water. The microbial growth control in DWDSs represents a great challenge because the survival of microorganisms is based on interaction of many variables. The present case study quantifies the effect that water chlorination and flow velocity had on culturable bacteria number and biomass of the biofilms formed in a model DWDS in 626 days. Although the influence of a pair of factors was considered, it contributes to understanding the biofilm behavior at various water flow velocities and changing chlorination regimes and could assist the DWDS management for ensuring safe drinking water to the consumers.

## Appendix



Quantification of biocide effectiveness and biomass removal at different chlorination regimes is important for the management of DWDS from technological and health-related point of view, since the viable cells in drinking water biofilms may represent a water safety problem when entering in water, while the devitalized but unremoved biofilm biomass may favor a further microbial contamination of a DWDS. It was demonstrated that biofilm formation can be managed within DWDS through operational decisions concerning the parameters that can be changed and controlled, such as flow velocities, chlorination and combined effects of hydrodynamics and chlorine dose. The obtained data indicated that changing the chlorination regimes exerts effects of varying degree on the culturable bacteria density and total biomass of the biofilms, and these biofilm changes did not occur simultaneously over the time. A better understanding of the biofilm behavior in DWDSs caused by occasional or intended changes of the disinfection regimes could raise effectiveness of the management practices of the water companies operating the DWDSs for provision of clean and safe drinking water and safeguard human health.

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The biofilm morphology after 33 days of water chlorination to 0.14 mg/l chlorine content (after 460 days of total operation of the model DWDS): 1—at 0.3 m/s, 2—at 0.5 m/s, 3—at 0.7 m/s and 4—at 1 m/s.



The biofilm morphology after 80 days of water chlorination to 0.14 mg/l residual chlorine (on the 506th day of the model operation): 1—at 0.3 m/s, 2—at 0.5 m/s, 3—at 0.7 m/s and 4—at 1 m/s.

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