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The control of biofilm formation by hydrodynamics of purified water in industrial distribution system

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ABSTRACT

Systems for storage and distribution of purified water at ambient temperature are highly susceptible to microbial contamination. The water flow, microbial content and chemical quality of the purified water in an industrial water system have been simulated in a biofilm annular reactor (BAR) to study the impact of different hydrodynamic conditions on biofilm development. Our results reveal the potential of stagnant purified water at total organic compounds (TOC) below 50 ppb to develop biofilm that allows detachment of planktonic bacteria and colonization of new surfaces within 24 h. However, under constant water flow over 7 days, the growth of initial biofilm was 40 times less, fewer bacteria were detached, and new surfaces were colonized to a lesser extent. Heterotrophic plate counts (HPCs) in biofilm were highly positively correlated with numbers of detached planktonic bacteria in effluent water. The study shows that the hydrodynamic conditions and level of planktonic HPC in water are critical for the development of biofilm at very low TOC. The results in the BAR agreed well with those from regular industrial microbial monitoring of purified water. To conclude, the BAR successfully simulates biofilm growth and can be used to establish an effective biofilm control strategy. However, the microbial quality of purified water in industrial system is a constant challenge; any increase of HPC in effluent water is a sign to take steps against excessive microbial growth.

1. Introduction

Purified water is one of the most widely used raw materials in pharmaceutical areas. Deterioration of the microbial quality of water can affect the quality and safety of the products (Hallam et al., 2001; Adley and Saieb, 2005; Simoes et al., 2010). Microorganisms can live and proliferate as individual cells or they can attach to surfaces, where they grow as highly organized multicellular communities or biofilms. Biofilm is the predominant mode of microbial life in nature as well as in persistent chronic infective diseases (Costerton et al., 1999; Davey and OToole, 2000; Estrala et al., 2009; Meng-Ying et al., 2009; Ammons, 2010). Biofilm infections on indwelling devices or implants are difficult to eradicate because of their much better protection against macrophages and antibiotics, compared to free living cells, leading to severe clinical complications often with lethal outcome (Estrala et al., 2009).

The transition of microorganisms from free-swimming cells to a surface-attached community-based lifestyle proceeds via distinct steps, culminating in the formation of a complex structural arrangement of cells: planktonic, attached, structured in microcolony or macrocolony, and detached (Monds and OToole, 2009). Communication between cells via cell–cell signalling is suggested to play a significant role in coordinating cell attachment and detachment (Dunny et al., 2008; Irie and Parsek, 2008).

Biofilm constitutes a protected mode of growth that allows microorganisms to survive, even in hostile environments, their phenotypes, physiology and behaviour being significantly different from their planktonic counterparts (Stewart and Franklin, 2008; Heffernan et al., 2009). Not only do bacteria in biofilm differ from planktonic bacteria of the same species, but also bacteria in mono species biofilm demonstrate vast heterogeneity in terms of metabolism, gene expression and physiology due to the different conditions on different microlocations (Koh et al., 2007; Vlamakis et al., 2008). Much progress has been made in the last decade in elucidating the molecular mechanisms of bacterial adhesion and understanding the structure and composition of biofilm (Karen, 2008; Monds and OToole, 2009).

Biofilm in water systems act as a reservoir of microorganisms – including pathogens, if present in the water (Attman et al., 2009) – that are released sporadically into the water, causing strong increase of cell density. The biological, chemical, and physical factors that drive detachment are complex and incompletely understood (Chambless and Stewart, 2007). Multiple factors are probably associated with attachment and detachment processes,

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depending on the availability of nutrient or oxygen (Chandy and Angles, 2001; Rice et al., 2005), shear-stress (Guillemot et al., 2006; Lee et al., 2008), environmentally controlled exopolysaccharide biosynthesis (Thormann, 2006), an erosive process in which individual cells are lost from the biofilm cell cluster (Bester et al., 2005; Duddu et al., 2009), etc. Recently it has been reported that biofilm structures that reflect changes during its growth, greatly influence the detachment process (Garny et al., 2008; Böl et al., 2008) that is responsible for deterioration of water quality. It is known that cells in biofilm display increased resistance to antimicrobials and environmental stress, causing microbial contamination of water in industrial settings of various processing industries (Hamilton, 2002; Russo et al., 2008; Simoes et al., 2010).

Most studies include disinfection of drinking water, but few are concerned with purified water at ambient temperature (LeChevallier et al., 1996; Sharp et al., 2001; Florjanič and Kristl, 2010; Manuel et al., 2010). Biofilm in systems for storage and distribution of purified water is difficult to detect, inactivate, and remove. In our previous study we reported the impact of two regimens for disinfection of a purified water system with ozone as a function of concentration and time 70 ± 20 ppb in the reservoir in a production regime and 250 ± 20 ppb in the whole system during weekly sanitization. The number of heterotrophic plate counts (HPCs) and the concentration of total organic compounds (TOC) were measured (Florjanič and Krist, 2006). Over four years, 94–98% of water samples exhibited HPC in the category 0–5 CFU/ml, and none in the category $\geq$50 CFU/ml. In spite of increased TOC in the inlet water up to 40 ppb, the microbial counts in purified water in the distribution loop were unaffected. It was emphasized that the critical points regarding microbial contamination of the purified water system are user point valves and the tubes used for transferring water to equipment. The specified ozone level prevented microbial growth and formation of biofilm in the distribution system to an extent that could endanger the water quality and even cause sporadic release of microbes to the water.

Our understanding of biofilm physiology and micro-ecology originates from experiments using *in vitro* biofilm models. Broadly speaking, such models may be used to replicate conditions within the laboratory or to focus on selected variables such as the impact of fluid hydrodynamics, nutrient concentration and antimicrobials on biofilm growth. Today biofilm models, including microtitre plate systems, flow cells, different biofilm fermentors, annular reactor, miniaturized calorimetry, are commonly used (Böl et al., 2008; Duddu et al., 2009; Lee et al., 2008; Karen, 2008; Lerchner et al., 2008; Russo et al., 2008).

Although the use of antimicrobial agents is widespread in biofilm control (Simoes et al., 2010), there are no standardized quantitative methods for selecting antimicrobials or for the design of efficient biofilm control protocols of pharmaceutical waters. Even in current United States and European pharmacopoeias, no biofilm sampling is defined or required for microbial quality determination of purified water. Consequently, the approach to design the system and operating characteristics should be developed for each particular case separately (Russo et al., 2008). With this in mind, we reviewed the experimental approaches used for drinking water (LeChevallier et al., 1996; Sharp et al., 2001; Ndiongue et al., 2005). A relatively new laboratory method for investigating biofilm in drinking water involves the biofilm annular reactor (BAR). No study of biostability of purified water using a BAR has been published.

In this study the impact of water hydrodynamics and residence time on biofilm growth, detachment of bacteria, colonization of new surfaces and dependence on numbers of planktonic and biofilm bacteria initially present in water, have been investigated. The industrial purified water hydrodynamics were simulated in a BAR as closely as possible. Additionally, the results obtained with the BAR were compared with the past 2 year's HPC results of regular microbial monitoring of purified water in an industrial system. The results focus on the impact of water hydrodynamics on biofilm growth and behaviour, and on possible quality assurance strategies.

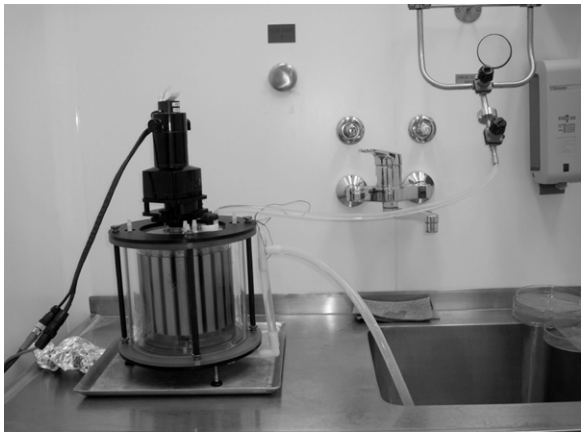

Fig. 1. Biofilm annular reactor (BAR) connected to the industrial purified water system.

2. Materials and methods

2.1. Design and operation of the biofilm annular reactor (BAR)

The BAR, model 1320 (BioSurface Technologies, Bozeman, Montana), consists of two cylinders: a stationary outer made of glass and a metal inner cylinder rotating on its vertical axis at up to 200 rpm (Fig. 1). The inner cylinder is equipped with 20 removable coupons with surface area 15 cm^2, of the same quality as the material of the water system. The BAR has a working volume of approximately 1 L and a high surface area to fluid volume ratio. Before experiments the tubs and the BAR were thoroughly cleaned by laboratory detergent, assembled and sterilized by moist heat at 121 °C for 1 h. The stainless steel coupons were sterilized by dry heat at 300 °C for 2 h and installed in the reactor aseptically. The BAR was connected by a tube directly to the industrial purified water distribution system. Sanitization of an industrial purified water storage and distribution system was performed with ozone in 7 days periods (Florjanič and Krist, 2006).

Purified water enters through the inlet opening on the top and, after moving in the region between both cylinders, leaves the equipment through the outlet at the bottom. While passing between cylinders the cells can attach and form biofilm on the sterilized coupons. In operation, the flow rate of the inlet water was 10 or 120 ml/min, which enable residence times less than 2 h (dilution rate of <0.5/h). During experiments samples of influent and effluent water and as well as the biofilm on coupons were monitored at known TOC, conductivity, temperature and pH of water.

2.2. Impact of hydrodynamic conditions on biofilm growth in BAR

The impact of four different incubation times and two different operation regimes on biofilm growth in BAR was investigated. Each experiment consists of a period in which water is stationary in the BAR (incubation time) and a period in which the water flows through the BAR and the inner cylinder is rotating (operating regimes) (Table 1). Incubation times in EXP1, EXP2, and EXP3a were 96, 72 and 24 h followed by operation regime 1 for 7 or 14 days. In EXP 4, water flowed constantly through the BAR in operation regime 2 without prior incubation. The indicators of biofilm growth were: density of HPC on the surface of coupon (CFU/coupon)

Table 1
Operating parameters in the BAR for studying the impact of different hydrodynamic conditions on biofilm growth and detachment. The temperature of the water in the BAR during study was 22 ± 1 °C, the mean TOC was 35 ppb (range 29–42 ppb) and conductivity less than 1.3 μS/cm at 25 °C.

Experiment	Incubation		BAR operating regimes	Duration of the study
	Time	BAR stopped		
EXP1	96 h	Yes	*Regime 1* • In operation: 8 h • BAR stopped: 16 h • Inlet water flow: 10 ml/min • Residence time: 100 min • Rotation: 200 rpm	7 days
EXP2	72 h	Yes	*Regime 1* (as defined above)	7 days
EXP3a	24 h	Yes	*Regime 1* (as defined above)	14 days
EXP3b	24 h	Yes	*Regime 1* (as defined above) Primary coupons were replaced	14 days
EXP4	0 h	No	*Regime 2* • In operation: 24 h • Inlet water flow: 120 ml/min • Residence time: 8.3 min • Rotation: 40 rpm	14 days

and differences in numbers of HPC between the effluent and influent water ($\Delta HPC = HPC_{eff} - HPC_{inf}$, CFU/100 ml). HPC in inlet water was always below 5 CFU/100 ml.

2.3. Colonization of new surfaces by existing biofilm

Each working day, after 24 h of incubation, two primary coupons were removed from the BAR and replaced immediately by sterile secondary ones (EXP3b) to study colonization ability of the new surfaces from existing biofilm (Table 1).

2.4. Microbial sampling and methods

Samples of influent and effluent purified water from the BAR were collected in a sterile flask. Total viable counts were determined by water filtration through a 0.45 μm membrane filter. The volume of filtered water depended on the bioburden of the investigated water: 100 ml for influent water with low microbial counts and 1–100 ml for effluent water with higher microbial counts. The membrane with total viable counts was incubated on R2A-agar (Oxoid, Basingstoke, Hampshire, England) for 5 days at 30–35 °C. A medium control plate was always prepared to eliminate error due to medium contamination.

At the sampling time, coupons were removed aseptically from the BAR. Biofilm was scraped with a sterilized scraper from the surface into a sterile baker. The coupons were rinsed twice with 100 ml of sterile peptone solution and homogenized by shaking. Total viable counts were determined by membrane filtration as described above. Colonies were counted and expressed as CFU per coupon or per 100 ml.

2.5. Sampling for chemical analysis

TOC and conductivity measurements were performed in compliance with the current U.S. and EU pharmacopoeias. TOC was measured on line with an Anatel monitor (Anatel corporation, Loveland, USA), calibrated once a year by the manufacturer. Conductivity and temperature were measured on line with an Endress + Hauser conductivity sensor (Reinach, Switzerland). The system was calibrated according to the requirements of the current U.S. and EU pharmacopoeias.

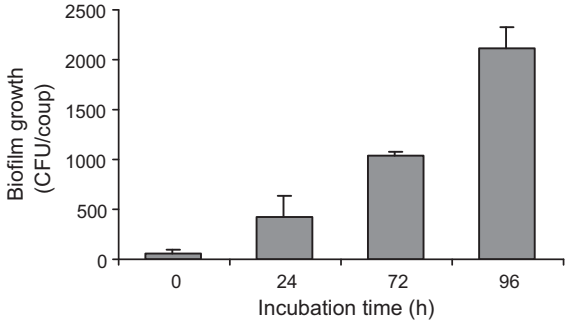

Fig. 2. Comparison of biofilm growth on coupons in 7 days after different incubation times: 0 h in EXP4, 24 h in EXP1, 72 h in EXP2, and 96 h in EXP3a. The means ± SD for at least three replicates are shown.

2.6. Statistical data analyses

Statistical data analyses were performed by SPSS version 17 (SPSS Inc, Chicago, IL, USA). Descriptive statistics were calculated and results are reported as means and standard deviations (SD). Correlation between HPC numbers in water and in biofilm was also assessed. Paired samples *t*-test was used for comparison of mean values between the two dependent samples. ANOVA with post hoc Bonferroni test was used for comparison of multiple groups data. The significance level was set at $p < 0.05$.

3. Results

The effect of incubation time variations combined with the action of hydrodynamic conditions on biofilm development has been investigated. Purified water contained on average 10-fold less TOC than allowed in pharmacopoeias to hinder growth of biofilm by an indigenous bacterial population. Using constant or variable water flow in BAR, results of 5 groups of laboratory experiments are presented.

3.1. Impact of incubation time on the amount of biofilm in BAR

Biofilm growth was measured at different incubation times (Fig. 2, Table 1). After 7 days less biofilm was formed in experiment EXP3a with the shortest incubation time (420 ± 210 CFU/15 cm^2) than in EXP2 (1040 ± 40 CFU/15 cm^2) and EXP1 (2123 ± 210 CFU/15 cm^2) (ANOVA, $p < 0.05$). However, in EXP 4 without incubation with constant water flow, even less biofilm (55 ± 40.5 CFU/15 cm^2) was formed than in experiment EXP3a. These results indicate that the incubation time influences the population of the biofilm. If biofilm is developed it affects the microbial quality of water for a long time.

3.2. Impact of hydrodynamic conditions on biofilm growth in BAR

The impact of different hydrodynamic conditions on the rate of biofilm growth in the BAR was studied in EXP3a (24 h of water stagnation was followed by regime 1 as defined in Table 1) and EXP4 (constant water flow). The development of biofilm and of HPC in influent and effluent water was monitored every working day for 14 days. In EXP3a, in the first 24 h of incubation, the planktonic bacteria multiplied from 10 to 4900 CFU/100 ml in effluent water (Fig. 3). In parallel, biofilm was formed at the coupon surface (27 CFU/15 cm^2). In the next 24 h the number of planktonic bacteria returned to 10 again and an increase of cells in biofilm was

Table 2
HPC in influent and effluent water (CFU/100 ml) and in biofilm (CFU/15 cm^2) at different hydrodynamic conditions. Indicated values are means ± SD, $n = 3$.

Days	EXP3a			EXP4			EXP3b
	Influent water in BAR_{inf}	Effluent water in BAR_{eff}	Biofilm on coupon means ± SD	Influent water in BAR_{inf}	Effluent water in BAR_{eff}	Biofilm on coupon means ± SD	Biofilm on secondary coupon means ± SD
0	0	10	0	0	0	5 ± 5	–
1	0	4900	27 ± 17	1	0	1 ± 1	1620 ± 1620
2	0	10	55 ± 15	0	0	5 ± 5	1215 ± 135
3	1	40	234 ± 167	0	0	5 ± 5	–
4	–*	–	–	–	–	–	–
5	–	–	–	–	–	–	11700 ± 900
6	0	500	2505 ± 435	0	10	1 ± 0	10350 ± 450
7	0	1080	420 ± 210	0	31	55 ± 2	12150 ± 1350
8	0	1510	700 ± 380	–	–	–	9965 ± 835
9	4	2940	5150 ± 1150	9	30	1 ± 0	5745 ± 2355
10	13	1760	984 ± 645	0	30	525 ± 25	–
11	–	–	–	–	–	–	–
12	–	–	–	–	–	–	6500 ± 2500
13	1	1710	2465 ± 55	3	210	330 ± 170	3060 ± 1440
14	0	8100	8620 ± 520	1	200	340 ± 60	4305 ± 195

* – means non working day.

revealed (Table 2, Fig. 3). Additionally, from the 1st to the 14th day, HPC in effluent water was significantly higher than in influent water (Table 2). EXP3a shows similar trends of increasing numbers of HPC in biofilm and in effluent water from 2nd to the 14th day of the study (Fig. 3). Furthermore, after 14 days, less biofilm and lower numbers of heterotrophic bacteria in effluent water were detected in experiment EXP4 (340 ± 60 CFU/15 cm^2; 200 CFU/100 ml) than in EXP3a (8620 ± 520 CFU/15 cm^2; 8100 CFU/100 ml) (Figs. 4 and 5). Biofilm growth in EXP4 was smaller than in EXP3a (Fig. 4). Significantly less biofilm was formed on coupons with faster than with slower water flow. Visual observations at the biofilm sampling have established that faster water flow leads to the formation of a thinner, smooth and dense biofilm structure, which is mechanically more stable. Hydrodynamic conditions in the BAR have practical implications for predicting the content of developed biofilm.

3.3. Correlation between HPC in biofilm and in effluent water

One of the objectives of this study was to investigate how the number of microorganisms in biofilm influences the number of planktonic cells in water. It is expected that any increase in the number of bacteria in effluent water of the reactor compared to the influent is predominantly the result of detached biofilm bacteria. Therefore, samples of effluent water and biofilm were taken at the same time and total viable count determined in each. Significantly fewer detached bacteria were observed in effluent water in BAR with a thinner biofilm (Fig. 5). Almost all the biofilm bacteria remained adhered in EXP4. Conversely, the larger amount of HPC in biofilm in EXP3a enables larger number of detached bacteria in the effluent water (paired samples t-test, $p < 0.05$). In experiments EXP3a and EXP4 a highly positive correlation between the

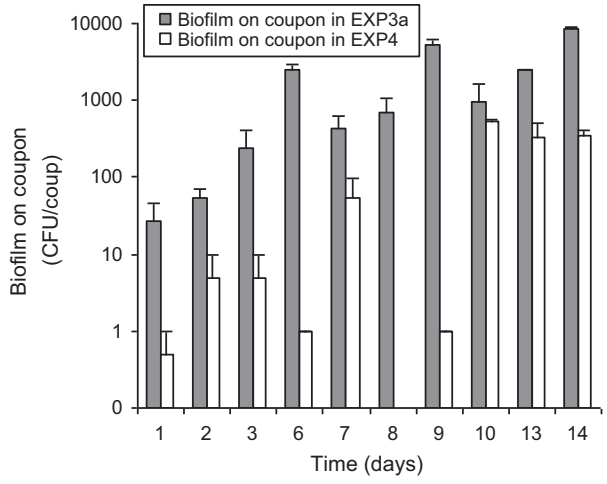

Fig. 4. Biofilm growth in different hydrodynamic conditions in experiments EXP3a and EXP4 presented as means ± SD, $n = 3$. In 4th and 5th day as well as 11th and 12th sampling was not performed.

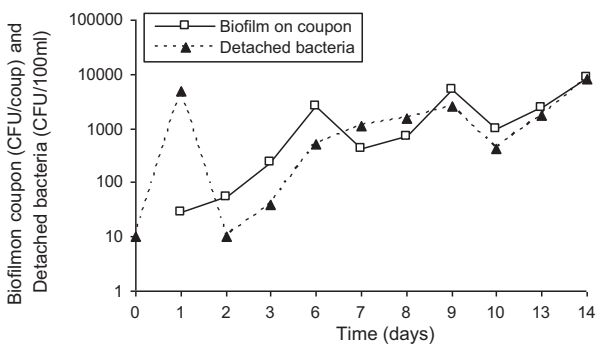

Fig. 3. Number of bacteria in biofilm at coupons and number of bacteria in effluent water, mostly detached from biofilm in EXP3a experiments. Results are presented as means ± SD, $n = 3$.

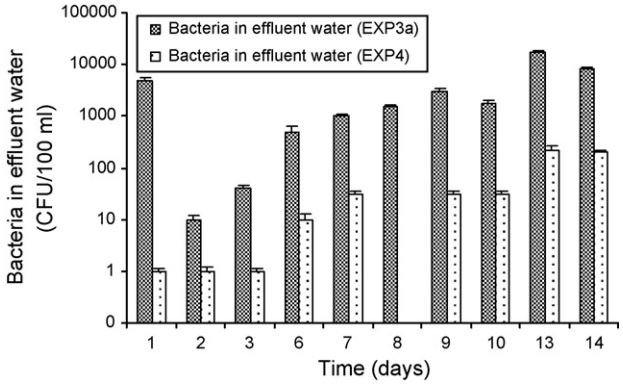

Fig. 5. Number of bacteria in effluent water under different hydrodynamic conditions in experiments EXP3a and EXP4. Indicated values are means ± SD, $n = 3$.

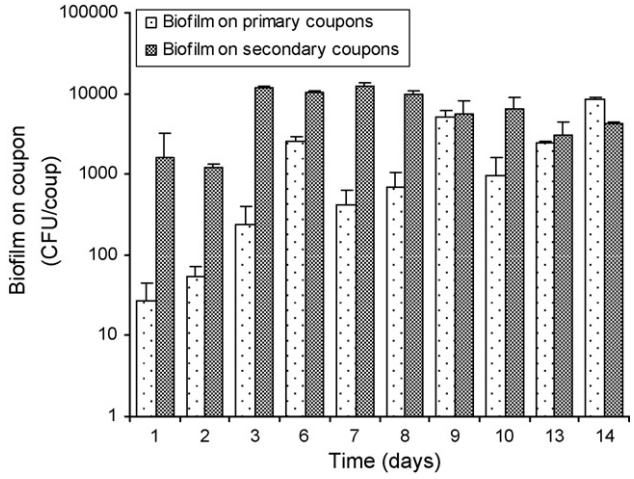

Fig. 6. Biofilm growth on primary and secondary coupons in BAR at equal exposure time in EXP3b.

number of planktonic cells (HPC of effluent water–HPC of influent water; CFU/100 ml) and the cell number in biofilm (CFU/15 cm^2) was determined. A stronger correlation was established in experiment EXP4 (R^2 = 0.99) than in EXP3a (R^2 = 0.84).

3.4. Colonization of new surfaces by existing biofilm

Colonization of new surfaces was studied in EXP3b. Parameters of the study are shown in Table 1. Each working day two coupons with primary biofilm (primary coupons) were removed and replaced with sterile ones (secondary coupons). Bacteria were found to occupy free surfaces very quickly. The biofilm developed on secondary coupons after exposure for 24 h in BAR exhibited 1620 ± 126 CFU/15 cm^2. The amount of biofilm in the same growth time is initially higher on secondary coupons and, approximately after one week, very similar on the two coupons (Fig. 6). The development of thicker biofilm on the secondary than on the primary coupon can be attributed to better conditions for production in the early stage of biofilm formation on new surfaces and to increased ability of detached cells from primary coupons to form biofilm. Thus, decrease in response over longer times may be explained by the fact that the biofilm community dynamics alters. Adhered cells at new, free surfaces diverge from the initial composition during early biofilm growth to converge towards the same composition as the matured biofilm. It is expected that the division of cells in a similar microenvironment is similar. Therefore, the effects of new surfaces and the growth rate of secondary biofilm seemed to be reduced with time.

3.5. Comparison of laboratory results with those in a real purified water system

The experimental results in EXP4 in the BAR were compared to the mean HPC collected during regular microbial monitoring of purified water in an industrial storage and distribution system in the past 2 years. A risk analysis of the purified water system was performed, using laboratory results from EXP4 and taking into consideration the length and diameter of the distribution piping where the biofilm is distributed. We assumed that in the ozonized storage tank no biofilm is present.

The amount of biofilm that developed after 7 days (between two sanitizations) in experiment EXP4 was 55 CFU/15 cm^2, corresponding to 3.7 CFU/cm^2. If all the surface of the distribution system is covered uniformly with biofilm to the extent determined in EXP4, and if the biofilm was detached and distributed at once in the total water volume, the HPC level in water would be 18 CFU/100 ml. The geometric mean of the results collected over the past two years, representing the HPC for our purified water storage and distribution system, was 7 CFU/100 ml (0.07 CFU/ml) or less. The results show satisfactory biofilm simulation in a BAR under working conditions. Since the worst case calculation was used to predict the higher HPC level in an industrial storage and distribution system we can conclude that the calculated number of bacteria (18 CFU/100 ml) is in good agreement with the actual HPC numbers in water of our industrial system.

4. Discussion

Microbial growth needs to be controlled in many microbiologically sensitive environments in which conditions are favourable for their proliferation and biofilm formation. Biofilm control methods must take into account the knowledge of the constitutive microflora and their responsive behaviour. The synergistic effect of ozone treatment and water hydrodynamics for maintaining very low numbers of bacteria in purified water system will be discussed.

The biofilm observed in a BAR environment simulating an industrial purified water storage and distribution system demonstrated that purified water with TOC even below 50 ppb is able to support biofilm growth. The HPC and TOC values in the studied inlet water samples were at least 10-fold lower than are allowed in United States and European pharmacopoeias. Within 24 h, an indigenous planktonic bacterial population formed biofilm, which allows detachment of bacteria and colonization of new surfaces. Even more biofilm was formed at longer incubation times. This is the reason why it is important to follow biofilm growth in the same water and under identical biotic and abiotic conditions. Incubation time affects the amount of developed biofilm in the BAR, starting at a very low cell density in inlet water and under nutrient-poor conditions. The CFU in biofilm increased by approximately 5-fold, from 420 to 2123 CFU/15 cm^2, as the incubation time was prolonged from 24 to 96 h (EXP1, EXP2 and EXP3a).

Water flow has a significant impact on biofilm growth and colonization in the low nutrient environment of a purified water system (Duddu et al., 2009). In a 7 day study, the amount of HPC observed in biofilm in stagnant and flowing water was time dependent. Surprisingly, almost 40-times less biofilm was formed in constantly flowing water at higher velocity and lower residence time than in experiments with a longer period of stagnant water, slower water flow and longer residence time (Fig. 2). A similar trend was obtained by Manuel and co-workers, who reported that increased stagnation of drinking water in the system promoted biofilm accumulation (Manuel et al., 2010).

The 14 day study of biofilm development under two hydrodynamic regimes revealed that biofilm is detected after 24 h in EXP 3a and scarcely at all after 7 days in EXP4 (Table 2). The trend of increasing difference between HPC in effluent water and in biofilm was ascertained through the whole investigated period for both experiment groups. Even so, HPC was constantly higher in effluent than in influent water. Due to slower water flow in EXP3a, allowing a retention time in the BAR of approximately 2 h, at least 25 times more biofilm was developed than in EXP4 with faster water flow and shorter residence time. It is suggested that faster water flow through BAR and consequently lower residence time delayed the initial growth, resulting in lower amounts of biofilm (Fig. 4). These experiments lead to the conclusion that less biofilm is formed in more dynamic, constantly flowing water with shortest residence times than in different combinations of stagnant and flowing water.

In contrast, it has been reported that higher HPC numbers are observed in biofilm at higher flow of drinking water. The findings were explained by higher transport of nutrients, planktonic cells

and oxygen that accelerated microorganism proliferation (Gillis and Gillis, 1996; Chandy and Angles, 2001; Simoes et al., 2007; Manuel et al., 2010). This apparent contradiction with our results could be explained by the, at least hundred-fold, lower level of organic carbon in purified water than in drinking water. Critical concentrations of organic nutrients have to be available to support biofilm growth, otherwise the starved cells detach from the biofilm (Stoodley et al., 2001; Ndiongue et al., 2005). The critical nutrient concentration in flowing water could be achieved by increased residence time due to decreased water flow at a given TOC, or by increasing TOC at a given water flow. Our results suggest that a TOC of approximately 40 ppb is close to the critical concentration of nutrients in constantly flowing water, as in EXP4. Due to the short residence time in the BAR, the majority of nutrients were continuously flushed out of the system and only a fraction, adhered to the surfaces, is available. By decreasing water flow or even stopping it for several hours, as in EXP1, EXP2 and EXP3a, the water remained longer in BAR. Therefore, fewer nutrients were flushed out and a larger fraction is available to support biofilm growth.

The explanation for the different HPCs in effluent water shown in Fig. 5 can be sought in the development of biofilm. The attachment of microorganisms to surfaces and subsequent biofilm development includes complex processes that are affected by several variables (Garny et al., 2008; Simoes et al., 2010). The driving force in biofilm development is self-organization and cooperation between cells, in which cell–cell signalling has been demonstrated to play a role in their attachment and detachment. High cell density results in a high concentration of signals inducing detachment of cells from biofilm. In our experiments, more cells were released from biofilm in water in EXP3a samples, where the CFU is higher.

Additionally, we have proved in EXP3b that biofilm serves as a source of bacteria that continuously colonizes new surfaces. Even high rotation of the purified water at 200 rpm in the BAR did not prevent colonization of new surfaces and formation of biofilm. The results show that, in general, more biofilm was formed per day on the secondary than on primary coupons (Fig. 6). It is expected that bacteria in biofilm with poor growth conditions look for better ones. The findings may be explained by the preference of detached cells for unoccupied surfaces, where they can form colonies enabling biofilm formation.

The larger amount of biofilm on secondary coupons may also be explained in terms of the development of genetic variants in primary biofilm with enhanced biofilm formation capability. It is generally accepted that the metabolic activity of bacteria within biofilm results in heterogeneities in the chemical and physical parameters of the biofilm interstitial fluid. Chemical gradients of nutrients, waste products and signalling compounds develop, and bacteria within biofilm respond to these local environmental conditions by increased frequency of genetic variations. Variants differ in several characteristics, such as attachment, swarming motility and biofilm formation, that are important for surface colonization. Thus, biofilm may form dispersal cells that differ from the parents' strain. They can have enhanced capability for adaptation to the microenvironment and increased capability for biofilm formation (Koh et al., 2007; Stewart and Franklin, 2008). These facts support our finding that, at higher HPC on coupons representing biofilm, the number of planktonic microorganisms in water is higher.

To minimize colonization it is therefore important to diminish continuously the numbers of planktonic cells in water. This is possible by recirculation of water back to a reservoir where it is continuously disinfected by ozone or heat. Our separate laboratory study revealed that biofilm bacteria *Stenotrophomonas malthophilia*, which represent the majority of biofilm cells in our purified water system, are more than 533 times more resistant to ozone than planktonic cells. Ozone at 70 ppb killed 99.98% of planktonic cells; however, 450 ppb of ozone for 120 min had almost no impact on biofilm cells. After ozonization, secondary biofilm formed even faster than primary, due to the presence of dead microorganisms that constitute nutrient for surviving cells (Florjanič and Kristl, 2010).

The increased HPC in biofilm showed a high positive correlation with HPC in effluent water (experiments EXP3a and EXP4). Higher flow rate decreases residence time from 100 min in EXP3a to 8.3 min in EXP4, together with continuous water flow, simulated better the actual hydrodynamics in our industrial purified water system. Continuous erosion of the individual cells or the smaller portion of biofilm in effluent water could explain the positive correlation observed between HPC in biofilm and effluent water. The correlation was also reported for a drinking water system. The numbers of cells detached from biofilm in the effluent water could therefore serve as an indicator of the biofilm growth rate (Bester et al., 2005). It has been reported that spontaneous detachment of cells from bacterial biofilm can be divided into two process, erosion (detachment of individual cells or smaller fragments of biofilm) and sloughing (rapid, massive loss of biofilm) (Stoodley et al., 2001).

The positive correlation between HPC numbers in biofilm and in the water phase underlines the importance of maintaining a low HPC in water. The biofilm bacteria are more resistant to antimicrobials than are planktonic bacteria. The excellent microbial results of water in our industrial system could be attributed to a constant flow of water (recirculation) through a reservoir with 70 ppb of ozone that efficiently kills planktonic microorganisms, and to the low content of total organic carbon.

The validity of the laboratory results obtained in a BAR has been confirmed by comparison with the geometric mean of HPC of our industrial storage and distribution system over the last few years. These conclusions confirm that the BAR successfully simulates biofilm growth in the hydrodynamic environment in a purified water system. During the whole period the quality of the purified water was in compliance with European pharmacopoeia.

5. Conclusions

Our laboratory study revealed that biofilm can develop in 24 h from less than 5 CFU/100 ml of planktonic cells in purified water and at a TOC less than 50 ppb. Hydrodynamic conditions greatly influence biofilm development. At a constant flow velocity, enabling short residence time, the development of initial biofilm was delayed, little biofilm was formed, and low numbers of bacteria detached from biofilm into the water. The primary biofilm acts as a constant reservoir of cells that are able to occupy new surfaces very quickly. The positive correlation observed between HPC numbers in the water phase and in biofilm confirmed that the increasing HPC in water is the consequence of biofilm formation. The results obtained in the BAR correlate with the mean HPC level in an industrial purified water system over a period of years, confirming the suitability of the BAR for investigating biofilm development, as well as for the design of control strategies. This approach enables directed action for prevention and control. The model used is sensitive for early detection of biofilm formation. Following the pharmacopoeial water parameters – TOC below 500 ppb and number of microorganisms below 100 CFU/ml – does not provide any guarantee of water quality regarding biofilm dangers.

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