Dynamics of biofilm formation in a model drinking water distribution system

Rasmus Boe-Hansen, Hans-Jørgen Albrechtsen, Erik Arvin and Claus Jørgensen

ABSTRACT

The dynamics of biofilm formation in non-chlorinated groundwater-based drinking water was studied in a model distribution system. The formation of biofilm was closely monitored for a period of 522 days by total bacterial counts (AODC), heterotrophic plate counts (R2A media), and ATP content determinations. The biofilm grew at a rate of 0.030 ± 0.002 day^{-1} reaching quasi-stationary state at 2.6×10^6 cells/cm^2 after approximately 200 days. The low substrate level in the bulk phase (AOC at approximately 6 μg ac-C/l) most likely caused the relatively slow biofilm formation rate observed. During the maturation of the biofilm, the bacterial community changed properties in terms of cell-specific ATP content and culturability of the bacteria.

Key words | biofilm, drinking water, formation, growth, stationary phase

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INTRODUCTION

Bacteria can and will colonise the pipe surfaces of drinking water distribution networks, and the biofilm accounts for a major part of the biomass of such systems (e.g. Keevil *et al.* 1995). A quasi-stationary state is prevalent in the biofilm since most drinking water pipes, from a microbiological perspective, can be considered as fully matured. The true stationary condition is never achieved, since selection is constantly occurring: even a slight change in environmental conditions will favour the growth of different organisms.

Exposing test surfaces to drinking water under controlled conditions allows the dynamics of biofilm formation to be studied. Investigations by Pedersen (1990), Donlan *et al.* (1994) and Van der Kooij *et al.* (1995), focused on the rate of biofilm formation, however none of these investigations established quasi-stationary conditions. It has been shown that composition and morphology change during the maturation of the biofilm. This implies that the properties of the mature biofilm may be very different from the young biofilm (Kalmbach *et al.*, 1997; Sternberg *et al.*, 1999).

The objective of this study was to quantify the rate of biofilm formation and to track changes in biofilm properties during biofilm maturation, under conditions that represent drinking water networks at low nutrient levels and no residual disinfectant. This information will provide us with a better understanding of the dynamics of biofilm formation, which ultimately will help us to construct better models of the water quality changes caused by the microbial growth.

METHODS

The model distribution system was comprised of two identical loops of 50 mm square stainless steel pipes connected in series (Figure 1). Each loop had a total length of 12 m with an active volume of 21 l. The system was continuously fed with drinking water from a municipal distribution network. The water originated mainly from a nearby waterworks where groundwater is treated without use of disinfectants (selected water quality

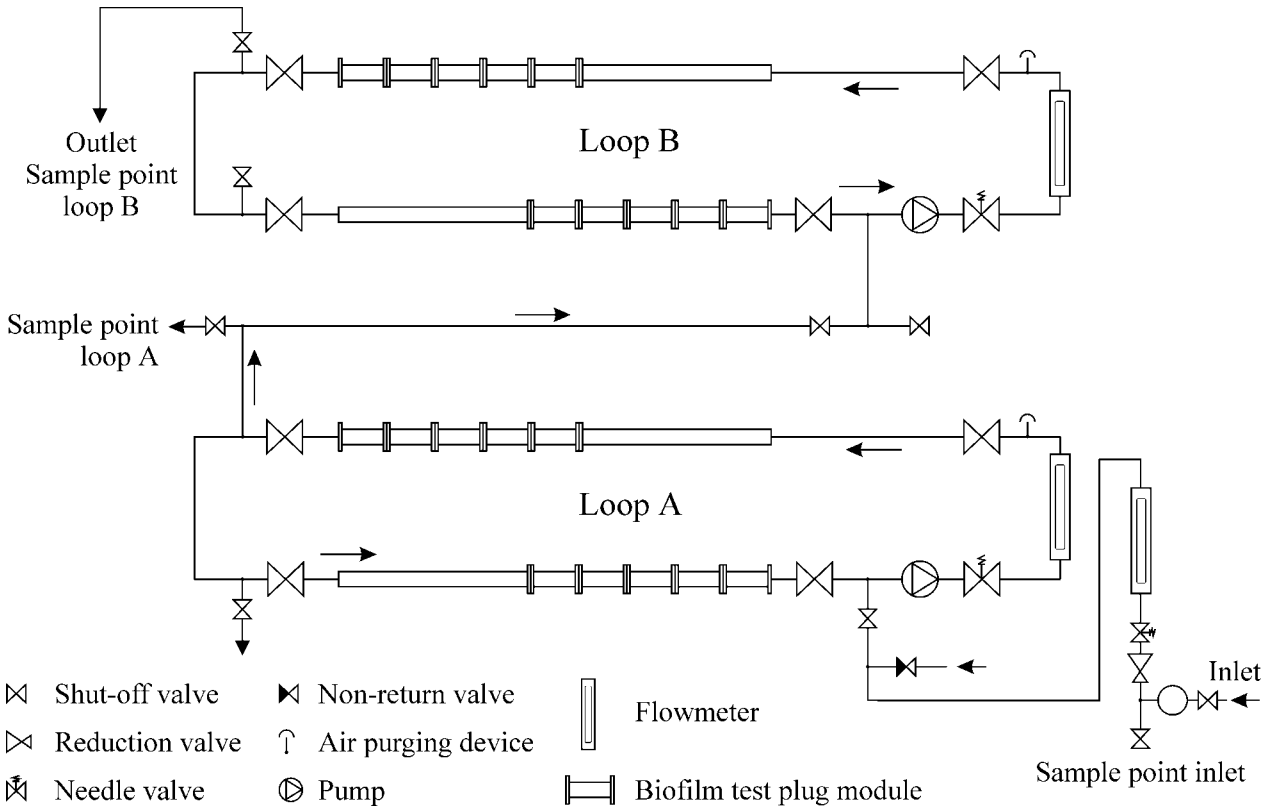

Figure 1 | The model distribution system.

parameters are listed in Table 1). The recycling flow rate was 0.5 m^3/h (velocity: 0.07 m/sec, Reynolds number: 690) with a hydraulic retention time of 1 h per loop. At this recycling rate, the model system was hydraulically similar to two mixed reactors in series allowing for only thee water sampling points: at the inlet, and at the outlet of each of the two loops. In order to monitor biofilm development 140 removable test-plugs were mounted even with the inner side of the pipe wall of the two loops. The test-plugs were stainless steel and they had an exposed area of 7.1 cm^2. Before insertion, the test-plugs were thoroughly cleaned (and sterilised) with 10% HNO_3^- for 1 day, rinsed (with Milli-Q water), and heated to 220°C for 5 h.

Experiments performed

The experiments began with a recycling of a hypochlorite solution (~15 g/l) for 24 h, which disinfected the system. This was followed by insertion of cleaned and sterile test-plugs. Two experiments were performed: (1) A biofilm-formation experiment considered the situation where bacteria colonise sterilised surfaces simulating a virgin distribution system. Test-plugs ($n = 61$) were successively harvested throughout a period of 522 days from the initial disinfection. (2) A re-colonisation experiment considered the colonisation of sterilised surfaces in a pre-colonised system. Whenever a test-plug was removed from the system, it was replaced by a new test-plug (clean and sterile), which was consequently inserted into a system already colonised by microorganisms. Since the originally inserted test-plugs were harvested and replaced at different times, the age of the biofilm present in the system varied. On two different occasions test-plugs supporting biofilm of different ages were simultaneously harvested ($n = 20$ test-plugs). These test-plugs represented "real life" situations where only a part of a distribution network (i.e. a pipe section) has been replaced. Water samples were

Table 1 | Basic water quality parameters at the waterworks

Parameter (unit)	Value*
pH	7.6
Turbidity (FTU)	0.28
Total hardness (°dH)	17.8
NVOC** (mg/l)	2.3
Ca^{2+} (mg/l)	93
Mg^{2+} (mg/l)	21
Na^{+} (mg/l)	65
K^{+} (mg/l)	4.2
NH_4^{+} (mg/l)	0.1
Fe, total (mg/l)	0.04
Mn, total (mg/l)	<0.005
HCO_3^{-} (mg/l)	362
Cl^{-} (mg/l)	109
SO_4^{2-} (mg/l)	11
NO_3^{-} (mg/l)	2.5
NO_2^{-} (mg/l)	0.03
P, total (mg/l)	<0.02
F^{-} (mg/l)	0.40
O_2 (mg/l)	7.5

*Average of two samples.
**Non-volatile organic carbon.

taken regularly from the system during the entire period of the studies.

Biofilm sampling

Biofilm was sampled by removing test-plugs from the system. The attached bacteria were collected by swabbing with a sterile cotton bud. The cotton bud was transferred to 10 ml sterile filtered water (0.2 μm, Millipore) and vortexed viscously for 1 min to release and suspend the bacteria. The swabbing efficiency was tested by direct microscopy of the test-plugs, which revealed that more than 95% of the biomass (AODC) was removed by the procedure. Subsamples from each test-plug were analysed by different microbial methods (HPC, AODC, and ATP analysis).

Heterotrophic plate counts

Biofilm and water samples were analysed by heterotrophic plate counts (HPC) on R2A agar (Remel Inc.) within 3 h of sampling. Plates were incubated at 15°C and counted after 7 days.

Total bacterial count

Samples were preserved by addition of a buffered formaldehyde solution to a final concentration of 2% and stored at 5°C. The total bacterial count was obtained using Acridine Orange Direct Count (AODC) (Hobbie *et al.*, 1977). A fraction of the sample (1–10 ml) was filtered through a black 0.2 μm polycarbonate filter (Osmonics Inc.), previously wetted with a 2% solution of Tween80. The bacteria on the filter were subsequently stained for 3 min with a 0.001% Acridine Orange solution followed by 20 sec of treatment with a 3% DABCO solution (1.4-diazbicyclo-(2.2.2)-octane). Finally, the bacteria were counted by epifluorescence microscopy (Olympus BH-2), where a minimum of 200 bacteria and at least 10 different areas were counted.

ATP (Adenosine-tri-phosphate)

The concentration of the energy carrier molecule ATP was determined as an estimate of the size and activity of the microbial community. A 1 ml sample was stored at −80°C for the ATP analysis. After thawing, 100 μl of the sample was pipetted into cuvettes and measured in a luminometer (Celsis Advance Coupe) which added 100 μl NRB reagent (Celsis) followed by 100 μl LuminATE-PM reagent (Celsis) 10 sec later. The light emitted by the enzyme

Table 2 | Water quality during experiments

	Inlet Mean± st. dev.	Outlet Mean± st. dev.	*n*	Pairs significantly different*?
Temperature (°C)	12.3 ± 1.7	13.0 ± 2.0	18	No ($P = 0.067$)
Conductivity (mS/m)	78 ± 10	79 ± 10	20	No ($P = 0.49$)
Oxygen (mg/l)	5.9 ± 1.0	5.7 ± 1.2	18	No ($P = 0.058$)
AOC (μg ac-C/l)	6.1 ± 3.3	4.1 ± 2.3	18	Yes ($P = 0.0017$)**
HPC (CFU/ml)	259 ± 317	245 ± 304	19	No ($P = 0.16$)**
AODC (10^5 cells/ml)	1.1 ± 0.4	1.3 ± 0.6	14	No ($P = 0.16$)**

*0.05 level of significance.
**Log transformation.

reaction was measured during 10 sec after a 2 sec delay and was proportional to the ATP amount of the sample.

Assimilable organic carbon (AOC)

A modification of the method described by Van der Kooij & Veenendaal (1995) was used. Triplicate water samples (200 ml) were collected directly into Teflon-capped glass flasks, which had been previously rinsed with acid, and heated to 220°C for 6 h (the caps were heated to 180°C). The samples were pasteurised (at 60°C for 40 min), inoculated by the two bacteria, *Aquaspirillum* sp. strain NOX and *Pseudomonas fluorescence* strain P17, and after 10 days incubation at 15°C, the number of bacteria was determined by HPC (R2A, 21°C, 3 days).

RESULTS AND DISCUSSION

Bulk phase

The AOC value, approximately 6 μg ac-C/l was lower than the value of 10 μg ac-C/l proposed by Van der Kooij (1992) as a limit for biologically stable drinking water. Some variations in the inlet water quality were observed during the 522 days of the experiment (Table 2), which were probably caused by the water works shifting the water abstraction between wells and seasonal temperature change.

Water samples taken simultaneously from the inlet and outlet were compared using paired *t*-tests. The 2-hour retention time affected the AOC content of the water significantly ($P = 0.0017$). The AOC declined by approximately 2.0 μg ac-C/l through the model system. This was in agreement with an observed increase in total bacterial counts of 0.2×10^5 cells/ml between inlet and outlet samples, although this difference was not statistically significant ($P = 0.16$). The results indicated that microbial activity reduced the level of available substrate and gave rise to bacterial cells in the bulk water phase. However, the heterotrophic plate counts failed to show an increase in bulk phase bacteria.

The oxygen concentration and temperature showed vaguely significant decreases of 0.2 mg/l ($P = 0.058$) and 0.7°C ($P = 0.067$), respectively. As expected, no change in conductivity was observed between inlet and outlet samples ($P = 0.49$).

Colonisation of the disinfected system (virgin system)

Approximately 200 days after initial disinfection, the attached bacteria reached the quasi-stationary phase (Figure 2). At this state, different removal processes such

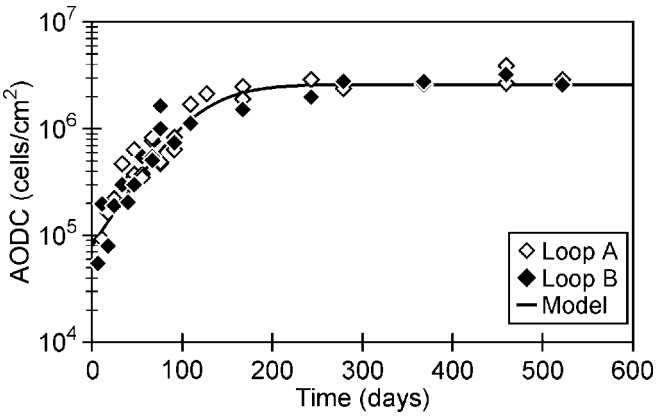

Figure 2 | Attached bacterial numbers (AODC) during colonisation of a virgin system.

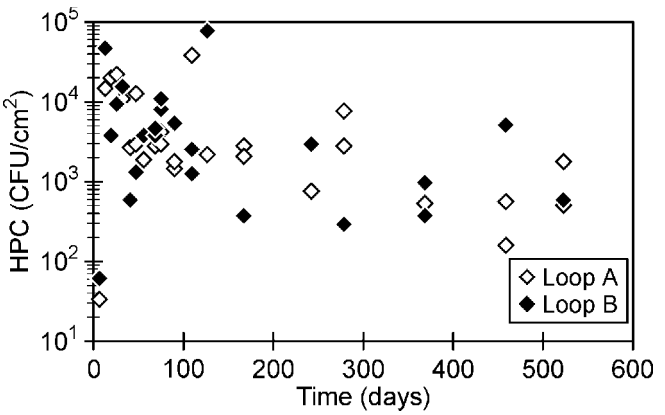

Figure 3 | Attached bacterial numbers determined by HPC during colonisation.

as detachment and grazing balanced the growth of attached bacteria. Direct microscopy revealed that the bacteria at this state had formed micro-colonies and visible EPS (exopolymeric substances). In spite of a lower AOC content of Loop B, a paired *t*-test of the log-transformed data showed no significant difference ($P = 0.53$) between the amounts of attached bacteria in the two loops. The result implies that the size of the biomass was controlled by other factors than the concentration of nutrients, such as the rate of detachment and/or grazing by protozoa.

The HPC measurement varied greatly (Figure 3), which was most likely caused by heterogeneity of the attached community and formation of single species micro-colonies.

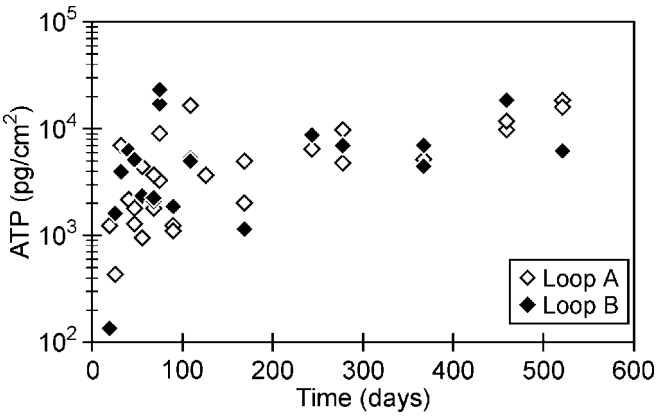

Figure 4 | ATP content in the biofilm during colonisation.

The ATP content of the biofilm (Figure 4) increased similarly to the direct microscopic counts. At quasi-stationary state, the ATP content of the biofilm was up to 10 times higher than values reported by Van der Kooij *et al.* (1995), where biofilm grown for 125 days in treated groundwater (AOC concentration of 7.5 μg ac-C/l) only reached values of 1400 pg/cm^2.

Modelling

A simple model using only bacterial growth and removal (detachment and grazing pooled) was applied to the total bacterial counts (AODC). The rate of biofilm formation was modelled by the computer program AQUASIM v.2.0 (Reichert 1994) using the following equation:

$$\frac{dX}{dt} = \mu_{obs} X - k X^2 \quad (1)$$

where X is attached total biomass (AODC) (cells/cm^2); t is time (days); μ_{obs} is observed growth rate (day^{-1}); k is removal rate constant. Using the model, it was possible to achieve a good estimation of the growth parameters of the system (Figure 2). The growth rate of the system (μ_{obs}) was estimated to be 0.030 ± 0.002 day^{-1}, equivalent to a doubling time of 23 days. At quasi-stationary state, the maximum number of attached bacteria obtained from the model was 2.6×10^6 cells/cm^2. Pedersen (1990) previously

reported a doubling time of only 11 days in drinking water produced from surface water containing a chlorine residual, the same study reported attached bacterial numbers of 4.9×10^6 cells/cm^2 (AODC) in a non-stationary biofilm. A higher nutrient level combined with chlorine tolerance might explain the shorter doubling time and the higher level of bacteria observed in the chlorinated surface water.

It has to be emphasised that the growth rate reported in this study was merely a rate of growth during biofilm-formation, and that the growth rate of the mature biofilm may vary significantly due to changes in the density, community and nutritional state.

Method comparisons

The culturable fraction (HPC/AODC) of attached bacteria varied between 0.004% and 24%, where the highest percentages were achieved in the young biofilm. The decrease in culturability throughout the maturation of the biofilm could be partly due to accumulation of non-active bacteria on the surfaces. Van der Kooij *et al.* (1995) observed a similar level of culturability and variability.

The HPC-specific ATP content (ATP/HPC) is depicted in Figure 5B. From the figure, it can be inferred that the amount of ATP per culturable cell is increasing as the biofilm matures. This is probably due to the fact that the culturability of the microorganisms declines while the overall activity of the biofilm remains relatively stable. From the results of this investigation it was concluded that the HPC (R2A, 15°C, 7 days), was inadequate in quantifying microorganisms collected from low-nutrient drinking water.

The cell-specific ATP content (ATP/AODC) varied between 0.75×10^{-15} g/cell and 30×10^{-15} g/cell (Figure 5C). The highest cell-specific ATP content was observed in the early growth phase, possibly due to a high bacterial metabolism indicating a higher growth rate. In a subsequent study using the same model distribution system, it was shown that the growth rate of the mature biofilm was significantly lower than the immature biofilm (Boe-Hansen *et al.* 2002). The increased growth rate during the early biofilm formation is probably caused by the bacterial utilisation of organic compounds adsorbed to the pipe wall and a lower competition between the bacteria. As a result, the total bacterial production may actually be larger for the immature biofilm compared to the mature biofilm, which could lead to a higher number of microorganisms in the drinking water. This might

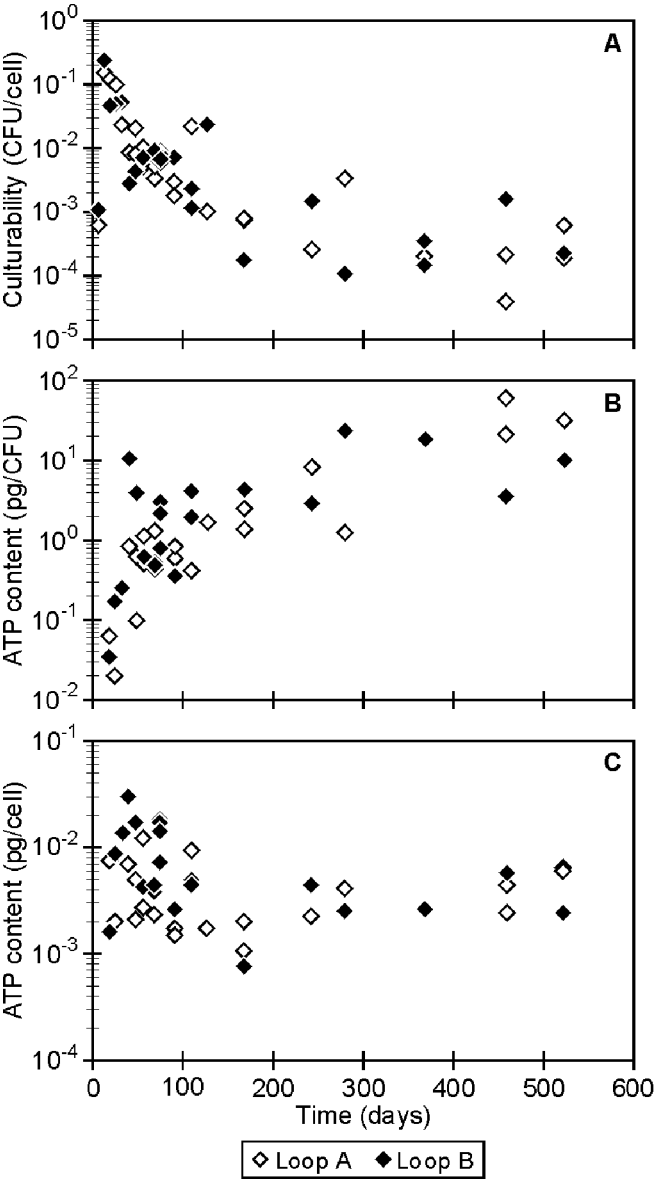

Figure 5 | Properties of attached bacteria during colonisation. (A) Culturability (HPC/AODC). (B) Culturable cell-specific ATP (ATP/HPC). (C) Cell-specific ATP (ATP/AODC).

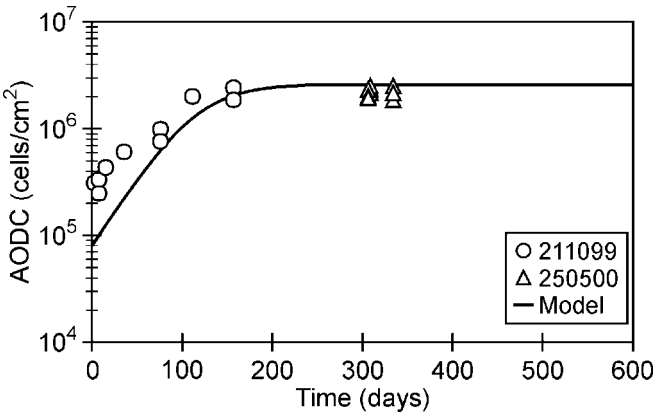

Figure 6 | Attached bacterial numbers (AODC) during colonisation in a pre-colonised system.

explain why elevated bacterial numbers often occur in new pipe installations.

The pre-colonised system

The initial biofilm formation was faster in the pre-colonised system than in the virgin system (Figure 6); it appeared that the presence of already colonised surfaces nearby enhanced the colonisation rate of new surfaces. The higher rate ceased after approximately 100 days, and subsequently there was agreement between the two methods of culturing biofilm. However, considerable time (>100 days) was still needed for the biofilm to stabilise at quasi-stationary state, and this might prove important for the practical situations where pipe sections have been replaced, since the bacterial production for the immature biofilm is different from the mature biofilm as discussed in the previous section.

CONCLUSIONS

The experiments performed in this study allowed us to closely study the dynamics of biofilm formation under low nutrient conditions.

- AOC decreased significantly throughout the model system, however the increase in bulk phase bacterial concentration through the system was not significant.
- The biofilm formation on the surfaces in the model drinking water system was slow with an estimated growth rate of 0.030 ± 0.002 day^{-1}, equivalent to a doubling time of 23 days.
- At least 200 days were needed for the biofilm to establish a quasi-stationary phase at 2.6×10^6 cells/cm^2.
- The culturability and cell-specific ATP content was higher during the early stages of biofilm formation compared to the mature biofilm.
- The presence of pre-colonised surfaces enhanced the biofilm formation rate of neighbouring virgin surfaces.

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REFERENCES

Boe-Hansen, R., Albrechtsen, H. J., Arvin, E. & Jørgensen, C. (In press) Bulk water phase and biofilm growth in drinking water at low nutrient conditions. *Wat. Res.*

Donlan, R. M., Pipes, W. O. & Yohe, T.L. 1994 Biofilm formation on cast iron substrata in water distribution systems. *Wat. Res.* **28**(6), 1497–1503.

Hobbie, J. E., Daley, R. J. & Jasper, S. 1977 Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **33**(5), 1225–1228.

Kalmbach, S., Manz, W. & Szewzyk, U. 1997 Dynamics of biofilm formation in drinking water: Phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and in situ hybridization. *FEMS Microbiol. Ecol.* **22**(4), 265–279.

Keevil, C. W., Rogers, J. & Walker, J. T. 1995 Potable-water biofilms. *Microbiology Europe* **3**(6), 10–14.

Pedersen, K. 1990 Biofilm development of stainless steel and PVC surfaces in drinking water. *Wat. Res.* **24**(2), 239–243.

Reichert, P. 1994 AQUASIM—a tool for simulation and data analysis of aquatic systems. *Wat. Sci. Technol.* **30**(2), 21–30.

Sternberg, C., Christensen, B. B., Johansen, T., Nielsen, A. T., Andersen, J. B., Givskov, M. & Molin, S. 1999 Distribution of bacterial growth activity in flow-chamber biofilms. *Appl. Environ. Microbiol.* **65**(9), 4108–4117.

Van der Kooij, D. 1992 Assimilable Organic Carbon as an Indicator of Bacterial Regrowth. *J. Am. Wat. Wks Assoc.* **84**(2), 57–65.

Van der Kooij, D. & Veenendaal, G. 1995 *The AOC Manual*. Report KIWA, SWE 95.022, pp. 1–35.

Van der Kooij, D., Veenendaal, H. R., Baars Lorist, C., Van Der Klift, D. W. & Drost, Y. C. 1995 Biofilm formation on surfaces of glass and teflon exposed to treated water. *Wat. Res.* **29**(7), 1655–1662.

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