Monitoring biofilm formation and activity in drinking water distribution networks under oligotrophic conditions

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Abstract In this study, the construction a model distribution system suitable for studies of attached and suspended microbial activity in drinking water under controlled circumstances is outlined. The model system consisted of two loops connected in series with a total of 140 biofilm sampling points. The biofilm from the system was studied using 11 different microbial methods and the results were compared and discussed. The methods were used for biomass quantification (AODC, HPC and ATP determination), visualisation of structure (CLSM), activity measurement (leucine incorporation, AOC removal rate, respiration of benzoic acid, CTC and live/dead stains), and microbial diversity profiling (clone libraries and DGGE).
Keywords Activity; biofilm; biomass; diversity; drinking water; sampling

Introduction

Studies of drinking water networks have shown that the major part of the biomass is attached to pipe surfaces in a biofilm, which affects the water quality and increases the maintenance cost of the distribution network (van der Wende and Characklis, 1990). However, it is difficult to examine the attached microbial population in distribution networks. There is generally limited access to the biofilm, and additionally it can be difficult to repeat experiments, since pipe sections have to be replaced after sampling. These technical hindrances call for new approaches that facilitate routine biofilm monitoring as well as studies in the laboratory. A solution could be to construct special sampling devices that allow for *ex situ* as well as *in situ* investigation of the attached microbial community. Different attempts have been made to culture biofilm under controlled but realistic conditions e.g. the Robbins device (McCoy *et al*., 1981), where glass slides or metal coupons have been installed in the water flow. Such removable coupons provide an easy access to biofilm and satisfy the need for a plane surface for microscopic studies. However, the conditions around such a slide reflect to only a limited degree the hydraulics at the inner surface of drinking water pipes. A different approach has been the RotoTorque reactor (Van der Wende *et al*., 1989), and flow cells (Caldwell and Lawrence, 1988), which allow culturing of biofilm under controllable and well-defined hydraulic conditions. However, these systems are not fit for studies of biofilm and water phase interactions, because they contain a relatively large unaccounted surface area, where the hydraulic conditions are poorly defined.

Another challenge is the choice of suitable technique(s) that allow us to monitor and understand the dynamics of the bacterial populations, indigenous as well as pathogenic. The water industry routinely uses heterotrophic plate count (HPC) to monitor the hygienic water quality. However, in order to fully understand the dynamics of the microbial community, information regarding the population size (biomass quantification), the spatial organisation (structure), the activity of the present microorganisms and the distribution among various (active) groups of organisms (diversity) is also needed. Based on this information,

an insight can be gained regarding the relation between operational parameters and the microbial community, which will help the water industry to improve the quality of the drinking water in the future. Selection of proper microbial characterisation method is very important since the type of result highly depends on the methods used, and often a combination of different methods is needed to obtain a complete picture. Microbial measurements in drinking water pose special difficulties, mainly related to the low amount of bacteria present and their low activity; hence the techniques best suited are often the most sensitive available.

Danish drinking water is generally characterized by an extremely low nutrient content and the absence of disinfectant residuals; this imposes special demands and constraints for the monitoring tools. The purpose of this study was to identify and test appropriate methods for monitoring biofilm in drinking water at low nutrient conditions. In our study, we compared results obtained by 11 different methods used in a model drinking water distribution system studied during 3 years.

Model system

In order to produce a large number of biofilm samples grown under comparable conditions a model drinking water distribution network system was constructed to be operated under conditions similar to the drinking water distribution networks in Denmark, and under constant conditions where the flow velocity and retention time was controlled independently. Some selected properties are shown in Table 1.

The model distribution system consisted of two identical pipe loops connected in series. In each loop, an adjustable centrifugal circulation pump (UPS25-40K, Grundfoss) recycled the water. The recycle-flow rate was controlled by a needle valve installed immediately downstream of the pump and was measured by a flowmeter (BM134, Rotameter). The two loops contained two strings of biofilm test-plug modules made from square pipes (width: 50 mm, gauge: 3 mm); each string consisted of a row of 5 biofilm test-plug modules each with 7 test-plugs i.e. in a total of 140 test-plugs was distributed throughout the system. To prevent turbulence induced by pumps, valves, and bends to affect biofilm formation in the system, a 2 m square pipe was inserted just upstream of the test plug modules to stabilise the flow in the modules. This construction should guarantee that the velocity distribution was identical from cross-section to cross-section, and that all test plugs within a loop were exposed to identical hydraulic conditions. The hydraulic retention time in the system was controlled at the inlet by a needle valve and monitored by a flowmeter.

The model system was constructed of stainless steel (grade 316) to prevent galvanic corrosion in the system with a limited number of brass fittings and valves. Teflon tape was used in a few cases to seal joints. The biofilm test-plug modules were mounted with flanges at each end, were joined by screws, and tightened by O-rings. The biofilm test-plug mod-

Table 1 Properties of the model distribution system

	Loop A	Loop B	Total
Active volume (L)	21	21	42
Inner surface area (cm^2)	19,000	19,000	42,000
Number of test-plugs	70	70	140
Total test plug area (cm^2)	450	450	990
Operational pressure (10^5 Pa)	0–6	0–6	–
Hydraulic retention time (h)	0.5–12.0	0.5–12.0	1–24
Recycle flow rate (L/h)	200–2,000	300–2,500	–
Flow velocity (m/s)*	0.029–0.286	0.043–0.359	–
Reynolds number*	280–2,770	420–3,460	–

* At the biofilm test-plug modules

ules were constructed in square pipes with a plane surface suitable for microscopic studies. The test-plugs were discs with a diameter of 3 cm (area: 7.1 cm^2), positioned on the upper side of the pipes secured by a screw (Figure 1). The test plug was mounted even with the inner surface of the pipe to prevent local turbulences.

The surfaces of the test plugs were preconditioned by grinding with emery cloth (180 J) followed by 6 hours of acid corrosion (1 L of 1 M HCl and 0.5 M HNO$_3$ to 40 plugs). An O-ring sealed the joint between the pipe and the test-plug.

Bulk water samples were collected from taps at three different sites: at the inlet, and at the outlet of each of the two loops. Biofilm was sampled from any of the 140 mounted biofilm test-plugs. After a test-plug was removed, a clean and sterile one replaced it. The test-plugs were immersed in 10% HNO$_3$ for 24 hours, rinsed in Milli-Q water, and finally heated to 220°C for 5 hours before insertion in the system.

The system was operated at temperatures between 10–15°C, which is typical in Danish drinking water. The temperature was controlled by a cooling system, which consisted of square brass pipes coiled around the pipes, recycling water from a thermo-regulated water bath. The entire system was covered by 13 mm insulation (Turbulit, Armacell).

Methods

The model distribution system was continuously fed with drinking water from a municipal distribution network. The drinking water was produced from groundwater without disinfectants with an inlet AOC content of 6.1 ± 3.3 µg ac-C/L (n = 18). The hydraulic retention time and the flow velocity were fixed at 2 hours and 0.07 m/s respectively.

Biofilm samples were collected by removing test-plugs and subsequently swabbing the entire surface of the plugs with a sterile cotton bud to collect the attached bacteria. The bud was then transferred to cell-free water, and vortexed vigorously for 1 minute to release the bacteria.

The model system was operated at constant conditions for more than one year to allow a quasi-stationary biofilm to form, and during this period the biomass on the surfaces was frequently quantified using total microscopic counts (AODC), heterotrophic plate counts
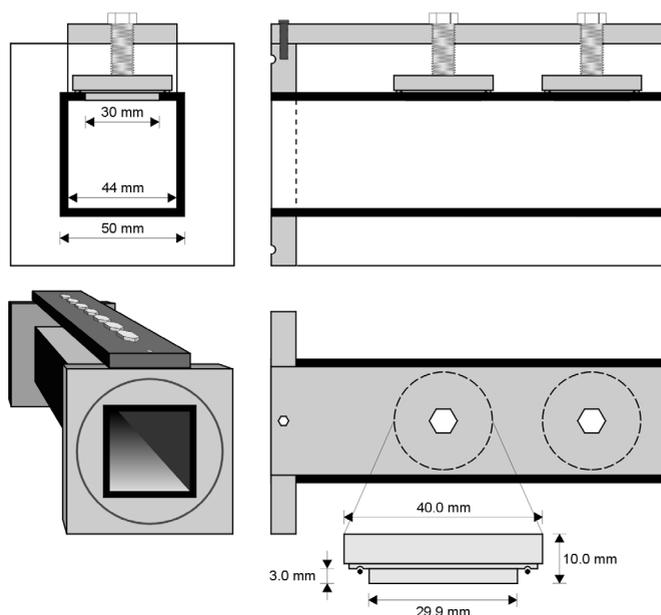

Figure 1 Biofilm test-plug module and test-plug

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(HPC, R2A, 15°C, 7 days) and ATP (adenosine triphosphate) measurement. Later, the mature biofilm (>1 year of age) was examined by a suite of techniques to evaluate the most suitable one for biomass quantification (HPC, AODC and ATP), activity measurement (leucine incorporation, AOC removal, CTC and live/dead stains), and microbial diversity profiling (clone libraries, DGGE). In addition some structural studies was performed directly on the test-plugs using a DAPI stain visualised by confocal laser scanning microscopy (CLSM). Additional information regarding the specific procedure for each technique is given in Boe-Hansen *et al.* (2001, 2002a, 2002b) and Martiny *et al.* (2002).

Results and discussion

Biomass quantification

In order to compare different biomass quantification methods and establish a mature biofilm in the model distribution system, the biofilm build-up was monitored by the AODC, HPC, and ATP techniques. The results showed that quasi-stationary conditions were reached in the biofilm after approximately 200 days (Boe-Hansen *et al.*, 2002a). The structure of the mature biofilm as visualised by CLSM was mainly characterised by a high degree of heterogeneity with a large number of micro-colonies separated by open areas (Figure 2).

Direct counts using a fluorescent stain (e.g. DAPI, Acridine Orange or Syto) are generally recognised as the "gold standard" for enumeration of bacteria. A limitation of this technique is the problem of separating living from dead cells as well as inorganic particles. This was also the case in our system where the small size of the cells and the low activity level made it difficult to identify the viable microorganisms. Direct counting of the bacteria is also a tedious process.

In contrast to direct counts, the ATP measurement is fast and easy to perform and generally has a high sensitivity, which makes the method well suited for biomass measurement at low nutrient conditions. The interpretation of the results can, however, be difficult, since the amount of ATP in the individual cell may vary depending on the nutritional state of the bacteria. In our study there was a good correlation ($p<0.0001$) between ATP and AODC (Figure 3), which indicates that the fast ATP determination method to some extent can substitute the AODC technique for routine monitoring of total biomass. A weak negative correlation was observed between HPC and AODC (Spearman correlation $= -0.27$, $p = 0.023$), which was probably caused by a higher culturability (HPC/AODC) in the young biofilm compared to the mature biofilm. Only a small amount of the bacteria visible in the microscope was able to form colonies on R2A agar, i.e. the culturability was generally low (approximately 0.04% for the mature biofilm). Additionally, a high coefficient of variance was observed in the mature biofilm (Table 2), which was probably caused by increased
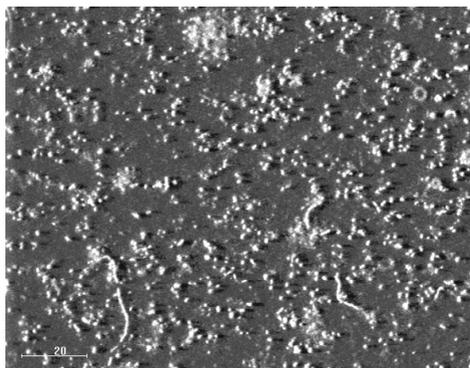

Figure 2 Mature biofilm structure visualised by CLSM (using Syto 62 stain) (scale bar: 20 µm)
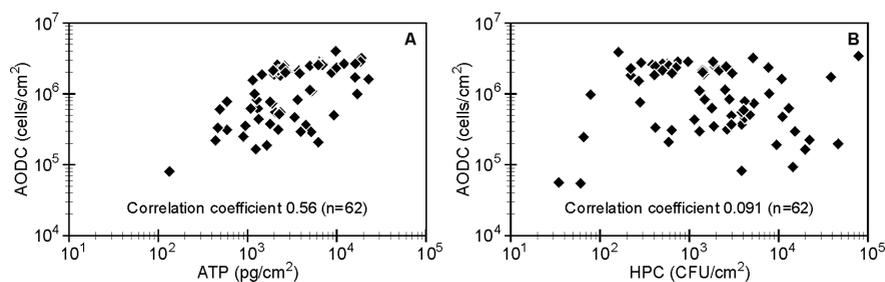
Figure 3 Comparison of methods during biofilm formation. a) AODC and ATP, b) AODC and HPC. (Non-parametric Spearman correlation coefficient and n = number of samples)

Table 2 Replicate measurements using different techniques applied to >300-day-old biofilm samples

Method	Unit	Range of measurements	Replicates	Coefficient of variation	Approx. sample size (cm^2)	Approx. time before result
Biomass quantification						
HPC (R2A, 15°C,7d)	CFU/cm^2	595–2,566	5	91%	0.5	7 days
AODC	10^6 cells/cm^2	1.8–2.6	5	14%	1	2 hours
ATP	pg ATP/cm^2	2,179–3,816	5	24%	0.1	1 hour
Activity						
Leucine incorporation	fmole/cm^2/h	3.5–6.1	5	22%	3	2 days
AOC removal	(g acC/L	4.2–6.7	5	19%	19,000	14 days
Diversity						
Clone libraries	# of species	49	1	–	10	7 days
DGGE	# of species	13	1	–	10	2 days

heterogeneity of the biofilm combined with a low culturability. Our study showed no significant correlation between HPC and the ATP measurement (Spearman correlation = 0.091, p = 0.48).

Activity

The biofilm formed on the pipe-surface is regarded as a potential reservoir of microorganisms that can detach into the bulk water, and reduce the hygienic quality of the water. The amount of biomass detached into the bulk water will roughly reflect the net growth of the attached population assuming steady-state conditions. However, when different perturbations are introduced to the system e.g. temperature up-shift at summer time or an increased substrate load, the activity of the present biomass will change according to the dynamics of the systems. The drinking water distribution networks are very dynamic systems where flow temperature, nutrient content etc. change within the hour. The activity of the biomass present is therefore an important parameter in the quality control of drinking water.

The bacteria of the biofilm are degrading bio-available organic substrates present in the drinking water; the amount of these substrates can be quantified by the assimilable organic carbon method (AOC) proposed by Van der Kooij et al. (1982). The model distribution system used in this study allowed for batch experiments, where the water was recycled, while the hydraulic conditions in the pipe were maintained. An AOC removal rate of 11.2×10^{-9} g C/cm^2/d was determined. (Boe-Hansen et al., 2002b). A series of experiments, where benzoic acid was spiked to the model distribution system showed a mineralisation rate of benzoic acid of 15.2×10^{-9} g C/cm^2/d, similar to the results obtained by measuring the AOC respiration rate. Addition of benzoic acid may therefore be used as a fast technique for measuring organic removal rates in the system.

Incubation of biofilm samples with ^{3}H-labelled leucine allows for the detection of very low levels of activity in the biofilm. As proposed by Kirchman *et al.* (1985), the leucine incorporation of the biofilm bacteria can be related to the protein synthesis rate, which again is related to the bacterial growth rate. Leucine constitutes approximately 7% of the bacterial protein (Simon and Azam, 1989). From the leucine incorporation rate measured in this study (7.9×10^{-12} g C/cm^2/d, n = 5) an estimated protein synthesis rate can be calculated (1.1×10^{-10} g C/cm^2/d). The AOC consumption rate was 100 times larger than the protein synthesis rate, which suggests that the respiration of substrate is large compared to the biomass growth (i.e. the biofilm yield coefficient is small). This observation was in agreement with an experiment where a small amount [^{14}C]-labeled benzoic acid was injected into the model distribution system, and which showed that the respiration was approximately 40 times higher than the incorporation (yield coefficient of 0.024 g C/g C) (Boe-Hansen *et al.*, 2002c).

The CTC stain provides a direct way of measuring respiratory activity. When applied to the biofilm of this system, it revealed no respiring cells (data not shown). In contrary to these results the live/dead staining showed that a very low number (<1%) of the bacteria had a compromised membrane. The lack of signal from the CTC stain is probably caused by the low activity of the bacterial cells and has led to the conclusion that the CTC stain is unfit for studies in an extreme oligotrophic system. The live/dead staining failed to show any discrimination of the present cells in the drinking water biofilm, which has led us to the conclusion that virtually all the biofilm cells are potentially viable.

Diversity

Various techniques can be used for measuring the diversity among the attached bacteria in both a qualitative and a quantitative way. Denaturing gradient gel electrophoresis (DGGE) can give a qualitative overview of an abundant sub-population. Each strain present in the sample will show as a distinct band on an acrylamide gel (or that is the assumption) and the result will be a community profile. The different bands can then be excised for further identification. The technique is limited by the risk of bias introduced during PCR amplification, which makes it difficult to quantify the different populations. Also, since the accumulation of biomass on the surface is low in this oligotrophic system (Martiny *et al.*, 2001), a large sampling area is needed, which can sometimes be a problem.

In contrast to DGGE, which mostly gives information about abundant species, one can also analyse individual strains or clones. This can either be done by restriction analysis (e.g. amplified rDNA restriction analysis (ARDRA), pulse-field gel electrophoresis (PFGE) and many others) or sequence analysis. This type of analysis gives a higher resolution of the diversity since many smaller sub-populations will be measured. This type of analysis will also allow for an estimate of diversification in the system and how this is influenced by perturbations. The two approaches were compared using a biofilm sample from the model system. It was possible to identify some of the intense bands from the DGGE analysis in the clone library, but the distribution among the different clones gave a more precise picture of their relative abundance.

The DGGE is a suitable and fast technique to detect changes in the microbial community but requires a priori knowledge of the different groups of organisms. This could be obtained by phylogenetic analysis of clone libraries or strains and then comparing this with the different bands on the DGGE gel. The information regarding the distribution of various groups of organisms is still very complex and difficult to correlate to operational parameters of the distribution system. Future studies will provide more insight into this subject.

Conclusions

The model distribution system and the biofilm sampling modules used in this study provided an easy access to a large number of biofilm samples. The system allowed biofilm to be grown under controlled conditions comparable to those prevalent in the drinking water distribution network. The retention time, the flow rate and temperature were independently controlled in the system, and furthermore it allowed chemicals or specific microorganisms to be added.

The results from this study show the type of results, which can be obtained by applying and evaluating different techniques for monitoring bacterial fouling in distribution networks. We suggest the use of either AODC or ATP quantification for monitoring biofilm build-up. If one wishes to use ATP quantification, it is important to relate it to direct counts since ATP measurements only provide you with a relative number. The structure of the biofilm was successfully visualised by CLSM revealing a high degree of heterogeneity on the micro-scale. Direct staining (CTC and live/dead) did not work as a tool for assessing microbial activity in this oligotrophic system. The leucine incorporation, the AOC removal and spiked benzoic acid methods are potential tools to provide us with a more complete picture of the microbial dynamics in drinking water, however these methods are generally too tedious for routine measurements. For monitoring the microbial diversity and dynamics in biofilms formed at pipe-surfaces, DGGE would be a useful tool after an initial mapping of the most abundant groups of organisms. The results are usually obtained quickly and the outcome is a profile of the total community.

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