Biofilm formation in a hot water system

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Abstract The biofilm formation rate was measured *in situ* in a hot water system in an apartment building by specially designed sampling equipment, and the net growth of the suspended bacteria was measured by incubation of water samples with the indigeneous bacteria. The biofilm formation rate reached a higher level in the hot water distribution system (2.1 d^{-1} to 2.3 d^{-1}) than in the hot water tank (1.4 d^{-1} to 2.2 d^{-1}) indicating an important area for surface associated growth. The net growth rate of the suspended bacteria measured in hot water from the top, middle and bottom of the hot water tank, in the sludge, or in the water from the distribution system was negligible. This indicated that bacterial growth took place on the inner surfaces in the hot water system and biofilm formation and detachment of bacteria could account for most of the suspended bacteria actually measured in hot water. Therefore, attempts to reduce the number of bacteria in a hot water system have to include the distribution system as well as the hot water tank.

Keywords Bacterial growth; biofilm formation; distribution systems; heterotrophic plate counts; hot water systems and indigeneous bacteria

Introduction

In hot water systems, problems such as bad taste and odour, skin allergies and increased heat transfer resistance in heating coils have coincided with the occurrence of a high number of bacteria (Ovesen *et al*., 1994). Some specific pathogenic bacteria such as *Legionella* species have been observed and investigated in hot water systems (Yee and Wadowsky, 1982; Brundrett, 1992; Szewzyk and Stenström, 1993) but the knowledge of the occurrence of other bacterial species is limited. The number of heterotrophic bacteria has been shown to be higher in hot water than in cold water (Szewzyk and Stenström, 1993; Ovesen *et al*., 1994; Bagh, 1998), and often 10^3–10^4 CFU/mL at 37°C was observed in hot water. In most cases the number of thermophilic bacteria detected at 55°C or 65°C was even higher. The steady state number of bacteria in the biofilm reached a 5-fold higher level per unit of surface area in hot water pipes than in cold water pipes and most of the bacteria were located in the biofilm (Bagh, 1998).

In order to achieve a good microbiological hot water quality attempts have been made to reduce the number of bacteria in hot water systems by cleaning of the tank, temperature variations, UV-treatment, micro-filtration etc. (Ovesen *et al*., 1994). However, in most cases the long-term effect was limited, possibly because the biofilm bacteria were protected in the biofilm and recolonized the hot water system by release of bacteria from the biofilm to the water phase. This is described for drinking water systems, especially in the presence of a disinfectant residual (Donlan and Pipes, 1988; Van der Wende *et al*., 1989; Van der Kooij, 1992).

The knowledge of bacterial growth in hot water systems is limited. Therefore, it is important to study biofilm formation and bacterial net growth rates in order to solve the problems associated to bacterial growth. The purpose of this work was to study the biofilm formation rates in the hot water tank and in the distribution systems for hot and cold water in an apartment block.

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Methods

The hot water system

The investigated hot water system was located in a building with 100 apartments in Copenhagen, Denmark. The average hot water consumption was 11 m^3/d and the average water residence time in the hot water system was 8 hours. The hot water system consisted of two tanks in series, a preheater – a condensate cooling tank (2,000 litres, 7 years old) and a hot water tank (2,500 litres, 29 years old). Both tanks were made of low-alloyed steel and the pipes in the distribution system were made of galvanized steel. In the hot water system, corrosion was prevented by electrolytic corrosion protection using two sacrificial anodes of aluminium. Aluminium released from these anodes by impressed current (average 0.127 A) formed a protection layer on the inner surfaces of the pipes in the distribution system and precipitated at the bottom of the tanks as sludge, which was removed once a week through a tap.

The hot water tank

Biofilm samples were collected from test plugs (2 cm^2) of low-alloyed steel, which were flush with the inner surface of the tank (Bagh *et al.*, 1999). The hot water tank was equipped with 10 sampling ports at the top, 10 in the middle and 10 at the bottom. Water samples were collected through the sampling ports and sludge samples through a tap at the bottom of the tank.

The distribution system

The biofilm samples were collected from test plugs (2 cm^2) inserted in sampling ports in by-passes in the cold water pipe in front of the preheater and in the hot water pipe after the hot water tank (Bagh *et al.*, 1999). Each by-pass had 10 test plugs which were flush with the inner surface of the pipe wall.

Analysis

Biofilm samples were collected from the test plugs by a sterile cotton stick, which was transferred to 10 mL dilution buffer in a glass tube and vortexed as described previously (Bagh *et al.*, 1999). Water quality parameters were measured in water samples collected from the cold water pipe, the hot water pipe and the hot water tank by *Standard Methods*. Heterotrophic Plate Count (HPC) were enumerated by the spread plate procedure with R_2A agar (Reasoner and Geldreich, 1985) and incubated in duplicate for 7 days. The plates were incubated at 25°C for samples from cold water and at 55°C for samples from hot water. Acridine Orange Direct Count (AODC) samples were preserved in formaldehyde (2%) and bacteria were collected on a 0.2 μm cellulose acetate black filter, stained by acridine orange (0.001%) before they were counted by an epifluorescence microscope (100x objective). The ratio of culturable bacteria was defined as the HPC obtained at 55°C divided by the AODC.

Experiments

Biofilm formation rates and bacterial growth rates were determined by various methods as explained in the following. The rates were defined as the relative increase of bacterial counts per day.

The long term biofilm formation rate was determined by exposing the test plugs in the system up to 42 days. Biofilm samples were collected after 4, 9, 21, 28, and 42 days and the steady state number of bacteria in the biofilm was expressed as the geometric mean of the HPC obtained from the biofilm samples collected after 21, 28, and 42 days of exposure.

Biofilm formation was also measured by exposing the test plugs in the system up to 10 days where biofilm samples were collected after 1, 2, 4, 7, and 10 days.

The initial biofilm formation rate was defined as the initial increase of bacteria obtained during the first 4 days of the exposure period. *The initial biofilm formation over 24 hours*. The samples were collected from the test plugs in the hot water tank (middle) and in the hot water pipe after 1, 2, 4, 8, and 24 hours and preserved in formaldehyde (2%) before direct AODC enumeration of the bacteria on the test plugs. This test was performed in order to study whether the the biofilm formation was a result of attachment rather than growth.

The bacterial net growth in the water phase was measured by incubation of water samples (1,000 mL) with the indigenous bacteria in the dark. Hot water samples and sludge were incubated at 55°C and the cold-water samples at 25°C. The indigenous bacteria were enumerated as HPC just after sampling and after an incubation time of 1, 2, 4, 7, and 10 days. The steady state number of bacteria was expressed as the geometric mean of the HPC obtained after a constant level was reached during the exposure period.

Results

The water quality

Copenhagen Water Supply delivered mainly non-disinfected groundwater for the hot water system. In the preheater, the cold water was heated from 11°C to 31°C. Water from the preheater and recycled water from the hot water system were mixed before entering the hot water tank and were heated to approximately 60°C. The pH was 7.2–7.3 and the concentration of TOC was 2.4 mg/L. The total concentration of aluminium was 0.04 mg/L in cold water but increased to 0.27 mg/L in hot water due to the electrolytic corrosion protection. Most of the aluminium occurred as flocs with a diameter larger than 0.2 μm (the filter size) since the total concentration of aluminium in the sludge was 12–13 mg/L and the concentration of dissolved aluminium was 0.5 mg/L. In the hot water system, the concentration of nitrate was 0.5 mg/L, the concentration of iron was 0.007 mg/L or less, and the average concentration of oxygen was 7 mg/L. The geometric mean of the HPC in the hot water (4.4×10^4 CFU/mL) in the distribution system was 100-fold higher than in cold water (0.04×10^4 CFU/mL). The HPC in the sludge from the hot water tank (2×10^5 CFU/mL) was 1.6- to 2.2-fold higher, than in the water samples from the rest of the hot water tank (top, middle and bottom), where the HPC was $8.9–12.5 \times 10^4$ CFU/mL.

Bacterial growth in the hot water tank

Already after one-day, bacteria could be detected on the surfaces of the test plugs in the hot water tank (Figure 1a). The initial biofilm formation rates ranged from 1.4 d^{-1} to 2.2 d^{-1} in the hot water tank (Table 1). The HPC in the biofilm was $1–2 \times 10^5$ CFU/cm^2 after an exposure period of 21 days. No significant differences were observed between the biofilm formation at the top, middle, or bottom of the hot water tank. No net growth could be measured in hot water since the number of suspended bacteria in the hot water tank (top, middle, and bottom) remained constant during the incubation time of 10 days (Figure 1b). In the sludge no net growth was observed either, and the number of bacteria (14×10^4 CFU/mL) was constant during the incubation time of 10 days (Table 1).

Bacterial growth in the hot water distribution system

HPC in the biofilm in the hot water pipe were 10^2 CFU/cm^2 after one day and increased to $10^4–10^5$ CFU/cm^2 after two to four days (Figure 2a), and the steady state number of bacteria in the biofilm was 13×10^5 CFU/cm^2 after 21 days. The initial biofilm formation rate

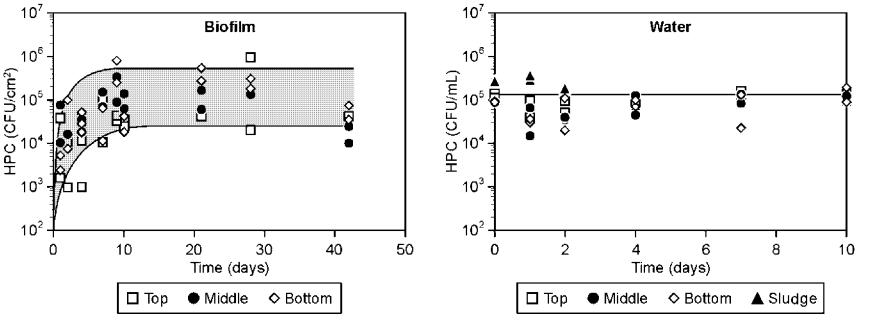

Figure 1 Bacterial growth in the hot water tank. (a) Biofilm samples, (b) Water samples. Hatched area represents the typical development pattern

Table 1 Initial biofilm formation rates measured in the hot water system in water and biofilm samples

Site	Water samples (10 days)			Biofilm samples (45 days)			Biofilm samples (10 days)	
	The steady state number of bacteria in water 10^4 (CFU/mL)	Suspended bacteria growth rate		The steady state number of bacteria in biofilm 10^4 (CFU/cm²)	Initial biofilm formation rate		Initial biofilm formation rate	
		μ^a (d^{-1})	$T_2{}^b$ (d)		μ (d^{-1})	T_2 (d)	μ (d^{-1})	T_2 (d)
Distribution system								
Cold water	28	4	0.2	28	1.0	0.7	1.1	0.6
Hot water	5	ng[c]	ng	130	2.1	0.3	2.3	0.3
Hot water tank								
Top	10	ng	ng	7	2.0	0.4	1.4	0.5
Middle	7	ng	ng	6	2.2	0.3	2.0	0.4
Bottom	7	ng	ng	17	2.2	0.3	1.9	0.4
Sludge	14	ng	ng	–[d]	–	–	–	–

[a] μ: The initial biofilm formation rate; [b] T_2: The doubling time; [c] ng: No growth; [d] –: No biofilm

was 2.1 d^{-1} in the experiment over 45 days and 2.3 d^{-1} in the experiment over 10 days (Table 1). The net growth in water samples from the hot water pipe was insignificant, because the number of bacteria remained fairly constant at 5×10^4 CFU/cm² during the incubation time of 10 days (Figure 2b).

Bacterial growth in the cold water distribution system

In the cold water pipe, the initial biofilm formation rate was 1.0–1.1 d^{-1} and the steady state

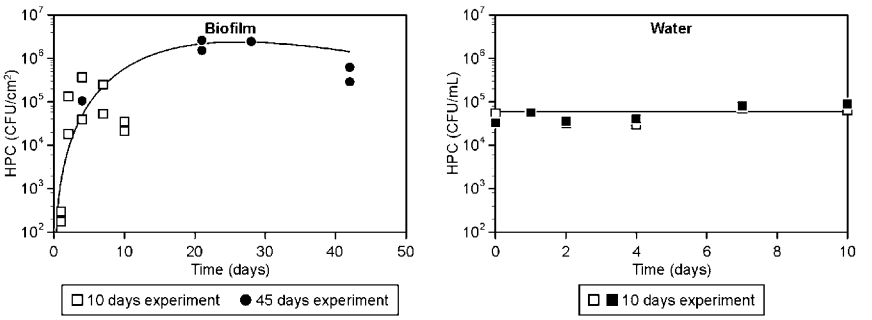

Figure 2 Bacterial growth in the hot water distribution system. (a) Biofilm samples measured over a growth period of 10 days and 45 days. (b) Water samples measured over a growth period of 10 days in two different batches

number of bacteria in the biofilm was 28×10^4 CFU/cm^2 after 21 days. The net growth was high in the cold water samples which resulted in an increase of bacteria from 0.04×10^4 to 28×10^4 CFU/mL during two days.

The initial attachment of bacteria over 24 hours

Microcolonies or dividing cells were not observed on the surfaces of the test plugs, but very long bacteria were observed on the test plugs from the hot water pipe (may be *Thermus* species) although they were not characterized. After one day, the direct AODC counts of bacteria on the test plugs were of the same order of magnitude as the AODC obtained by the scraping procedure (10^4–10^5 cells/cm^2). The initial biofilm formation was higher in the hot water pipe than in the hot water tank (Figure 3).

Discussion

The initial biofilm formation on the inner surfaces of the hot water tank and of the distribution system was fast as observed in other investigations of hot water systems (Walker *et al.*, 1991; Rogers *et al.*, 1994). The initial biofilm formation rates were approximately 2 d^{-1} in the hot water pipe, which was twice as high as in the cold water pipe, probably because the rates of biological processes increase with increasing temperature. However, the initial biofilm formation rate in the cold water distribution system (1.0–1.1 d^{-1}) was higher than reported in other investigations of drinking water systems where the specific growth rates of attached bacteria were from 0.06 d^{-1} to 0.144 d^{-1} (Van der Wende *et al.*, 1989) and 0.5 d^{-1} (Hermanowicz *et al.*, 1991).

The net growth rate of suspended bacteria in hot water was neglible compared to the net growth rate in cold water. However, in both systems biofilm formation took place. It seems that the biofilm bacteria in the hot water system were able to use the available substrate in the water phase. The growth rate in cold water was 4 d^{-1}, which was in the same order of magnitude as in waste water (Henze *et al.*, 1992). Since drinking water is an oligotrophic habitat, the observed high growth rates are surprising. However, if nutrients are plentiful, the population of *Legionella* can double every 3–4 hours (Brundrett, 1992), which is equal to a specific growth rate of 7 d^{-1}, and in general, the growth rates are influenced by many factors such as the dilution rates, differences in the organic matter, the bacterial population, temperature, etc. (Hermanowicz *et al.*, 1991; Van der Wende *et al.*, 1989).

Initially, we suspected the sludge in the hot water tank to be the major source of bacteria since the precipitation of aluminium was expected to co-precipitate phosphorus and organic matter. However, in the hot water system investigated, the number of bacteria in the sludge was only slightly higher than in the rest of the hot water tank and there was no net growth in the sludge. In many other hot water systems, the lower part of the hot water tank

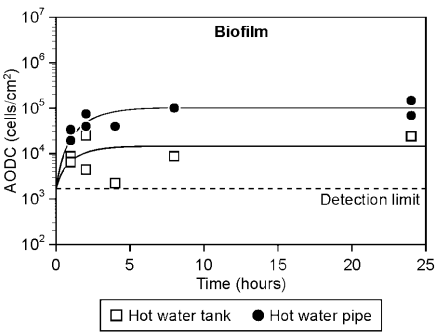

Figure 3 The initial biofilm formation measured by direct AODC countings of bacteria on the test plugs from the middle of the hot water tank and the hot water pipe over a period of 24 hours

contains brownish water with bad odour, a high concentration of organic matter, a high number of bacteria ranging from 10^5 to 10^6 CFU/mL at 70°C (Kristensen and Samsøe-Schmidt, 1989), and up to 10^7 CFU/mL at 37°C (Ovesen *et al*., 1994). The removal of sludge once a week from the bottom of the hot water tank investigated may be the reason for the low content of sludge and consequently the lower number of sludge-associated bacteria.

The biofilm formation in the hot water tank and in the distribution system was very fast and therefore problems associated with a high number of bacteria are probably difficult to eliminate. Most of the procedures developed to reduce the number of bacteria have concerned the hot water tank. However, since the major part of the bacterial growth occurs in the distribution system, focus on the hot water tank alone will not eliminate the problems. This is in accordance with other results where cleaning and additional chlorination of hot water tanks reduced the number of bacteria, but after one or two weeks the number of bacteria was in the same order of magnitude as before the cleaning procedure (Ovesen *et al*., 1994). This can be explained by bacterial inocculation from the biofilm on the inner surfaces of the pipes and fittings as reported in other investigations concerning the decontamination of *Legionella pneumophila* by UV light, chlorination and ozone (Muraca *et al*., 1987) or by heat flushing (Zacheus and Martikainen, 1996). This is especially a problem in hot water systems with stagnant water in dead ends and bendings that promote biofilm formation and bacterial growth in the water phase. In the apartment system studied, the water flow is recycled from the distribution system to the hot water tank. This assures an adequate hot water temperature at the taps, but at the same time, bacteria from biofilm and water are distributed in the entire hot water system.

Conclusions

This study has shown that the biofilm formation rate (i.e. the relative increase in bacterial counts per day) in the hot water tank and in the hot water distribution system in an apartment block supplied with groundwater was high, in the range of 1.4–2.3 d^{-1} (operation temperature, 60°C). A reduction of the number of bacteria in the hot water tank after cleaning of this tank would therefore only last for a short period since biofilms in the hot water distribution system continuously release bacteria to the water, which recolonize the hot water tank. An attempt to control the number of bacteria in the hot water tank should therefore include the whole system – for example by reduction of the growth potential (substrate) in the cold water supplying the system. This is important since the growth potential in cold water was high which probably promoted growth of bacteria and biofilm formation within the hot water system.

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