Effect of chlorine, biodegradable dissolved organic carbon and suspended bacteria on biofilm development in drinking water systems

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The influence of chlorine levels, the concentration of dissolved organic carbon and the abundance of bacteria in suspension, on the formation of biofilms on experimental glass surfaces were evaluated. Twelve reactors, packed with glass spheres, were continuously perfused with tap water. The properties of water were altered in different ways: chlorine was neutralized by the addition of thiosulfate, the levels of assimilable organic carbon were increased through the addition of acetate, and the bacterial load was modified by means of the continuous inoculation of a growing active culture of Pseudomonas aeruginosa. Continuous addition of bacteria to water containing 0.5 mg/l of free chlorine, did not result in the formation of detectable biofilms even after one month. When bacteria were added simultaneously with thiosulfate as a chlorine neutralizer, a community of attached bacteria appeared in less than 24 hours. Addition of acetate with the presence of 0.5 mg/l of chlorine did not stimulate the formation of biofilms. On the contrary, neutralization of chlorine with thiosulfate allowed the formation of biofilms with $10^6$ cfu/cm$^2$ in about two weeks.

The presence of biofilms in the conductions of water distribution networks constitutes one of the currently recognized hazards affecting the microbiological quality of drinking water (COSTERTON et al. 1995). Biofilms are constituted by a microbial community adapted to conditions of low nutrients and high chlorine levels. The characteristics of these biofilms vary widely, from sparsely colonized surfaces, to thick complex layers with a depth of several micrometers formed by a densely interwoven structure of extracellular polymers and microbial cells (LECHEVALLIER et al. 1987, STEWART et al. 1993). Microbial growth within the biofilm and release of the offspring into the overlaying water contributes to increase microbial counts in the water phase. On the other hand, the structure of the biofilms contributes to shelter occasional contaminants from the effects of chlorine, thus preventing adequate disinfection and potentially allowing regrowth of contaminants in the water distribution network (LECHEVALLIER et al. 1988, CAMPER 1994).

Development of biofilms seems to be governed by the interplay of several factors; the microbial load in the circulating water, the amount of nutrients available, the concentration of disinfectant, and the hydraulic regime of the system (CAMPER et al. 1999). At present, suppliers involved in the management of water distribution networks, aware of the problem posed by biofilms, are attempting to control their growth by a combination of several factors (BLOCK 1992). First, an adequate level of disinfectant is maintained throughout the distribution network by means of regular booster chlorination at stations placed at selected points of the network. Second, the presence of organic carbon in water is minimized through several procedures (activated carbon filtration, ozonization, UV treatment) in order to minimize the availability of substrates for growth. Third, low microbial levels at the input of the water

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distribution system are minimized through adequate disinfection treatments all along the potabilization sequence.

Implementation of biofilm control measures can require a substantial investment both in infrastructure and in the subsequent operating costs. Evaluation of the benefits derived from these measures has often been hindered by the lack of adequate biofilm monitoring techniques. This work attempts to evaluate the relative importance of several of the environmental variables involved in biofilm development and control in a well defined laboratory environment. An experimental system (MORATÓ 2001) formed by an inert support of spherical glass beads was subject to the effect of a stream of water, in which characteristics such as the suspended microbial load, the concentration of organic carbon or the levels of disinfectant were adequately controlled. The support was sampled at different times and analyzed for attached biomass. The results, although not directly comparable to the field, provide a good indication in order to help the control of biofilm formation in real systems.

Materials and methods

Experimental system: The study was carried out using a set of twelve polypropylene reactors with a internal diameter of 15 mm and a length of 610 mm, connected in parallel and packed with 5 mm diameter borosilicate glass beads. A diagram of the experimental setup can be seen in Fig. 1. Each of the reactors could be isolated and sampled independently. Water from the main water distribution system was fed to the set of reactors at a rate of 6 l/h (0.5 l/h for each reactor). Precise flow control was achieved using a needle valve. The water feeding to the set of reactors was modified through the addition of sterile solutions or bacterial suspensions as mentioned below. The operating parameters of the reactors are listed in Table 1.

Kinetics: Several experiments, in which the characteristics of the circulating water were modified in order to study the effect of the factors mentioned above on biofilm development, were run separately (Table 2). For each experiment, water was allowed to run through the biofilm monitoring system for periods of up to 30 days. Individual reactors were sampled at different intervals during this period and analyzed for attached biomass.

Chlorine levels and microbial load in the water supply were sampled on a daily basis. Water from the main supply usually contained chlorine levels averaging 0.6 mg/l. When required, chlorine was neutralized by the continuous addition of a sterile solution of sodium thiosulfate (0.13%). The levels of dissolved organic carbon were modified by pumping a sterile solution of sodium acetate (0.57%), at a final concentration of 30 mg/l.

![Fig. 1](image_url)  
Experimental system: a, medium tank; b, peristaltic pump; c, regulation valve; d, tap water inlet; e, packed bead reactor
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Table 1
Characteristics of biofilm reactor

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of reactor</td>
<td>6.1 cm</td>
</tr>
<tr>
<td>Diameter of reactor</td>
<td>1.5 cm</td>
</tr>
<tr>
<td>Cross sectional area of reactor, $A_{cs}$</td>
<td>1.77 cm$^2$</td>
</tr>
<tr>
<td>Volume of reactor, $V$</td>
<td>10.78 cm$^3$</td>
</tr>
<tr>
<td>Diameter of glass beads</td>
<td>0.5 cm</td>
</tr>
<tr>
<td>Specific surface area</td>
<td>12</td>
</tr>
<tr>
<td>Area of glass bead</td>
<td>0.785 cm$^2$</td>
</tr>
<tr>
<td>Total glass area</td>
<td>58.87 cm$^2$</td>
</tr>
<tr>
<td>Volume of voids, $V_v$</td>
<td>5.87 cm$^2$</td>
</tr>
<tr>
<td>Porosity, $\varepsilon = V_v / V$</td>
<td>0.54</td>
</tr>
<tr>
<td>Feed Flow rate, $Q$</td>
<td>500 cm$^2$/h</td>
</tr>
<tr>
<td>Detention time, $\theta = V_v / Q$</td>
<td>0.012 h</td>
</tr>
<tr>
<td>Superficial velocity of fluid, $\nu = Q / A_{cs}$</td>
<td>282.49 cm/h</td>
</tr>
</tbody>
</table>

Table 2
Variation of chlorine and acetate levels in the different kinetics

<table>
<thead>
<tr>
<th>Kinetic</th>
<th>Chlorine</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

$(+)$, presence; $(-)$, absence

Finally, in a separate experiment, modification of the concentration of bacteria suspended in the water phase was achieved through the continuous addition of a stream of *Pseudomonas aeruginosa* (ATCC 15442) at a final concentration of $1 \cdot 10^5$ cfu/ml. The stream of bacteria was drawn from a steady state continuous culture (BIOFL, New Brunswick co., New Jersey) running at a dilution rate of 0.1 h$^{-1}$. The organism was grown in a 1 liter culture vessel with agitation set at 600 rpm, a temperature of 25 °C and 500 l/h of aeration.

The culture medium used was a synthetic formulation with the following composition per liter: sodium glutamate 0.5 g, KH$_2$PO$_4$ 2.0 g, MgSO$_4$ · 7H$_2$O 1.0 g. Use of a continuous culture as a source of bacteria was necessary in order to ensure that the input of cells had identical characteristics and physiological state throughout the duration of the experiment.

Sampling: Sampling of attached biomass required isolation and removal of individual reactors. After isolation, the reactors were flushed with 100 ml of sterile saline solution (NaCl 0.9%) and the packaging beads were transferred to plastic vials containing 15 ml of sterile saline solution. Attached microorganisms were released by sonication (LEVY 1997, PIRIOU 1997) in a water bath (3 min, 40 W). After sonication samples were shaken for 30 sec in a vortex mixer, diluted in saline solution and counted in agar plates.

Analyses: Water circulating through the system was sampled at regular intervals to determine the concentration of suspended microorganisms and the chlorine levels. Chlorine (total and free) was measured by the DPD method according to Standard Methods (CLESCERI et al. 1998). Viable counts enumeration were performed by filtration through 0.22 µm pore-size membrane filters (Millipore) plated onto duplicate R2A agar (DIFCO Laboratories Inc., Detroit, Mich) plates and incubated at 22 °C during 7 days in semi-closed plastic bags and sealed with parafilm.

Biodegradable dissolved organic carbon (BDOC) analysis were performed according to procedure described by LEVI and JORET (1990).
For attached biomass analysis, different replicates were subsampled, and viable counts were performed by membrane filtration through 0.22 μm pore-size membrane filters (Millipore) or by the spread plate method, using in both cases R2A agar (Difco Laboratories Inc., Detroit, Mich). The plates were incubated for 7 days at 22 °C in semi-closed plastic bags and sealed with parafilm.

*Pseudomonas aeruginosa* was enumerated in Plate Count Agar (PCA) plates (Cultimed, Spain) incubated at 37 °C for 24 h.

**Bacterial identification:** At the end of kinetics, the predominant colonial morphologies were selected and isolated for further identification according to the scheme proposed by Lechevallier *et al.* (1980). The characteristics of identified strains were confirmed according to the typical characteristics given in the Bergey’s Manual of Systematic Bacteriology (Butler 1986).

**Results**

**Tap water characteristics**

The average concentrations of free chlorine and total chlorine were 0.59 mg/l (n = 41, SD = 0.08) and 0.73 mg/l (n = 41, SD = 0.09). BDOC levels had a mean of 0.39 mg/l (n = 5, SD = 0.17) and represented the 22% of total organic carbon (1.75 mg/l).

The average heterotrophic plate count was 59 cfu/100 ml (n = 37, SD = 40) with a proportion of pigmented colonies which ranging from 24 to 90% of the viable count. It has been proposed that percentages of chromogenic bacteria higher than 25% of the heterotrophic plate count are indicative of nonpolluted water samples (Guthrie *et al.* 1974). In the case of drinking waters, except for anomalous cases, all samples should be considered un-polluted. Even so, Reasoner *et al.* (1989) suggested that sharp changes in the percentage of total pigmented bacteria could indicate changes in water quality such as post-treatment contamination event or a change in disinfectant residual.

**Effect of the levels of suspended bacteria on biofilm formation**

In order to evaluate how the concentration of bacteria in suspension affected the kinetics of biofilm formation, two separate experiments were performed. In both experiments, the continuous addition of an exponentially growing culture of *Pseudomonas aeruginosa* (μ = 0.1 h⁻¹) from the overflow of a continuous culture artificially increased the concentration of bacteria in the water stream perfusing the reactors, from the average value of 59 cfu/100 ml up to 1 · 10⁷ cfu/100 ml through. In one of the experiments chlorine was neutralized by the continuous addition of a Na₂SO₃ solution. In the second experiment, chlorine levels were between 0.5–0.6 mg/l, the values usually found in the water supply. In both cases, the increase in attached biomass was followed at regular intervals for a period of one week. The results, shown in Fig. 2 indicate that in the presence of chlorine, significant biofilm forma-
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Fig. 3
Kinetics of biofilm formation in different conditions

tion could not be detected even after 170 h of continuous addition of bacteria. When chlorine was neutralized, colonization of the glass surface occurred very quickly, reaching a stable level of $10^5$ cfu/cm$^2$ in only 24 h.

Effect of organic matter and chlorine on biofilm formation

In order to evaluate the effect of both factors, four different kinetics were performed. The different experiments are summarized in Table 2. In the first one, chlorine levels were neutralized and organic matter levels increased. In the second experiment, chlorine levels were also neutralized but organic matter levels were not increased. In the third experiment, chlorine levels were not neutralized and organic matter levels were increased. The last one was a control, in which the tap water was not modified. Results are represented in Fig. 3.

In tap water, without any modification (chlorine +, acetate –) it was not possible to detect a significant community of attached cells after 22 days (<1 cfu/cm$^2$). Biofilm formation was detected when chlorine was neutralized with and without acetate addition. In these cases, after 15 days the density of culturable microorganisms remained stable between $1 \cdot 10^6$–$1 \cdot 10^7$ cfu/cm$^2$. 
When acetate was added without neutralization of the chlorine, it was not possible to detect a significant biofilm formation (<1 cfu/cm$^2$). This result showed the role of chlorine. When chlorine levels are correct, biofilm formation is inhibited.

Kinetics of biofilm formation seems to be faster without acetate addition than with acetate addition. But, since the different experiments corresponding to different kinetics were not done at the same time, this difference may be due to variations in bacterial charge of inlet water during biofilm development.

**Bacterial identification**

All bacterial strains isolated from biofilms were gram negative non-glucose fermenters. In kinetics 1 (Chlorine −, Acetate +) 28 strains were isolated and all of them were identified as *Pseudomonas* spp. In kinetics 2 (Chlorine −, Acetate −) 22 strains were isolated, 15 of them were identified as *Alcaligenes* spp., 5 were identified as *Pseudomonas* spp., 1 was identified as *Moraxella* spp. and the remaining one was identified as *Acinetobacter* spp.

**Discussion**

In presence of normal levels of chlorine it was not possible to detect biofilm formation after one month, even when bacterial charge and organic matter levels were increased. When chlorine was neutralized the biofilm began to develop in a few days and after 15 days the density of culturable microorganisms remained stable between $1 \cdot 10^6$–$1 \cdot 10^7$ cfu/cm$^2$. Although most of the bacteria present in oligotrophic aquatic systems are metabolically active (Kuznetsov *et al.* 1997), only less than 1% can be cultured (Staley and Konopka 1985). Therefore, plate counts of biofilm bacteria on R2A agar has been estimated as 0.1% or less of total cell counts (Guthrie *et al.* 1974). However, bacteria attached to surfaces are more active than planktonic bacteria (Manz *et al.* 1993) and in this case it has been postulated that dominating bacteria were culturable on R2A medium (Kalmbach *et al.* 1997). For this reason our isolated strains could be considered as representing the dominant bacteria from biofilm.

In our study, acetate addition in the absence of chlorine seems to have had effect in the selection of HPC dominant bacteria. When a easily biodegradable substrate such as acetate was added, *Pseudomonas* spp. was the dominant genus. *Pseudomonas* group has an important role in water systems and has been proposed as an indicator of potential regrowth in water distribution systems (Ribas *et al.* 2000). For this reason it is not surprising that one strain of *Pseudomonas fluorescens* (P17) was selected as a test microorganism for the assimilable organic carbon assay (Van der Kooij 1983). In absence of acetate however, and with a low content of biodegradable organic carbon, different dominant genera were detected. Without nutrient limitation it is probable that microorganisms with best capacity to grow during the process of biofilm formation become predominating. It could explain the fact that when acetate was added, only one predominating genus was detected.

Biofilm development is due to growth from attached cells and by cell recruitment from the bulk fluid phase. As different kinetics were not performed at the same time, characteristics of inlet water could be different in each experiment and obviously the final diversity of biofilm could be affected by changes in the type of initial attached populations and by the recruitment of different cells. For this reason, although the predominance of β-subclass of proteobacteria in biofilms has been documented (Wagner *et al.* 1993, Weiss *et al.* 1996) it is reasonable to think that percentages of each genus, in multi-species biofilms, could vary.

In normal conditions chlorine levels are able to prevent bacterial proliferation and biofilm development in spite of an accidental entry of non-autochthonous microorganisms or the increment of BDOC levels in the distribution system. However, in all water distribution
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systems biofilms exist. In presence of chlorine, biofilm formation may appear but probably more slowly than in its absence.

The role of chlorine is very clear, but the speed of biofilm formation, microorganisms diversity and density may also be modulated by surface shear stress (DUDDRIDGE et al. 1982), pipe material (VAN DER KOOIJ and VEEENDAAL 1993), temperature (ROGERS et al. 1994) and BDOC (ROMPRÉ et al. 1995). Among these parameters, the levels of organic matter have been postulated not to be the most influential (VOLK and LECHEVALLIER 1999). However, reduction of BDOC has to be considered with other perspectives. In fact, all bacterial regrowth in drinking water systems will not be stopped by the limitation of the nutrient levels, although it is well known that the occurrence of coliform bacteria is dependent upon a complex interaction of several factors, and one of them is nutrient levels (LECHEVALLIER et al. 1996, 1991). For this reason, although BDOC has only a little impact on biofilm, it may be a key point in order to prevent undesirable growth. Moreover, a reduced level of organic matter reduces chlorine demand and increases disinfectant stability, allowing optimization of chlorine dosage, minimizing disappearance during distribution. Consequently, it improves the ability of chlorine to act against free or attached microorganisms; although the limited capacity of chlorine on biofilms is a known fact.

Although it is not a universal premise, nowadays in some European countries (e.g. The Netherlands) there is a tendency to reduce or eliminate the use of chlorine, and this doesn’t have any dramatic impact on public health. Nevertheless, it is necessary to consider that a drinking water distribution system is a complex structure with kilometers of pipes, and it is very difficult to ensure correct chlorine levels in all points. If problems exist with chlorine levels, biofilm formation may be allowed or accelerated. Without chlorine addition and with a biofilm presence, the capacity to control microbial quality is reduced. For this reason, it is important to ensure sufficient chlorine levels in all points in order to prevent biofilm formation and to guarantee the microbial quality of water. It is well known that in drinking water systems of big buildings (such as hospitals), especially in hot water conductions, colonization by Legionellaceae involves a serious hazard, and the need to implant strategies to control bacterial growth is evident.

The results of this study indicate that an adequate level of chlorine (around 0.5 mg/l) is enough to ensure the absence of biofilms even after microbial contamination events or input of organic carbon in the system. On the contrary, reduction of chlorine levels, even without adding carbon or increasing the suspended microbial load allows the development of an attached community in approximately two weeks.

Work with packed bead reactors is an easy method to study drinking water biofilms and allows the securing of considerable samples of biofilms. It is uncomplicated and fast to develop biofilms in laboratory when chlorine is neutralized. These biofilms could be a useful tool to improve our knowledge about drinking water biofilms and help us understand the behavior of biofilm in front of the different incidences that could appear in a drinking water system.

References


identification of bacteria in drinking water and adjoining biofilms by hybridization with Pseudomonas fluorescens to stainless steel under defined flow conditions. Biotech. and Bioeng., 24, 153–164.


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