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Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials

Miriam M. Moritz, Hans-Curt Flemming, Jost Wingender*

Biofilm Centre, Department of Aquatic Microbiology, Faculty of Chemistry, University of Duisburg-Essen, Geibelstraße 41, D-47057 Duisburg, Germany

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ABSTRACT

Drinking water biofilms were grown on coupons of plumbing materials, including ethylene-propylene-diene-monomer (EPDM) rubber, silane cross-linked polyethylene (PE-X b), electron-ray cross-linked PE (PE-X c) and copper under constant flow-through of cold tap water. After 14 days, the biofilms were spiked with *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Enterobacter nimipressuralis* (10^6 cells/mL each). The test bacteria were environmental isolates from contamination events in drinking water systems. After static incubation for 24 h, water flow was resumed and continued for 4 weeks. Total cell count and heterotrophic plate count (HPC) of biofilms were monitored, and *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* were quantified, using standard culture-based methods or culture-independent fluorescence in situ hybridization (FISH). After 14 days total cell counts and HPC values were highest on EPDM followed by the plastic materials and copper. *P. aeruginosa* and *L. pneumophila* became incorporated into drinking water biofilms and were capable to persist in biofilms on EPDM and PE-X materials for several weeks, while copper biofilms were colonized only by *L. pneumophila* in low culturable numbers. *E. nimipressuralis* was not detected in any of the biofilms. Application of the FISH method often yielded orders of magnitude higher levels of *P. aeruginosa* and *L. pneumophila* than culture methods. These observations indicate that drinking water biofilms grown under cold water conditions on domestic plumbing materials, especially EPDM and PE-X in the present study, can be a reservoir for *P. aeruginosa* and *L. pneumophila* that persist in these habitats mostly in a viable but non-culturable state.

Introduction

In drinking water distribution systems, all surfaces in contact with water can be colonized by microorganisms (Kilb et al., 2003; Servais et al., 1995; Wingender and Flemming, 2004). Certain types of plastic and elastomeric materials can promote biofilm formation due to the release of biodegradable compounds providing favourable nutrient conditions for microorganisms (Keevil, 2002; Kilb et al., 2003; Rogers et al., 1994b). It has been estimated that about 95% of all microbial cells present in drinking water distribution systems exist as biofilms on pipe surfaces and only 5% occur in the water phase (Flemming et al., 2002); similarly, in a domestic hot water system, most of the culturable bacteria (72%) were found to be surface-associated (Bagh et al., 2004). Drinking water biofilms are formed predominantly by microorganisms of the autochthonous aquatic microflora without any relevance to human health. However, drinking water biofilms have the potential to harbor opportunistic pathogens which can harm human health, especially in immunocompromised people (Flemming et al., 2002; Keevil, 2002). Once integrated in a drinking water biofilm, these organisms are protected from external stresses such as the action of disinfectants and can thus persist and possibly multiply inside the biofilm. Contamination of drinking water occurs when opportunistic pathogens are released from a biofilm as a consequence of physical disturbance or active detachment of infectious cells, which then pose a potential threat to human health (Flemming et al., 2002; Szewzyk et al., 2000).

Important opportunistic pathogens which can be involved in biofilm-associated contamination of domestic plumbing systems are *Pseudomonas aeruginosa* and *Legionella pneumophila* (Eboigbodin et al., 2008; Keevil, 2002). *P. aeruginosa* appears sporadically in drinking water distribution systems, for example as a consequence of contamination during construction works (Clark et al., 1982; Hambsch et al., 2004), but these bacteria seem to occur at a higher frequency in domestic plumbing systems compared to water mains (Wingender et al., 2009). In drinking water biofilms, *P. aeruginosa* has been observed to be occasionally present (Emtiazi et al., 2004; Kilb et al., 2003; Lee and Kim, 2003). The occurrence of *L. pneumophila* has been associated with biofilms in warm water plumbing systems, where the bacteria persist and can replicate in association with free-living protozoa (Lau and Ashbolt, 2009). Growth of *L. pneumophila* occurs between 25 °C and 45 °C. How-

* Corresponding author. Tel.: +49 203 379 1938; fax: +49 203 379 1941.
E-mail address: jost.wingender@uni-due.de (J. Wingender).

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ever, there are indications that *L. pneumophila* can also survive at lower temperatures in drinking water environments. Thus, the persistence of *L. pneumophila* in drinking water biofilms cultivated at 15 °C or 20 °C has been reported (Gião et al., 2009; Lehtola et al., 2007; Rogers et al., 1994a). Coliform bacteria, which also include opportunistic pathogens such as certain *Citrobacter*, *Enterobacter* and *Klebsiella* species, are occasional contaminants of drinking water distribution systems and have been found in the biofilms on pipe walls and rubber-coated valves in these systems (Kilb et al., 2003; LeChevallier et al., 1987; Wingender and Flemming, 2004; Feuerpfeil et al., 2009). However, their distribution and hygienic relevance in domestic plumbing systems is largely unknown.

The detection of opportunistic bacteria in drinking water biofilms has usually been performed by methods based on the culturability of the organisms. However, bacteria may enter a viable but non-culturable (VBNC) state as a response to some form of environmental stress (Oliver, 2005). In the VBNC state the bacteria fail to grow on routine bacteriological media, but they are still alive and typically demonstrate low levels of metabolic activity. Viability markers of VBNC cells may be respiratory activity, cytoplasmic membrane integrity or the presence of ribosomes detected by fluorescence in situ hybridization (FISH) using oligonucleotide probes targeted at specific sequences of 16S rRNA molecules. Oliver (2005) listed more than 60 bacterial species which have been described to be capable of entering the VBNC state, including *P. aeruginosa*, *L. pneumophila* and coliform species. Investigations of the VBNC state are usually performed using planktonic cells, so it is largely unknown if the VBNC state can also be induced in biofilm environments.

The effect of pipe materials on biofilm formation as well as on the persistence of pathogens in biofilms has mainly been studied on materials which make up drinking water distribution systems, while domestic plumbing materials that usually differ from those of distribution systems have been considered less frequently (Eboigbodin et al., 2008; Rogers et al., 1994a, 1994b). Common plumbing materials are copper and plastics, but other material types such as elastomers can also be found as components of plumbing systems (WHO, 2006).

The aim of the present study was to investigate the possibility that the integration and persistence of *P. aeruginosa*, *L. pneumophila* and the coliform species *Enterobacter nimipressuralis* in pre-established drinking water biofilms is influenced by the type of plumbing material. The focus was on copper, plastic and elastomeric materials that occur as relevant components of plumbing systems in Germany. The fate of the bacteria introduced into the biofilms was traced by both cultural methods and the culture-independent FISH technique in order to recognize a possible VBNC state of the target organisms on the different plumbing materials.

Materials and methods

Bacterial strains

P. aeruginosa AdS was a water isolate from an automatic shut-off valve of a shower in a plumbing system of a school building. Identification was performed, using the API 20 NE system (bioMérieux) and GN Microplates (Biolog) according to the manufacturer's instructions. *L. pneumophila* AdS (serogroup 1) was a biofilm isolate from the same automatic shut-off valve. Species and serogroup determination of *L. pneumophila* was performed, using a commercially available latex agglutination test kit (Oxoid). Species identity of both *P. aeruginosa* AdS and *L. pneumophila* AdS was confirmed by 16S rDNA sequencing. *E. nimipressuralis* 9827 clone A was isolated from an elevated tank of a drinking water supply system; the isolate was kindly supplied by Prof. Exner, University of Bonn, Germany.

Plumbing materials

Four different materials were employed: copper, silane cross-linked polyethylene (PE-X b), electron-ray cross-linked polyethylene (PE-X c) and ethylene-propylene-diene-monomer (EPDM) rubber. The PE-X b, PE-X c and EPDM fulfilled the physical and chemical specifications of German recommendations for plastic or rubber materials (Anonymous, 1977, 1985) as well as the microbiological specifications of the German Gas and Water Association (Anonymous, 2007) as a prerequisite for their use in contact with drinking water. The materials were employed in the form of coupons (26 mm × 76 mm), which were treated with 70% (v/v) ethanol for 10 min, washed in deionized water and air-dried for 24 h before use.

Preparation of bacteria

For cultivation of *P. aeruginosa* AdS or *E. nimipressuralis* 9827 clone A, 20 mL of Lenox broth (per L: 10 g tryptone, 5 g NaCl, 5 g yeast extract, pH 7.0) in a 100-mL Erlenmeyer flask were inoculated with a single colony pre-grown on nutrient agar (Merck) at 36 °C for 24 h, and the culture was incubated at room temperature (approximately 23 °C) with shaking at 180 rpm for 24 h. For cultivation of *L. pneumophila* AdS, 20 mL of yeast extract broth (Ristroph et al., 1980) were inoculated with a single colony pre-grown on BCYEα agar (Oxoid) at 36 °C for 72 h, and the culture was incubated at 36 °C with shaking at 180 rpm for 24 h. Bacteria from the liquid cultures were harvested by centrifugation (15 min, 1912 × *g*, 10 °C), washed twice in 20 mL of filter-sterilized tap water and suspended in 200 mL of filter-sterilized tap water to a concentration of approximately 3×10^6 cells/mL. The bacterial suspensions were incubated statically at 20 °C (*P. aeruginosa* and *E. nimipressuralis*) or 30 °C (*L. pneumophila*) for 24 h and subsequently combined.

Cultivation and inoculation of drinking water biofilms

Drinking water biofilms were grown in a 200-L stainless steel tank connected to a cold water laboratory tap and perfused with drinking water at a flow rate of 20 L/h. The concentration of assimilable organic carbon in the drinking water was approximately 6 μg C/L (G. Schaule, personal communication). Up to 32 coupons of the plumbing materials were introduced vertically into the tank and fixed with stainless steel clamps to a stainless steel bar. After 14 days of perfusion with drinking water, coupons were transferred to 100-mL stainless steel flow-through reactors. Six coupons of each material were vertically placed in a reactor with a distance of 3 mm between the coupons. The reactors were filled with a suspension of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* (10^6 cells/mL of each organism) by injecting 100 mL of the bacterial suspension into the tubing at the reactor inlet. After static incubation at room temperature for 24 h, the reactors were connected to a cold water laboratory tap and continuously perfused with drinking water for 4 weeks.

Microbiological analysis

Samples for microbiological analysis were biofilm suspensions and effluent from the biofilm reactors. For biofilm analysis, the biomass from both sides of two coupons was scraped off, using a sterile rubber scraper and suspended into 20 mL deionized water. For biofilm dispersion, the suspensions were vortexed for 2 min. Serial dilutions of the suspensions were prepared in deionized water. The water phase was sampled by collecting reactor effluent in sterile 250-mL glass flasks.

Determination of the total cell count in biofilm supensions was performed by staining the cells with the fluorochrome 4′,6-