Survival of pathogens in drinking water plumbing systems: impact factors and sanitation options

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ABSTRACT

The objective was to determine impact factors that would allow *Pseudomonas aeruginosa* and *Legionella pneumophila* to survive sanitation measures of household drinking water systems. Therefore, different disinfectant concentrations were tested under different operating conditions in close-to-practice experiments. Particular attention was paid to the viable but non-culturable (VBNC) state of the pathogens. *Legionella pneumophila* was able to survive disinfection with ClO₂ in a culturable state in the biofilm at 37°C while successful sanitation could be achieved at 11°C, however non-culturable cells were still present. Culturable *P. aeruginosa* outlasted disinfection in biofilms of pipes at elevated nutrient concentrations and in built-in parts at 37°C. Overall, the VBNC state was not the predominant factor for its survival. Additional experiments showed that the lack of an autochthonous biofilm may promote the growth of *P. aeruginosa*. This emphasizes the importance of localization and elimination of contamination sources in a plumbing system before disinfection is performed and the need for compliance with operating conditions and construction requirements defined by generally recognized standards of good practice in Germany.

Key words | biofilms, disinfection, drinking water, opportunistic pathogens, premise plumbing

INTRODUCTION

Domestic drinking water plumbing systems play a central role in the case of drinking water contamination with opportunistic pathogenic bacteria like *Legionella pneumophila* and *Pseudomonas aeruginosa* (Flemming *et al.* 2014). A nationwide survey in Germany with 4,600 public buildings inspected between 2003 and 2009 and 30,000 evaluated drinking water samples showed that 12.8% of the household installations exceeded the technical action value for *Legionella* spp. (100 cfu in 100 ml) and 2.9% exceeded the guideline value for *P. aeruginosa* (0 cfu in 100 ml) (Völker *et al.* 2010). These opportunistic pathogens can be found in drinking water biofilms of domestic plumbing systems from where they can contaminate the planktonic phase of drinking water (Flemming *et al.* 2002; Eboigbodin *et al.* 2008; Moritz *et al.* 2010) and in that way the entire plumbing system. In such a biofilm, pathogens are able to persist in a viable but non-culturable (VBNC) state and, thus, cannot be detected with standard culture based methods (Juhna *et al.* 2007; Benölken *et al.* 2010; Moritz *et al.* 2010).

For sanitation, chemical or thermal disinfection are usually used. In practice however, shock disinfection of a facility often causes only a transient disappearance of the pathogens suggesting that they survived the procedure (Benölken *et al.* 2010). Thus, for a successful sanitation it is crucial to understand the interactions between operating conditions, biofilm, growth characteristics of the pathogens and disinfection.

The objective of this study was to determine impact factors that would allow *P. aeruginosa* and *L. pneumophila* to survive the sanitation measures of plumbing systems. Therefore, different disinfectant concentrations under different operating conditions such as temperature, pipe material,
and nutrient concentration in the water were tested in close-to-practice experiments. Particular attention was paid to the relevance of the VBNC state of the bacteria. The results should help to improve recommendations for secure operating conditions and successful sanitation strategies for drinking water plumbing systems.

MATERIAL AND METHODS

Bacterial strains

Wild type strains of *L. pneumophila* AdS and *P. aeruginosa* AdS were obtained from the Biofilm Centre, Aquatic Microbiology, University of Duisburg-Essen, Germany. Cells were harvested from liquid culture, washed two times in filter sterilized water of the same quality as used in the subsequent experiment and starved for 24 h at either 11 or 37 °C.

Close-to-practice long-term experiments

Two test rigs containing eight parallel pipelines each were operated simultaneously. One test rig was operated at 37 °C and one at 11 °C. Each pipe simulated separate drinking water plumbing (Figure 1). A consumption program with standardized flow (in total 29 min per day) and stagnation times (in total 23 h 31 min per day) according to the German norm DIN EN 15664-1 (2014) (145 l/d) was applied. Each line was operated under different conditions (temperature, pipe material, nutrient concentration, and disinfection method) (Meier & Bendinger 2014).

The test rigs were installed in a local waterworks and operated with non-chlorinated drinking water that had been produced from anaerobic groundwater by closed pressure aeration with technical oxygen followed by a two-step rapid sand filtration. Selected parameters describing the water quality are given in Supplementary Material, Table 1 (available with the online version of this paper).
The water quality in one line of a test rig could be modified by dosing of a nutrient solution. The final concentration in the feed water was 1.74 mg/l sodium phosphate, 13.7 mg/l sodium nitrate, and 0.34 mg/l sodium acetate (equals 100 μg/l ac-C).

The four pipe materials used were copper (half-hard), stainless ferritic chrome steel 1.4521, high density electron-ray cross-linked polyethylene (PE-Xc) approved for use in drinking water according to DVGW code of practice W 270 (2007) and KTW-recommendations category A (Anonymous 1985), and ethylene propylene diene monomer (EPDM) not approved for use in drinking water. All pipes had an inner diameter of 13 mm. One line of a test rig contained two parallel pipes of each material.

Experiments were carried out over a period of 5 months. The timeline of an experiment is shown in Supplementary Material, Figure 1 (available with the online version of this paper). The test rigs were operated with drinking water for 6–7 weeks in order to establish an autochthonous biofilm with a near-constant total cell count. Then, the test rigs were filled with a suspension of the starved pathogens (10⁶ cells/ml each) for 24 h and subsequently flushed. After six more weeks of operation, the test pipes were mechanically cleaned and disinfected according to DVGW code of practice W 557 (2012). Cleaning was performed by air impulse flushing (5 bar water pressure, air impulse every 2 sec for 1 sec with 5 bar, 60 impulses per pipe). Different disinfection methods and disinfectant concentrations were tested: 50 mg/l NaOCl; 140 mg/l H₂O₂; 0.3, 2, 7.5 and 20 mg/l ClO₂. The most intensive procedure tested was a sequential combination of one thermal (70–80 °C for 3 min at sampling valve) and two chemical disinfections (70 mg/l ClO₂) (chemical–thermal–chemical, CTC). After 24 h exposure time the disinfectant had been consumed completely on copper and EPDM. In order to fulfil the recommendations of DVGW code of practice W 557 (2012), disinfection was repeated two times before the pipes were set back to the consumption program. In PE-Xc and stainless steel pipes, disinfectant was still measurable after 24 h exposure.

Right before the disinfection event the test pipes were disassembled and mounted again in test lines with heat sterilized peripheral plumbing of the test rig and built-in parts such as pipe junctions, check valves and sampling valves. That way it was assured that no pathogen from outside the test pipe biofilm could contaminate the test system. Afterwards the test rigs ran for 6 more weeks.

**Experiment in test rig to evaluate the influence of built-in parts on the survival of *P. aeruginosa***

For disinfection contaminated test pipes operated at 37 °C with water enriched with inorganic nutrients (1 mg/L ortho-phosphate and 10 mg/l nitrate) were treated with 140 mg/l H₂O₂ or 20 mg/L ClO₂ as described above. In contrast to the disinfection procedure described above no disassembly and exchange of the built-in parts (valves and junctions) and transfer into heat sterilized peripheral plumbing was performed. Forty to 42 days after the disinfection measure the built-in parts of one of the parallel pipes of each material were replaced by heat sterilized ones. After 3 days of operation, the bulk water of the pipes with and without exchanged built-in parts were analyzed for *P. aeruginosa*.

**Sampling**

Bulk water and biofilm were sampled at the end of an 8 h overnight stagnation phase. Sampling was carried out on specific days: 6 weeks after start, 24 h, 4 weeks and 6 weeks after contamination event, 24 h, 3 weeks and 6 weeks after a cleaning and disinfection measure. In total seven samples were taken from each pipe. The non-contaminated reference pipe (EPDM) was sampled in a similar manner to rule out cross contamination among pipes.

Before taking 700 ml water samples, the sampling valves were sterilized by flaming and the first 50 ml were discarded. Biofilm samples were taken by cutting off 25 cm long sections from the rear end of a test pipe. Biofilm from 102 cm² was isolated by mechanical abrasion with zirconium oxide beads (average diameter of 0.4 mm) in filter sterilized mineral water as described by Meier & Bendinger (2014).

**Laboratory experiments for evaluating the effect of autochthonous biofilm on the proliferation of *P. aeruginosa***

Twenty-five centimetre long pipe sections carrying a 6 week old drinking water biofilm were taken from the 37 °C test rig. Subsequently, half of the pipes were disinfected with 50 mg/l
NaOCl for 24 h and half of them were not treated. Duplicate pipes were filled with a suspension of starved *P. aeruginosa* (2,000 cells/ml) and incubated at 37 °C for 16 h. After that, the concentration of *P. aeruginosa* in the biofilm (102 cm²) and water phase (62 ml) was analysed by cultivation and quantitative polymerase chain reaction (qPCR).

**Culture detection and quantification of index organisms**

Colony forming units (cfu) of *P. aeruginosa* were determined on selective cetrimide agar according to ISO 16266:2006. The quantification and identification of *L. pneumophila* was done as described in ISO 1731-2:2004 on selective GVPC agar. Triplicates were prepared of at least 3 decimal dilution stages. The enumeration of cfu was done according to ISO 8199 (2005). The weighted average was calculated according to the equation \( C_S = \frac{Z}{V_{tot}} \times V_S \) with \( C_S \) being the calculated cfu in the reference volume \( V_S \), \( Z \) is the total amount of positive colonies found in all dilutions. \( V_{tot} \) is the total volume analyzed. Only plates with <200 cfu were included. Standard deviations were calculated for error bars.

**qPCR detection and quantification of index organisms**

The PowerBiofilm™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) was used to isolate DNA from water and biofilm samples according to manufacturer’s information. Cells in water samples as well as biofilm samples were retained on polycarbonate filters (0.2 μm) for DNA-extraction. Biofilm samples that could not be filtered (i.e. from EPDM pipes) were centrifuged at 10,000 g for 5 min. TaqMan® qPCR was done in duplicate according to the specifications from Shannon *et al.* (2007) using primers and probes designed by Lee *et al.* (2006) (sequences are given in Supplementary Material, Table 2, available with the online version of this paper). For quantification five dilutions of standards were carried along in each run. Standards were prepared according to Röder (2011) and their sequences are given in Supplementary Material, Table 3 (available online). Calculation of gene copies per microlitre standard was carried out according to Röder (2011) as well. Real-time PCR reactions were executed in a Step One™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using the TaqMan® Gene Expression Master Mix (including ROX as a reference dye) with the following temperature profile: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s. Results are given in gene copies (gc) per ml or per cm². The detection limit was 10 gc per μl of the standard for both genes. Amplification plots and standard curves are depicted in Supplementary Material, Figures 2–5 (available online).

**Evaluation of the success of facility disinfection**

A disinfection procedure was considered successful if the cell concentration was below the respective detection limit. For culturable *L. pneumophila* and *P. aeruginosa*, the detections limits were 1 cfu in 100 ml bulk water or 1 cfu per 100 cm² surface area. For qPCR the detection limits for both pathogens were 1,000 gc in 100 ml and 1,000 gc per 100 cm².

**RESULTS AND DISCUSSION**

Figures 2 and 3 show the impact of the parameters pipe material, water quality, operating temperature and disinfection procedure on the effectiveness of the respective sanitation attempt for *L. pneumophila* and *P. aeruginosa*, respectively. This way the favorable conditions for a successful sanitation are illustrated.

In all experiments the pathogens detected after disinfection in either biofilm or bulk water must have originated from the biofilm in the test pipe. Other options could be ruled out, since the pipes were removed from the ‘contaminated’ line and connected to a line with heat sterilized (autoclaved) peripheral piping and built-in parts.

**Effects of cleaning and disinfection on *L. pneumophila***

At 11 °C no culturable *L. pneumophila* (0 cfu in 100 ml) were detected in the bulk water (with and without added nutrients) after all disinfection methods applied (Figure 2). In the corresponding biofilm, the bacteria were only detected on EPDM or steel after disinfection with ClO₂, but not after CTC disinfection. However, in 15 out of 16 pipe biofilms and in six out of 16 bulk water samples *L. pneumophila* was detected by qPCR in considerable numbers 24 h after disinfection. Thus, at 11 °C, *L. pneumophila*
can survive even very intensive disinfection measures in biofilms probably in a VBNC state.

At 37°C, culturable *L. pneumophila* had decreased significantly on all materials 24 h after disinfection. Their numbers were below or slightly above detection limit and qPCR indicated always higher concentrations of *L. pneumophila* in biofilm and stagnating bulk water samples. However, an increase in numbers of culturable cells was observed 3 weeks after the disinfection procedure on all materials (Supplementary Material, Figure 6, available with the online version of this paper). Thus, *L. pneumophila* could not be eliminated from the biofilms by disinfection with any of the applied ClO₂ concentrations. Also 140 mg/l H₂O₂ and 50 mg/l NaOCl were ineffective in earlier experiments (data not shown). Even the most intensive CTC disinfection procedure, combining chemical disinfection

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**Figure 2** | Matrix for evaluation of the effects of different disinfection procedures on *L. pneumophila* in biofilm and bulk water with different pipe materials, nutrient concentrations (N, P, and AOC), and temperatures. Displayed are the maximum numbers of colony forming units (cfu) and gene copies (gc) of *L. pneumophila* in biofilm and stagnating bulk water obtained during 6 weeks after shock disinfection. n.d., not detected, ● pipe with cfu or gc below detection limit prior to disinfection.

**Figure 3** | Matrix for evaluation of the effects of different disinfection procedures on *P. aeruginosa* in biofilm and bulk water with different pipe materials, nutrient concentrations (N, P, and AOC) and temperatures. Displayed are the maximum numbers of colony forming units (cfu) and gene copies (gc) of *P. aeruginosa* in biofilm and stagnating bulk water obtained during 6 weeks after shock disinfection. n.d., not detected, ● pipe with cfu or gc below detection limit prior to disinfection.
and heat treatment, was found to be unsuccessful at 37 °C. *Legionella* survived in the biofilms in a culturable or VBNC state and recontaminated the bulk water, where they always reached numbers above the technical action value valid in Germany (100 cfu in 100 ml). Values up to $8 \times 10^6$ cfu/100 ml were reached in EPDM pipes. Every cultural detection of *L. pneumophila* was confirmed by qPCR.

Thus, a low operating temperature of 11 °C was effective in preventing regrowth of *L. pneumophila*. However, at a moderate temperature of about 37 °C none of the applied intensive disinfection methods was capable of preventing regrowth of the pathogen.

**Effects of cleaning and disinfection on *P. aeruginosa***

At 11 °C, culturable *P. aeruginosa* were not detected in bulk water or biofilm (Figure 3). The only exception was EPDM in water with added nutrients after treatment with 2 mg/l ClO$_2$. However, in 10 out of 16 biofilms in water with or without nutrient addition, non-culturable *P. aeruginosa* were detected by qPCR. The cells were still detected after the most intensive CTC disinfection on all materials. The observation that these cells did not give rise to culturable cells in the corresponding bulk water indicates that the qPCR signals might have derived from VBNC cells or dead cells with intact DNA. In all 53 biofilm or bulk water samples from 15 pipes where neither culturable nor non-culturable *P. aeruginosa* were detected after disinfection (green boxes) its number of cfu and gc in biofilm and bulk water had already decreased below detection limit before the disinfection procedure had been started (green boxes with dot). Thus, after initial integration into existing biofilms, *P. aeruginosa* was not able to survive under the given conditions for 6 weeks in the biofilm and didn’t show any regrowth after disinfection.

Also at 37 °C without added nutrients, culturable *P. aeruginosa* were not detected after disinfection in biofilms and bulk water. In 10 out of 16 biofilm and bulk water samples from eight pipes (green boxes with dot), culturable *P. aeruginosa* were not detected prior to disinfection (Supplementary Material, Figure 7, available with the online version of this paper), thus leaving the question open as to whether the disinfection was successful. At least no regrowth had been observed in these pipes after disinfection. However, if nutrients were added to the water the numbers of culturable *P. aeruginosa* were not reduced but instead had increased 24 h after disinfection in almost all pipes (Figure 4). In EPDM pipes the pathogen reached high concentrations of about $3 \times 10^6$ cfu per 100 cm$^2$ surface and about $3 \times 10^7$ cfu per 100 ml bulk water. Even by the most intensive CTC disinfection, culturable *P. aeruginosa* had not been eliminated from biofilm. Solely in the copper pipe treated by CTC disinfection, *P. aeruginosa* was not detected in biofilm by cultivation while qPCR of the biofilm sample and cultivation of the water sample gave positive results. This exception might suggest a survival of *P. aeruginosa* in the VBNC state on copper surface.

The sanitation success of pipes contaminated with *P. aeruginosa* at 37 °C is strongly influenced by the nutrient concentration of the water.

**Figure 4** | Transient increase of culturable *P. aeruginosa* in biofilm and bulk water in test pipes of the 37 °C test rig 24 h after disinfection with 2 mg/l ClO$_2$ (with added nutrients). Error bars indicate standard deviations.
Influence of autochthonous biofilm on proliferation of introduced \textit{P. aeruginosa}

In order to verify the increase of culturable \textit{P. aeruginosa} in biofilm and bulk water 24 h after disinfection, laboratory experiments were conducted with pipe sections taken from the 37 °C test rig. Untreated pipes carrying an autochthonous biofilm on their surface and pipes containing a biofilm that had been treated by disinfection were filled with a suspension of \textit{P. aeruginosa}.

Within 16 h at 37 °C, \textit{P. aeruginosa} multiplied to higher numbers in biofilm and bulk water of EPDM, PE-Xc and steel pipes treated by disinfection, compared to the untreated pipes of the same materials (Figure 5).

The observed growth was particularly pronounced on EPDM. The culturable and non-culturable \textit{P. aeruginosa} densities on treated surfaces were 1,000 and 10,000 times higher than on untreated surfaces. In PE-Xc and stainless steel pipes the numbers of culturable and non-culturable \textit{P. aeruginosa} on the treated surfaces were five and two times higher than on the surfaces with untreated biofilms.

For PE-Xc and steel the results of the stagnating water were even more significant. In treated pipes the concentration of culturable \textit{P. aeruginosa} was 100 times (PE-Xc) and 10 times (steel) higher than in pipes with an untreated biofilm. This experiment was repeated with pipes where the biofilm was mechanically removed (abrasion with zirconium oxide beads) instead of disinfection and gave comparable results (data not shown).

We assume that the growth of \textit{P. aeruginosa} in treated pipes was promoted since the autochthonous bacteria in biofilm were inactivated or at least heavily damaged by the disinfectant. Thus, there was no or much less competition, which allowed \textit{P. aeruginosa} to multiply fast. It can be excluded that only biodegradable organic compounds that were produced from organic material in biofilm during disinfection with NaOCl caused the multiplying of the pathogen, since a comparable growth was observed after only mechanical removal of the autochthonous biofilm (data not shown).

In copper pipes, no growth of \textit{P. aeruginosa} after disinfection was observed. Unlike the other test pipes, the culturable and non-culturable concentrations of introduced \textit{P. aeruginosa} on pipe surface and in bulk water of copper pipes treated by disinfection were significantly lower than in untreated pipes. This indicates a protective function of the autochthonous biofilm against the inhibiting impact of copper ions on culturable and non-culturable \textit{P. aeruginosa}. Pizarro \textit{et al.} (2014) showed that most of the copper is associated with the biomass which is attached to the inner surface of a copper pipe. Thus, a disturbance of the copper–biomass complexation by disinfection or mechanical treatment might lead to higher copper-concentration in bulk water and inner pipe surface affecting introduced \textit{P. aeruginosa} cells during the 16 h of the experiment. The decrease of the number of culturable \textit{P. aeruginosa} might suggest its entrance into the VBNC state induced by copper, as described by Bédard \textit{et al.} (2014) and Dwidjosiswojo \textit{et al.} (2011). However, this would not explain the concomitant decrease of the gene copy numbers of \textit{P. aeruginosa} and needs further investigation.

Role of built-in parts for the survival and persistence of \textit{P. aeruginosa}

In a separate experiment in the 37 °C test rig in which built-in parts had not been exchanged before disinfection with sterilized ones, \textit{P. aeruginosa} was detected in a cultivable state in the bulk water of all test pipes (except copper) even after a facility disinfection (140 mg/l H₂O₂ or 20 mg/l ClO₂). However, in the corresponding biofilm samples
P. aeruginosa was not detected by cultivation or qPCR after disinfection (data not shown).

Since the biofilm of the pipes was ruled out as source of contamination, the built-in parts (check valves, sampling valves and pipe junctions with EPDM sealing) were removed and their biofilms were analyzed. In at least one built-in part of each test pipe, culturable P. aeruginosa were detected. To verify the hypothesis that the built-in parts represented the source responsible for the persistent findings of the organism in the water samples, in one of the two parallel pipes of each material the built-in parts were replaced by heat sterilized ones 40–42 days after the disinfection event. After 3 days of operation the water and biofilm samples of the pipes with exchanged built-in parts were free of P. aeruginosa, while it was still detectable by cultivation in the parallel pipes, where the built-in parts had not been exchanged. This is illustrated in Figure 6 by means of the data for EPDM and stainless steel pipes.

CONCLUSIONS

The results show that pathogens were able to endure intensive sanitation measures under special operating conditions. However, in the course of the study it was possible to sanitize contaminated test pipes when the initial operating conditions were set according to the generally recognized standards of good practice in Germany. In the majority of the experiments, the survival of the pathogens in biofilm after disinfection and to proliferate again did not necessarily imply their occurrence in a VBNC state. The following impact factors were found to be critical for survival of the opportunistic pathogens after completed treatment with disinfectant:

Operating temperature

Even though it was not possible to eliminate non-culturable L. pneumophila with any disinfection measure applied, it was possible to suppress a regrowth of L. pneumophila in most biofilms and all bulk waters after disinfection at a temperature of 11 °C. At 37 °C all sanitation attempts failed and L. pneumophila survived in a culturable or VBNC state. Bej et al. (1991) showed that exposure of L. pneumophila to 100 mg/l of hypochlorite caused VBNC cells to form. Mathys et al. (2008) state that temperature of the hot water is probably the most important determinant for the multiplication of Legionella; 37 °C falls right within the temperature range for maximum growth of L. pneumophila between 30 and 46 °C reported by them. Legionella do not or very slowly multiply at temperatures below 20 °C (WHO 2007), which is in accordance with our observation at the operation temperature of 11 °C. Even at 24 °C Buse & Ashbolt (2011) observed no proliferation of L. pneumophila in the presence of amoeba. Thus, in cold water systems a low operating temperature around 11 °C appears to be an effective measure to prevent L. pneumophila proliferation.

At 11 °C, no culturable cells of P. aeruginosa were detected in biofilm and water either before nor after disinfection (with only one exception), while non-culturable cells were still detectable by qPCR in most of the biofilm samples. It is not known whether P. aeruginosa persisted in the biofilm in a VBNC state or was dead. However, under unfavorable operating conditions (37 °C, elevated nutrient concentration), P. aeruginosa was able to survive the applied disinfection measures in a culturable state.

Thus, at 11 °C, culturable and non-culturable P. aeruginosa seemed to be outcompeted by the autochthonous bacteria in biofilm after a couple of weeks and showed no
regrowth after ClO₂-treatments. At 37 °C, culturable *P. aeruginosa* only persisted in biofilm and water at an elevated nutrient concentration and survived even very intensive disinfection attempts.

**Piping material**

The organic material EPDM which released the highest amounts of biodegradable organic compounds for biofilm growth promoted the highest regrowth of surviving cells of *L. pneumophila* in biofilms after disinfection. This is in accordance with findings from Rogers *et al.* (1994) and Moritz *et al.* (2010) from inoculation studies. At 37°C, growth of culturable *L. pneumophila* after the disinfection attempts occurred on copper to the same extent as on stainless steel and, thus, no inhibiting effect of copper as described by Moritz *et al.* (2010) was observed.

In biofilms on the metal surfaces of copper and steel, generally lower numbers of culturable or non-culturable cells of *P. aeruginosa* were found after ClO₂-treatment compared to organic materials. Only in the single case of the 37 °C copper pipe treated by CTC-disinfection did the results give possible hints that the pathogen persisted in the biofilm in a VBNC state from where it disseminated into the bulk water and gave rise to culturable cells. The fact that *P. aeruginosa* enters into a VBNC state on a copper surface has been described by Moritz *et al.* (2010).

**Water quality (N, P, and AOC)**

A high nutrient level in the water strongly increased the growth of *P. aeruginosa* after the disinfection attempts at 37 °C in biofilm and bulk water in pipes of all materials. The effect of added nutrients on regrowth of *L. pneumophila* was less pronounced. Only in biofilms and bulk water of steel and copper pipes did the numbers of non-culturable cells slightly increase.

**Presence of low-flow niches or dead spaces in built-in parts**

*Pseudomonas aeruginosa* was able to survive disinfection in a culturable state inside built-in parts like pipe junctions or check valves and to recontaminate the system after disinfection. Thus, an exchange of built-in parts of contaminated plumbing systems is strongly recommended as a precondition for successful sanitation.

**Presence of autochthonous biofilm**

The lack or severe damage of an autochthonous biofilm, e.g. due to disinfection, may promote the growth of surviving or newly introduced *P. aeruginosa* in biofilm and bulk water. This might explain the observed growth of *P. aeruginosa* after the disinfection attempts during the long-term experiment in test rigs. Observations in practice, that a contamination with *P. aeruginosa* often occurs in newly built installations (Wingender *et al.* 2009), support this thesis.

The growth of introduced *P. aeruginosa* can be significantly inhibited when an autochthonous biofilm occupies large parts of the surface. A decrease in the number of culturable *P. aeruginosa* AdS after incorporation into established biofilms on EPDM, PE-Xb and PE-Xc over 28 days has already been described by Moritz *et al.* (2010). In another bench-scale study of Bressler *et al.* (2009) the mucoid strain *P. aeruginosa* SG81 was detected for no longer than 14 days under constant flow conditions in the biofilm on EPDM material in one of the triplicates. Once this native biofilm has developed, its microbial community might be more competitive in utilization of available carbon sources.

A reverse effect was observed on copper where the autochthonous biofilm may protect *P. aeruginosa* from an inhibiting impact of copper ions released by the material.

**Compliance with generally recognized standards of good practice**

For a long-term successful sanitation it is indispensable to localize and eliminate contamination sources in the plumbing system before suitable cleaning and disinfection procedures are performed. Additionally, plumbing systems have to comply with operating conditions (e.g. temperature specifications for cold (<25 °C) and hot (>55 °C) water and construction requirements (e.g. use of approved materials, avoidance of low-flow zones) of the generally recognized standards of good practice in Germany (e.g. VDI/DVGW Guideline 6023 (2015), DVGW code of practice W 551 (2004) and W 557 (2012)).
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