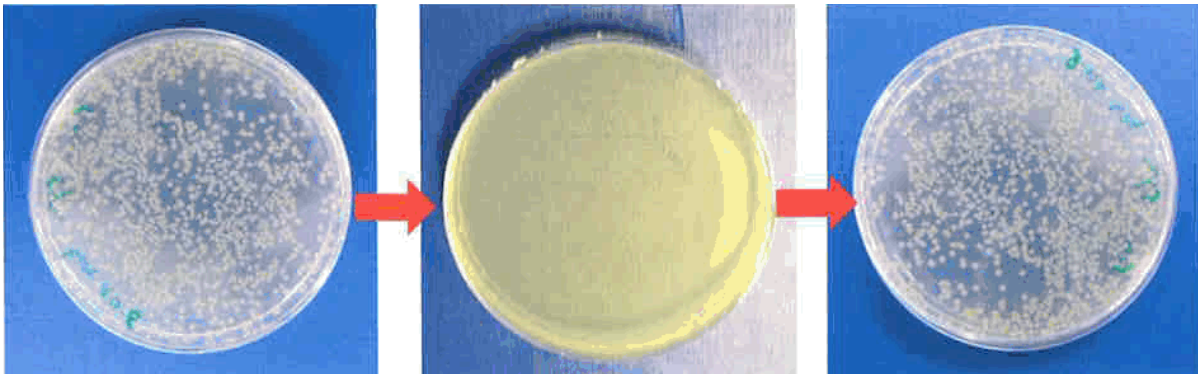
Executive Summary:
Results of the Collaborative Research Project Detection and Treatment of Transiently Nonculturable Pathogens in Drinking Water Installations ("Biofilm Management")



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Introduction

Biofilms in drinking water systems

Drinking water is not sterile and does not need to be so. Up to 10^5 cells per mL can be present without any hygienic relevance. These cells have a tendency to attach to surfaces and form biofilms. Practically all surfaces in contact with water serve as a substratum for microbial biofilms, which usually cover such surfaces in a patchy form, and usually do not represent any health risk to humans.

However, biofilms can harbour microorganisms of hygienic relevance. This can occur occasionally and transiently and they may be guests in established drinking water biofilms. Among these, *Legionella pneumophila, Pseudomonas aeruginosa* and others are reported (Flemming *et al.*, 2013). Under favorable conditions, such bacteria can persist and even multiply in biofilms and, as a consequence, contaminate the drinking water (Wingender, 2011). From a health perspective, it should be made certain that biofilms in drinking water distribution and installation systems are not the source of hygienically relevant bacteria and should not raise concerns for human health.

Within biofilms, microorganisms can tolerate much higher concentrations of disinfectants than when in suspended form in water (Davies, 2003). This tolerance can lead to persistent problems in decontamination and sanitation efforts, considerable costs and last for years. (Schauer *et al.*, 2013). These do not only include the direct costs for repeated disinfection measures and verification of success, but also labour and measures for compensating malfunction of the system. For example, in hospitals and retirement homes, point-of-use water filters may be employed at taps and shower heads which may represent an expensive solution, which does not address the cause of the problem.

The weakest link in the drinking water supply chain from catchment to consumer is the in-premise drinking water installation. This is the most complex and least controlled component in the chain. Pipe diameters can be very small, offering large surfaces in contact with water which can be colonized by biofilms; a great variety of plumbing materials are employed, not all of which comply with regulations; consumption patterns are irregular including long periods of stagnation (Flemming *et al.*, 2013).

In order to avoid biofilm problems and to provide safe drinking water, the concept of "Biofilm Management" was developed. Variables which can be controlled are water quality, plumbing materials and operation conditions. Manipulation of these variables is possible through process-engineering, physical and/or chemical measures which can limit biofilm growth. Furthermore,

these variables can be influenced by design planning and operation of the system; particular attention should be put on avoiding no- and low-flow areas and dead legs. Fundamentally important for effective biofilm management is surveillance by appropriate sampling and analysis in order to detect any risks in time and to assess the efficacy of measures and strategies in biofilm management. The major principles have been addressed in guideline VDI/DVGW 6023 and the key features of such management are:

(i) Stable drinking water,

(ii) Limitation of nutrients with particular attention to elastomeric plumbing materials which can leach biodegradable substances such as plasticizers, anti-oxidants, anti-statics, paraffin etc.,

(iii) Compliance of state-of-the-art drinking water installations, particularly after stagnation periods,

(iv) Representative surveillance.

Cases of persistent microbial contamination and long-term problems following sanitation measures are well known. In such situations, it has to be taken into account that microorganisms can enter a viable-but-nonculturable state (Oliver, 2005, 2010; Li *et al*., 2014). In this state, the organisms are not dead but cannot be detected by cultivation methods. They have the potential to resuscitate; therefore, they deserve special attention.

The viable-but-nonculturable state (VBNC)

Global gold standards for determination of living bacteria in drinking water are culture-dependent methods. They are based on the ability of microorganisms to multiply, grow in liquid media and form colonies on agar media. Conversely, it is concluded that bacteria which don´t grow in or on nutrient media, are dead or at least irreversibly inactivated. Cultivation methods are of central relevance in practice – they are employed to assess the hygienic quality of drinking water, food and beverage as well as the entire medical testing context. Their employment is extremely successful; they are the key for prevention of waterborne infectious diseases. However, these methods have limits. It is well known that bacteria which do not grow are not necessarily dead. They can escape the "radar of surveillance" when in a nonculturable state. In a specialized conference "How dead is dead" in Bochum, 2009, this state was defined, based on the work of Oliver (2005, 2010) in the following way:

"A bacterial cell in the VBNC state may be defined as one which fails to grow, but which is in fact alive and has still metabolic activity".

This is a purely operational definition, because it is based on the response of the cell to cultivation conditions under which it normally can be detected. The reason is an important phenomenon: in the VBNC state, microbial cells show practically no more growth metabolism. Therefore, they do not multiply and form colonies or cause turbidity in liquid media. But they still can keep their maintenance metabolism which may include replacement of cell components such as membrane, cell wall etc., or repairing DNA damage due to UV irradiation or to action of antimicrobials.

Entering the VBNC state can be understood as a survival mechanism. It may be a response to stress which might be detrimental to the cells if they continue to grow (Li *et al.*, 2014). Such stress can be generated by for example, disinfectants, toxic metal ions, nutrient depletion or unfavorable temperatures. Cultivation methods can lead to false negative results because the cells are not dead but only inactive. This is already well known already for *Legionella pneumophila* (Steinert et al., 1999; Alleron *et al.*, 2008). For *Pseudomonas aeruginosa*, a facultative pathogen with increasing relevance for drinking water hygiene, less is known. However, first investigations indicate clearly that the same pattern is to expect from that organism (Moritz *et al.*, 2010; Flemming *et al.*, 2013).

The problem with the VBNC state is that it can be transient and reversible. For *L. pneumophila*, it could be shown that the organism can return into the cultivable and also the infectious state (Steinert *et al.*, 1999), and more recently, the same is shown for *P. aeruginosa* (Dwidjosiswojo *et al.*, 2011).

A range of methods do exist to detect VBNC organisms. Vitality markers can be determined, e.g., the integrity of the cell membrane as determined by the live/dead-system, the presence of ribosomal RNA as an indicator for protein production or others. A "toolbox" of methods is shown in Table 1.

Table 1: Toolbox for detection of viability markers of VBNC organisms (Hammes and Egli, 2010, Rochelle et al., 2011, Hammes et al., 2011)

METHOD	PARAMETER	INTERPRETATION
DAPI, Syto 9, PCR	Nucleic acid	All cells, dead or alive
PI+Syto 9, PMA	Membrane integrity	Principially viable
Rhodamin 123	Proton motive force	Energy conservation
Resazurin, CTC	Fluorescent resorufin, fluorescent formazan	Metabolic activity
Fluorescein diacetate	Hydrolysis of FDA, fluorescein formation	Intracellular metabolic activity
rtPCR	Reverse transcriptase	Protein expression
Direct viable count	Cell elongation	Growth sign
FISH, DVC-FISH	ribosomal RNA	Protein production
ATP	ATP	Energetical state of cell
Ethidium bromide	Expelling of EB	Efflux pump activity
13C uptake	Cell-bound isotope activity	Assimilation, metabolic activity

Organisms in the VBNC state represent a significant and currently underestimated risk for the hygienic safety of drinking water. They are particularly relevant for drinking water installations within hospitals and other healthcare facilities, schools, retirement homes, nurseries, military barracks, hotels, universities and other large buildings. If immunosuppressed people are within the water user population and exposed to resuscitated pathogens, the risk becomes more problematic.

The reliability of spatio-temperal sampling strategies for the detection of microbial contaminations in drinking water installations of large buildings has been investigated. It became obvious that solid detailed knowledge of the local conditions is crucially important. In the same building, very different results were found at the different sampling points. Even at the same sampling point, large variations were found through the day. With such a variation, it is quite an achievement that a logistic regression model in combination with the potable hot water (PHW) constant temperature allows for a realistic assessment of the risk of a *Legionella* contamination (Völker *et al.*, 2013). In a laboratory system, the efficacy of disinfection measures was investigated. Interestingly, *P. aeruginosa* seemed to be controlled by the autochthonic biofilm, yet regrew after disinfection measures which eradicated the autochthonic biofilm. Disinfection may offer a selection advantage to fast-growing pathogens; thus, they may return particularly rapidly after disinfection. Copper induced the VBNC state in *P. aeruginosa*, which could be reversed by treatment with a copper chelator. After such treatment, infectivity was regained (Dwidjosiswojo *et al.*, 2011; Flemming *et al.*, 2013).

The results of the project are currently compiled in publications. The essence of the findings is available in German from the project website (biofilm-management.de)

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1. Viable but nonculturable (VBNC)

1.1 The capacity to form VBNC cells is widespread in water bacteria in oligotrophic habitats

For bacteria, drinking water represents an oligotrophic habitat. Typical biofilm forming microorganisms in drinking water systems include representatives of the group Aquabacterium-Ideonella-Sphaerotilus or pseudomonads. They occur in many drinking water systems, although in variable compositions and make up to 99 % of VBNC cells. In exemplarily experiments with *L. pneumophila* and Aquabacterium, which had been previously cultivated and exposed to drinking water, after 2-3 weeks 80 % of cells had turned into the VBNC state and could no longer be detected by cultivation– but were not dead. This adaptation to limiting factors in drinking water was found in six species of *Pseudomonas*, in *L. pneumophila* and in three species of Aquabacterium both in laboratory experiments and *in situ*. As Aquabacteria represent up to 60 % of the total population in drinking water biofilms, these are certainly representative biofilm formers. Other species can occur more frequently only where plumbing materials support significant biofilm growth. It was shown that in principle, all Aquabacteria have the capability of transition into the VBNC state, but that this property differs strongly even among tightly related species.

1.2 Even very low nutrient concentrations can be used by VBNC cells

Analysis of Aquabacterium cells grown under limited carbon concentrations revealed that those cells are physiologically active and respond to the addition of organic carbon in low concentration by cell division. Nevertheless, these cells could not be cultivated on the standard nutrient agar used in routine analysis. This could be demonstrated for *P. aeruginosa* as well, although they were no longer culturable on standard nutrient agar, but could utilize other substrates. Significantly lower intensity of FISH signals (less ribosomes indicate less protein production) of VBNC cells are a hint that they prefer to carry out maintenance metabolism rather than growth metabolism. In *P. aeruginosa*, an alteration of typical colony morphology on routine standard nutrient agar was additionally observed.

1.3 VBNC cells of Aquabacteria are found not only in the water phase but also in biofilms

Even in very thin biofilms (1-3 cell layers) within drinking water systems, next to the VBNC cells is almost always a certain proportion of cultivable cells. Some of these cells can get into the water phase as swarmer cells (“pioneers”) and colonize further surfaces. These swarmers

are in a different physiological state from the rest of the biofilm cells, and it is possible that they may adapt to conditions on standard nutrient agar. This can explain the observation that the proportion of VBNC cells in the natural flora of *Aquabacterium* spp. is higher in biofilm than in planktonic populations.

1.4 The transition into the VBNC state represents an adaptation to oligotrophic habitats for many environmental bacteria

The VBNC state for many water bacteria is likely the common state of life in order to survive in oligotrophic environments for extended periods of time. This adaptation results in the fact that those cells cannot be found under routine cultivation methods. Many pathogens can enter this state after exposure to the environment and some can survive for much longer periods of time than it was thought when using cultivation methods alone.

1.5 *P. aeruginosa*, a facultative pathogenic bacterium, can grow in biofilms even in oligotrophic drinking water systems

P. aeruginosa can grow in oligotrophic systems such as drinking water. Therefore, it is particularly interesting and important to know if this organism can enter the VBNC state and escape standard routine detection by cultivation. Experiments with pure and mixed cultures in oligotrophic media revealed contradictory results. Under substrate limitation, only a minor part of the population enters the VBNC state; in other investigations, the transition comprised several orders of magnitude. This points on the possibility that this bacterium has more than one adaptation mechanism to oligotrophic conditions. Interestingly, it could be shown that Aquabacteria who had settled in biofilms could stimulate pseudomonads into the VBNC state. This suggests the involvement of signaling molecules in the transition process.

1.6 Under the influence of copper concentrations common in drinking water plumbing systems, *P. aeruginosa* and *L. pneumophila* can enter the VBNC state

In the presence of copper ions (~ 60 µg L-1) in the water from installations made of copper materials (pipes and fittings), *P. aeruginosa* can enter a VBNC state (Dwidjosiswojo *et al.*, 2011). In experiments with deionized water spiked with copper in similar concentrations, *L. pneumophila* also enter a VBNC state.

1.7 By application of chelators, e.g., sodium diethyldithiocarbamate (DDTC), a return to the culturable state could be achieved in *P. aeruginosa*.

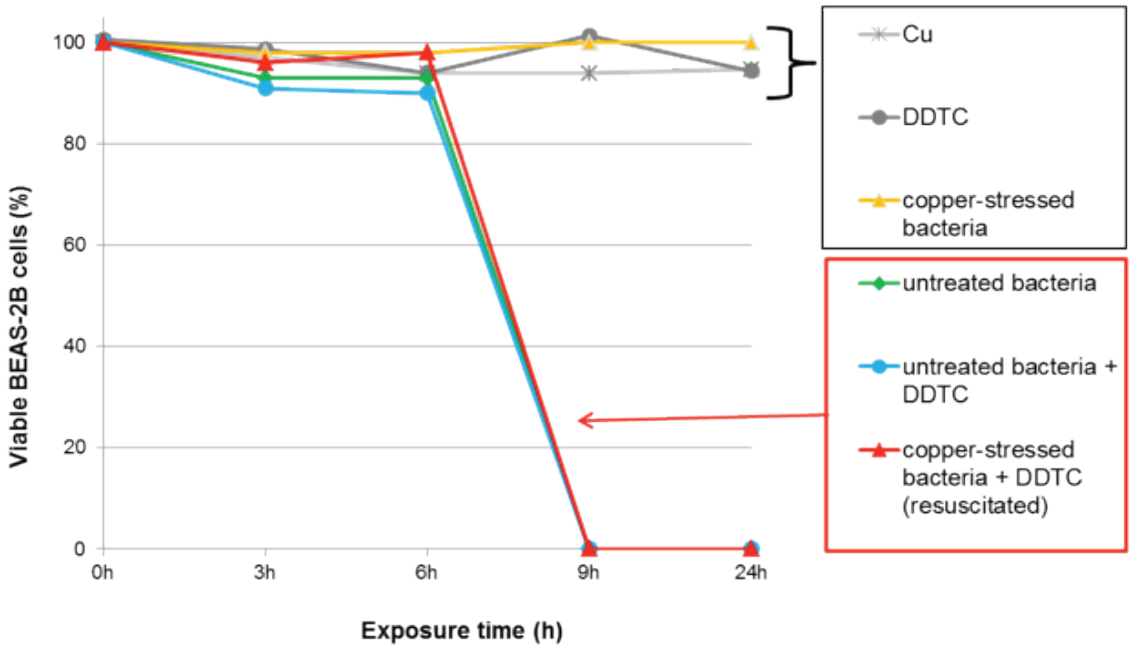
The copper stress, which turns *P. aeruginosa* into the VBNC state, can be lifted by application of the copper chelator sodium diethyldithiocarbamate (DDTC). After 7-14 days, the

organism returns into the culturable state (Dwidjosiswojo *et al.*, 2011), a process called "resuscitation". Conditions leading to resuscitation are specific: nature and duration of copper exposure, as well as the copper specific chelator, can influence the return to culturable state. It is important to keep in mind that the VBNC state is purely operational defined; it is a non-specific stress response and can be caused by a variety of different stimuli. Therefore, the conditions for resuscitation can also be various. The example of *P. aeruginosa* is presented in order to demonstrate that the VBNC state can be reversible.

1.8 After returning to the culturable state, *P. aeruginosa* regains cytotoxicity.

As demonstrated with human lung epithelium cells (BEAS-2B) it is only in the culturable state that *P. aeruginosa* have cytotoxic and DNA damaging effects on eukaryotic cells. In the copper-induced VBNC state, no cell damage by *P. aeruginosa* can be detected. When returned to the culturable state, *P. aeruginosa* regained cyto- and genotoxicity (see Fig. 1).

Figure 1: P. aeruginosa *after 14 days incubation at 20 °C (before and after resuscitation) with Na-diethyldithiocarbamate (DDTC) 100 µM; Cu 10 µM*



Neither copper-stressed bacteria, copper nor the copper chelator Na-diethyldithiocarbamate (DDTC) show cytotoxic effects but both untreated and resuscitated *P. aeruginosa* lead to death of the BEAS-cells within 9 hours.

The cytotoxicity of *P. aeruginosa* is strain-dependent. While isolates from drinking water systems (*P. aeruginosa* SG81 and its non-mucoid mutants) show strong cytotoxicity, the type

strain from the German Strain Collection showed only very moderate cytotoxicity. The effect is also influenced by the nutrient situation. While in oligotrophic environments, complete killing of BEAS-cells could only be observed after 10 hours. In drinking water with elevated nutrient concentrations, the effect was achieved after 6 hours. It is plausible that the cytotoxic effects of *P. aeruginosa* are based on the secretion of toxins: supernatant free of *P. aeruginosa* cultures led to damage of BEAS-cells.

1.9 *L. pneumophila* is a bacterium which typically multiplies inside amoebae. Growth in biofilms (without amoebae) probably occurs only in complex and metabolically highly active biofilms.

It is generally assumed that *Legionella* occur in drinking water biofilms only intracellularly within protozoa. If external multiplication occurs, this is only the case in water systems in which the biofilm community has high nutrient concentrations and is highly active. Nutrients can stem from the water but also from construction materials of the plumbing system. The latter has been observed particularly with elastomeric materials (Moritz *et al.*, 2010). Active mixed species biofilms can represent a suitable environment for settling and persistence of legionellae. It was shown that legionellae settle in clearly amoebae-free biofilms and remain detectable over weeks.

1.10 *L. pneumophila* can also enter a VBNC state, e.g. after extended nutrient depletion

Settling into drinking water biofilms has been demonstrated in laboratory experiments within a temperature range between 8 and 20 °C. The legionellae persisted over several weeks as shown by culture-independent methods. However, in these experiments no increase of legionellae could be observed, independent of temperature and nutrient conditions.

1.11 After passage through amoebae, *L. pneumophila* could return to the culturable state

Two strains of *L. pneumophila* (*L. pneumophila* German Strain Collection 7514 and the environmental isolate *L. pneumophila* AdS) which had been turned into a VBNC state by nutrient limitation were co-cultivated with *Acanthamoeba castellanii* at 30 °C for up to 7 days. Under these conditions, uptake of the legionellae in the amoebae could be verified microscopically. While the total cell number and the number of FISH-positive legionellae did not increase, the number of culturable legionellae using standard cultivation on GVPC agar increased significantly. Nutrient-rich water systems and their biofilms which usually contain amoebae can support the resuscitation of legionellae which persist in the VBNC

state over extended periods of time. This illustrates the role of nutrients, and thus the benefit in avoiding plumbing materials which support microbial growth.

1.12 The VBNC state can lead to an underestimation of the presence of hygienically relevant microorganisms and an overestimation of efficacy of sanitation measures

By entering a VBNC state, hygienically relevant microorganisms cannot be detected by standard cultivation-based methods. As VBNC cells are characterized by signs of viability (e.g. intact membranes, presence of ribosomal RNA, enzymatic activity, presence of ATP), they cannot be considered to be irreversibly inactivated. A return into a culturable state cannot be excluded. This results in a possible underestimation of hygienically relevant microorganisms and an overestimation of sanitation measures success. This may result in a hygienic risk which should not be underestimated.

2. Microbiological and molecular biological methods

2.1 Methods required by the German Drinking Water Regulation, based on cultivation methods, do not always indicate contaminations of hygienically relevant microorganisms

Analyses of 768 drinking water samples from 9 public buildings, performed in parallel by standard cultivation methods and quantitative polymerase-chain-reaction (qPCR), showed detection of *L. pneumophila* with qPCR which was positive although the cultural detection was negative (0 cfu per 100 mL).

Samples containing concentrations of lower than 200 CFU/100ml were mainly negative in qPCR, which means that the lower detection limit was higher than theoretically calculated. This is a phenomenon well known and often discussed in the literature. As an example, the comparative culture vs PCR results of 2 buildings are summarised below highlighting the importance of correctly interpreting results and problems with contamination.

BUILDING G			BUILDING H		
N total = 73	PCR +	PCR -	N total = 95	PCR +	PCR -
Culture +	15	32	Culture +	29	5
Culture -	2	7	Culture -	15	18

2.2 Molecular diagnostic methods cannot replace but can complement the classical cultivation-based detection methods used for routine drinking water installation monitoring

Quantitative PCR was shown to be a sensitive method which could be standardized for environmental samples. A quantification of the absolute numbers of pathogens was possible throughout a large range of concentrations with sensitivity for even very low numbers of bacteria. However, it has to be considered that qPCR cannot distinguish between dead cells and VBNC cells. By enhancing this method using propidium monoazide (PMA), it is possible to distinguish intact from strongly membrane damaged cells.

On the other hand, qPCR can give a negative result even if colonies of *L. pneumophila* are found on GVPC agar. This can be attributed to matrix effects of components of the drinking water. In contrast to laboratory experiments with pure cultures, there remains a proportion of "real" false-negative qPCR results. Therefore, qPCR cannot replace standard cultivation methods, but it can support them.

2.3 In cases of persistent and recurrent microbial contaminations it has to be taken into account that pathogens may temporarily convert to a VBNC state. Cultivation methods are insufficient and should be supported by other cultivation independent methods under these conditions

Advanced molecular biological methods can be supportive in detecting hidden contaminations. Positive signals of qPCR can be used as early warning signs, when the above mentioned limitations are considered. Principally, they can indicate the potential presence of hygienically relevant organisms.

⇨ **The Live/Dead system:** By application of the live/dead system, the viability state of a microbial population can be assessed. The system indicates the integrity of the cell membranes which is considered a life sign. In principle, the method is based on the application of two fluorescent dyes. Green fluorescing SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing Propidium iodide (PI) enters only cells with damaged cytoplasmic membranes. Damage of the cytoplasmatic membrane is interpreted as cell death. The method is not specific for hygienically relevant microorganisms but gives an overview on the viability of the total population in a sample, (i.e. Live/green; Dead/red).

⇨ **Membrane integrity using qPCR:** Can also be investigated. This is based on the retention of propidium monoazide (PMA) by intact membranes. PMA is a photoreactive dye with a high affinity for DNA. The dye intercalates into double stranded DNA and forms a covalent linkage upon exposure to intense visible light, resulting in chemically modified DNA, which cannot be amplified by PCR. Because PMA is designed to be cell membrane-impermeable, when a sample containing both live and dead bacteria is treated with PMA™, only dead bacteria with compromised cell membranes are susceptible to DNA modification. Thus, subsequent extraction of DNA and qPCR permits quantitation of viable cells.

- **Fluorescence-in-situ-hybridization (FISH):** Another viability indicator is the presence of ribosomal RNA. The gene probe can be specific for target cells, e.g. pathogens. In the ribosomes, synthesis of proteins takes place which is required by the cell for maintaining metabolism. This is a fundamentally important life process. If rRNA is present, it is a life sign for protein production. It is based on the assumption that dead cells do not have rRNA anymore as it is very instable. However, this method is not unequivocal because in some cases, rRNA can be preserved. The method can only cover a range of between 0.1 and 100 % of the total cell number. At high total cell numbers, the target cells make up only for a small proportion and are difficult to detect. This causes a high detection limit, with the consequence that a single FISH signal can cause an overestimation of target cells. Therefore, the method is particularly susceptible for false positive signals. On the other hand, low numbers of pathogens among high total cell numbers may not be detected.

3. Detection, monitoring and interpretation of microbial contamination

3.1 Drinking water installations contaminated with *L. pneumophila*, are prone to strong spatial and temporal variations in detection, both short-term and long term.

Nine buildings with known systemic contamination of *L. pneumophila* were sampled in a narrow spatial and temporal pattern. Even when the sampling sites were located next to each other, large fluctuations were observed. Within one building, negative results for *L. pneumophila* were observed at one point, whereas high concentrations of this organism were detected (> 1,000 – 10,000 cfu per 100 mL) at the same time at other sampling points. Over a period of 6 months, high fluctuation of *L. pneumophila* concentrations (up to 5 logs) could be found at the same sampling point. Even in the course of a day, the concentration could vary over 4 logs (e.g. measurements at 10.00 am: 11,900 cfu per 100 mL, at 8.00 pm: 18 cfu per 100 mL). No systematic pattern of contamination could be determined in any of the buildings sampled.

3.2 Established sampling strategies for systemic investigation of drinking water installations may detect microbial contaminations to a limited extent.

The selection of sampling points was performed according to relevant German guidelines (UBA 2012; DVGW Arbeitsblatt W 551, DVGW-Information Wasser Nr. 74). These guidelines require selection of representative sampling points in the drinking water installation such as

(i) at the calorifier exit for "potable water hot" (PHW),

(ii) at the end of circulation (recirculating hot water) before calorifier re-entrance PWH-C,

(iii) at representative sites of the ascending pipework and

(iv) preferably at ascending pipes supplying showers (if applicable).

The qualitative analysis revealed that selecting sampling points using the established sampling strategies in accordance to guidelines, rarely revealed a contamination over a time range of six month. In a quantitative analysis, the positive predictive value (PPV), the sensitivity and the correct classification rate (unprobability of prediction) of the respective buildings were calculated (Table 1). In some buildings with very high contamination (building K), the established sampling strategies yield good results with a high positive predictive value and a high sensitivity. In larger buildings with complex factors influencing

the contamination process (building F), sampling strategies complying with the guidelines detected only 29.9 % of all known contaminations. The sensitivity of a detection of a systemic contamination was 37.8 % and the correct classification rate of all samples was 54.5 %.

Table 1: Sensitivity, positive predictive value (PPV) and correct classification rate (CCR), when applying sampling in accordance to guidelines (incl. long-term sampling), ranked for sensitivity. N = number of samples analyzed.

BUILDING	NUMBER OF SAMPLING POINTS ACCORDING TO GUIDELINES	TOTAL NUMBER OF SAMPLING POINTS PER BUILDING	SENSITIVITY	PPV [%]	CCR [%]	N
K	5	7	77,8	84,0	68,8	32
A	11	34	59,5	44,9	60,0	105
B	9	44	50,0	27,9	60,9	110
G	16	31	44,4	16,3	49,5	101
E	6	22	28,6	16,7	55,9	68
D	8	27	20,5	28,6	52,8	108
F	7	29	16,1	14,7	47,6	105
H	5	35	0,04	3,6	50,5	101
Total (Average)	67	229	(37,8)	(28,9)	(54,5)	730

NB: Building C was removed from the data population after no *Legionella* contamination was found.

3.3 Investigation of the calorifier outlet and the recirculating water before calorifier re-entrance with regard to *Legionella* seems to have little significance in terms of identification of contamination in the PWH installation system

The established practice of surveillance of the calorifier outlet and PWH-C return loop gave limited results. Only one of eight contaminated PHW systems could be verified as contaminated using this practice (building K). Data for calorifier outlet and recirculating water before calorifier re-entrance samples in all other installation systems remained constantly below the technical threshold level (100 cfu per 100 mL) for legionellae. Therefore their significance for the prediction of contamination of the entire PHW installation system appears very limited. As a consequence, even if the values of *L. pneumophila* at the calorifier outlet and in the recirculating water before calorifier re-entrance (PHW-C) are below the technical threshold level at these points, the PWH system can still be contaminated by the same genetic strain of bacteria.

Nevertheless, the established practice of surveillance maintains its usefulness for focusing on the causes of contaminations of drinking water installations: positive results at the calorifier outlet indicate a contamination of the hot water reservoirs with *L. pneumophila* ahead of the entrance into the PWH and – combined with the data from PWH-C – the presence of *L. pneumophila* in the entire system can be indicated.

3.4 Total water consumption (PWH and PWC) in the drinking water installation of a building, and the hot-water exchange in the PWH-installation, proved to be inadequate indicators for the detection of stagnation and contamination events

The German guidelines VDI/DVGW 6023 recommend a complete exchange of water volume in the entire drinking water installation at least every three days (better: every day). The volume exchange of the PHW system was, when possible, determined centrally by a water meter. Furthermore, in all cases the total water exchange of the system was monitored by the water meter at the entrance to the household water system. A quantitatively sufficient exchange of the total water system volume according to the guidelines does not mean that *L. pneumophila* will not occur in the respective building (Table 2). A possible explanation may be water flow concentrating on specific selected outlets, leaving other outlets unflushed, or, recontamination by *legionella*-containing biofilms. The water use could flush selected piping areas preferentially while possible stagnation areas remain. These undoused outlets can be the source for subsequent contamination.

Table 2: Quantitative volume exchange of the PHW installation system and exceeding the technical threshold level in the buildings. (N.a. = not available)

BUILDING	VOLUME PHW SYSTEM INCLUDING STORAGE [L]	VOLUME EXCHANGE PHW SYSTEM [PER DAY]	SAMPLES EXCEEDING TECHNICAL THRESHOLD LEVEL [%]	TOTAL NUMBER OF SAMPLES FROM PHW
A	750	1,6	37,4	99
B	4000	2,1	25,0	104
D	4500	1,6	20,7	58
E	2900	0,4	23,4	64
F	n.a.	n.a.	25,0	136
G	n.a.	n.a.	18,4	98
H	2200	n.a.	27,7	94
K	2600	0,4	84,4	33

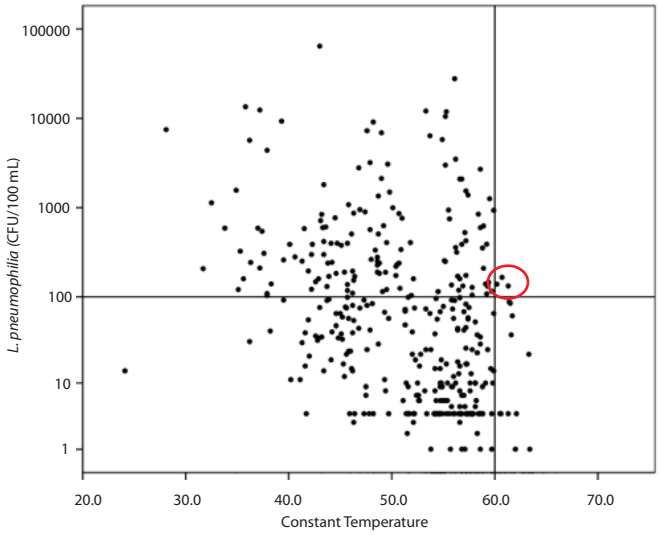
3.5 Qualitative information obtained during the assessment of drinking water installations, through inspections and surveys, provides meaningful data for the selection of suitable sampling points, particularly if stagnation areas and sparsely used water taps are included in the assessment

An individual stagnation risk could be determined for each sampling point through an on-site investigation. This was done by qualitative aspects and included the type of users, number of users, frequency and regularity of outlet use and general information from staff, operators and users. Hygienic trained and experienced observers are able to note evidence of stagnation through absence of indicators which would suggest regular use such as external contamination, or traces of water at the sampling point, and include such hints in the assessment. The parameter "stagnation (qualitative), limited usage" requires hygienic experience and includes findings from qualitative interviews with building managers, operators and users. Hygienic-microbiological relevant stagnation areas can be identified with microbial analysis. It had a sensitivity of 72.5 % for the detection of systemic contamination, and a correct classification rate of about 65 % (n = 544) can be achieved. However, the quantitative measurement of stagnation did not yield any valid results. The qualitative approach was the most successful tap-specific approaches for predicting a contamination with *L. pneumophila* in a building.

3.6 PHW-constant temperature is an important predictor for occurrence of *L. pneumophila*

The tap specific PHW high constant temperature (determined after extended flow during sampling) and the occurrence of *L. pneumophila* showed a highly significant negative correlation ($r = 0.36$, $p = <0.01$, $n = 541$): the probability for occurrence of *L. pneumophila* decreases with increasing PHW constant temperature. In only three samples of all buildings monitored (each with known systemic contamination of *L. pneumophila*)the value of *L. pneumophila* exceeded the technical threshold level in the case the temperature was above 60 °C (see red circle in Fig. 2).

Figure 2: Scatter diagram of culture occurrence of L. pneumophila (cfu per 100 mL) and PHW constant temperature of the same samples (n = 541)



3.7 Missing compliance with the 5 °C rule is a relatively good predictor for the occurrence of *L. pneumophila*.

The parameter "no compliance with the 5 °C rule" is based on the temperature difference between calorifier outlet temperature and the constant temperature measured at the tap (this also applies for the PWH-C return loop). The temperature-related parameter "no compliance with the 5 °C rule" as well as the parameter "constant temperature 35-45 °C, can predict a possible contamination with *L. pneumophila* compared to other parameters such as the calorifier outlet and recirculating water before calorifier re-entrance (sensitivity 37.7 and 38,9 % respectively, correct classification rate 62,1 and 68,6 respectively; n = 140 each).

3.8 A logistic regression model including the parameter stagnation and PHW-constant temperature provide a good estimation for risk of a *Legionella* contamination at single sampling points.

By stepwise exclusion of parameters, which were statistically not suitable, a logistic regression model was developed for a prognosis of a contamination with legionellae at single sampling points. With a very high significance, the metric parameter "PHW constant temperature" and the dichotomic parameter "stagnation (qualitatively), limited usage" (both $p<0.001$) were included in the final model. In this model, higher PHW constant temperature led to a lower risk for a contamination with legionellae (effect coefficient exp (B) = 0.89), while stagnation with low water use increases the risk more than threefold (effect coefficient exp(B) = 3.3). It is capable to predict exceeding of the technical threshold level for *L. pneumophila* with a positive prediction value of 61.1 %. The correct classification rate is 68.8 %. This model provides the possibility for a prediction of the contamination with *L. pneumophila* at each sampling site within a building.

4. Influence of plumbing material and water quality on *P. aeruginosa* and *L. pneumophila*

4.1 Under equal operating conditions, a lesser amount of culturable *P. aeruginosa* cells is found on copper surfaces compared to EPDM, PE-Xc (electron beam crosslinked ethylene-propylene-dien-monomer) and stainless steel. In biofilms on copper pipes, *P. aeruginosa* can enter a VBNC state.

In a laboratory model, drinking water operated at 37 °C with and without additional nutrients (1 mg L-1 phosphate, 10 mg L-1 nitrate and 100 µg L-1 acetate combined), after a single bolus shock contamination with *P. aeruginosa*, lower cfu numbers of this organism were found in biofilms on copper surfaces than in biofilms on EPDM, PE-Xc and stainless steel.

At an operation temperature of 10 °C, but otherwise identical experimental conditions, *P. aeruginosa* could not be detected by culture methods over a period of 6 weeks. At the same time, constantly high values from qPCR (> 10,000 gene copies cm^2) were found indicating the presence of *P. aeruginosa*. It has to be assumed that some of these bacteria have entered a VBNC state. The corresponding stagnant water (6 h stagnation before sampling) was free from *P. aeruginosa* as determined by cultivation and qPCR. The reason could be that the organism was either released to the water at levels below detection limit or it was effectively killed by elevated copper ion concentrations in the stagnant water.

Similar observations could be made under the same conditions for *L. pneumophila*: 6 weeks after contamination, the organism was not detectable in the biofilm on copper nor in the corresponding stagnant water samples, while qPCR indicated its presence until disinfection. In the biofilm, even after disinfection, gene copies of *L. pneumophila* were found although at low numbers. As with *P. aeruginosa*, no release into the water phase was detected, even by qPCR, following 6 h stagnation prior to sampling.

4.2 In copper pipes and with elevated phosphate concentrations (1 mg L-1) in the drinking water, biofilm growth increased with increasing concentrations of *P. aeruginosa* and *L. pneumophila*.

Phosphate is employed as a corrosion inhibitor. By reaction with copper, it forms a protective layer and prevents the release of copper to the water, resulting in lower copper concentrations. In a laboratory model, elevated numbers of *P. aeruginosa* and *L. pneumophila* were observed under these conditions. The presence of copper ions reduces the culturability of both *P. aeruginosa* and *L. pneumophila* (see 1.6). This effect could be masked by complexation of copper by phosphate.

On stainless steel, EPDM and PE-Xc, elevated phosphate concentrations did not lead to significant changes in total cell numbers, HPC or the numbers of *P. aeruginosa* and *L. pneumophila*.

4.3 An autochthonic biofilm on copper provides some protection to *P. aeruginosa* against copper stress.

After disinfection and the concomitant damage to the autochthonic biofilm, a subsequent contamination with *P. aeruginosa* led to lower numbers on the copper surface (determined both by cultivation and qPCR) than in the presence of an intact autochthonic biofilm. This leads to the assumption that the autochthonic biofilm provides some protective effect to *P. aeruginosa*.

5. Disinfection

5.1 Culturable and nonculturable states of *P. aeruginosa* and *L. pneumophila* in drinking water biofilms can be completely inactivated in principle. However, under unfavorable operation conditions, pathogens can survive disinfection

Laboratory model experiments showed that successful disinfection was possible under normal conditions and could be verified by culture independent methods. The concentration of culturable and nonculturable states of *P. aeruginosa* in biofilms were significantly reduced by oxidizing disinfectants, i.e. chlorine dioxide (ClO_2 - 50 mg L-1), sodium hypochlorite (40 mg L-1), and hydrogen peroxide (150 mg L-1). After treatment, the organism could neither be found by cultivation nor by culture independent methods.

At unfavorable conditions, however, they could survive even after application of intense disinfection conditions (e.g. sequential application of 50 mg L-1 of ClO_2 for 24 h) heating at 70 °C for 3 min and of 50 mg L-1 of ClO_2). Under these conditions, a survival and even regrowth of *P. aeruginosa* in biofilms was possible. The "unfavorable conditions" include:

- ⇨ Elevated nutrient concentrations in water or on plumbing material, e.g. 100 µg L-1 of acetate-C in water or on a material which leaches biologically degradable substances (EPDM, even if approved for use in drinking water). This leads to strong biofilm growth.
- ⇨ Non-compliant temperatures (37 °C), which can favour a multiplication of *P. aeruginosa* and *L. pneumophila*.

5.2 *P. aeruginosa* can survive intensive discontinuous disinfection (chemical or thermal) in biofilms on plumbing components and recontaminate the system after disinfection.

In laboratory experiments, *P. aeruginosa* was found in the water phase (but not in biofilm) after disinfection of the plumbing system both by cultivation dependent and independent methods. This organism could survive in the water phase in dead legs because it was protected from the disinfection and supported by synthetic polymeric materials in connections, back pressure or sampling valves. From these locations, recontamination of the system occurred. Only by removal of these nutrient parts and their sterilization or replacement, could the experimental plumbing systems be successfully sanitized.

5.3 In the presence of an autochthonic drinking water biofilm, multiplication of *P. aeruginosa* on the inner surfaces of pipes can be inhibited compared to non-colonised surfaces.

In 24-hour laboratory experiments it could be observed that a well-developed autochthonic biofilm could limit the multiplication of *P. aeruginosa*. This is possibly due to competition for nutrients and living space between the autochthonic biofilm and *P. aeruginosa*. This organism may survive in a biofilm, but not multiply as strongly as on surfaces which are not colonized by a drinking water biofilm. This observation was made on PE-Xc, stainless steel and most expressively on EPDM, but not on copper.

5.4 Discontinuous disinfection of the authochthonic drinking water biofilm on the inner surfaces of the pipes without simultaneous removal of the contamination source can lead to rapid regrowth of *P. aeruginosa* in a contaminated system.

A frequent observation is that after disinfection, a rapid regrowth of *P. aeruginosa* occurs. This was confirmed in a laboratory model at technical scale. The reason may be that some cells have survived cleaning and disinfection measures on inner surfaces (see 5.2) and re-entered the system after removal of the disinfectant. Furthermore, *P. aeruginosa* may have survived on pipe surfaces (see 5.1). The strong regrowth may have been favoured because the autochthonic biofilm was inactivated by the disinfection measure and could no longer inhibit the growth of *P. aeruginosa*. This effect was particularly strong on EPDM, stainless steel and PE-Xc.

5.5 For successful long-term sanitation of a contaminated drinking water installation, the following measures should have priority over intense disinfection measures:

- Elimination of contamination source
- Compliance to operation conditions according to accepted codes of practice

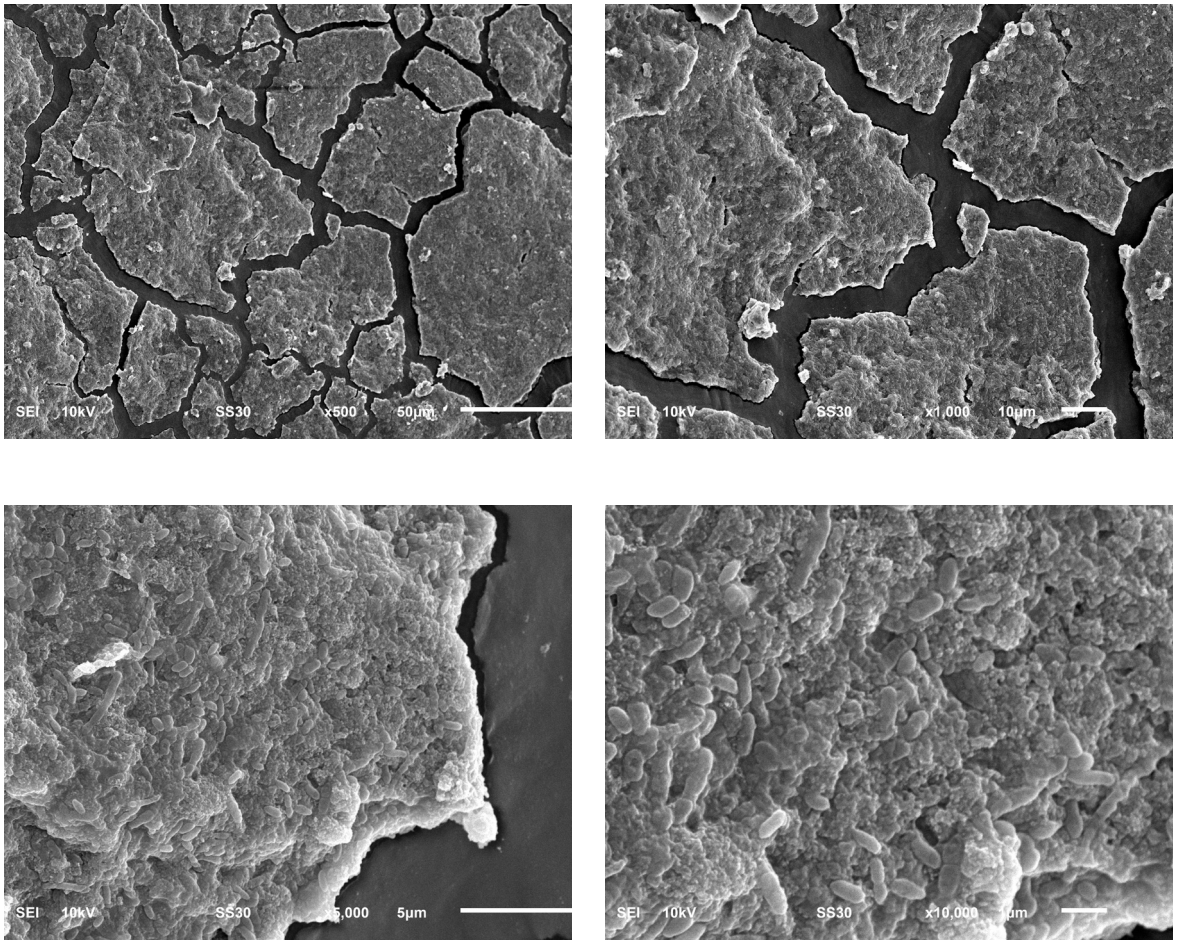
Whenever sanitation is necessary, a reliable localisation of the contamination source is mandatory. The accepted codes of practice have to be observed for the sanitation according to German guidelines DVGW working sheet W 551.

Discontinuous cleaning and disinfection measures can fail in some cases; it may even make the situation worse. Contaminated plumbing components, such as valves, have to be replaced if other measures are unsuccessful.

5.6 Underdosed disinfectants modify the population structure of biofilms and select for organisms with elevated disinfectant tolerance

After treatment of sublethal concentrations of various oxidizing chlorine-based biocides (sodium hypochlorite, electrochemically activated chlorine, continuous treatment with 0.15 mg L-1 chlorine or shock treatment with 10 mg L-1 chlorine over 6 h), cyclic regrowth of biofilm was observed. This may be attributed both to increased tolerance towards the disinfectant as well as to recolonization of inner surfaces. Disinfection drastically reduced the number of colony forming units per cm^2 while the total cell number was reduced only slightly (see Fig. 3). After insufficient disinfection, biodiversity of the biocoenosis decreases while some species thrive. These show an increased tolerance towards the disinfectant. Insufficient disinfection can encourage the recurrence of hygienically relevant microorganisms.

Figure 3: Scanning electron micrograph of a biofilm on EPDM material at various magnifications (see micrometer bar) after 130 days exposure to elevated concentrations of chlorine



5.7 Underdosed disinfectants can support the transition to VBNC state

L. pneumophila can enter the VBNC state. It is not clear if sublethally dosed electrochemically active (ECA-) chlorine or sodium hypochlorite influence the transition to the VBNC state. Laboratory studies with quantitative suspension experiments (similar to EN 13623) in combination with culture independent methods (membrane integrity and esterase activity) already strongly indicated such transition. The concentration range for inducing VBNC states was about 0.25 - 0.3 mg L-1 free chlorine and 1 h exposure time, applied to 3 day old cultures. With younger cultures, the concentration window is slightly higher (0.4 mg L-1).

With a newly established microtiter plate assay using PNA-FISH for membrane integrity, the VBNC state for *P. aeruginosa* was very probably induced by ECA chlorine, chlorine dioxide and sodium hypochlorite. After 24 h exposure time to concentrations relevant for drinking water (0.2 – 0.25 mg L-1) to all three chlorine products, positive PNA-FISH signals could be detected. At the same time, culture-based methods showed negative results. The combination of metabolic activity and membrane integrity without culturability indicates the occurrence of the VBNC state.

5.8 Efficacy of disinfection measures can be improved by parallel physical measures

Short-term disinfection measures bear the risk of only superficial inactivation of microorganisms, particularly biofilms associated with lime scale. At the same time, the disinfectant may react unspecifically and be consumed without antimicrobial effect, leading to sublethal concentrations and encourages the VBNC state. After disinfection, there is a possibility that VBNC cells return to the culturable state and cause elevated contamination. Intense removal of biofilms, such as with air -water scouring where most of the biofilm is removed, minimises the disinfectant demand. Combination of air-water scouring and disinfection can remove biofilms below the detection limit. Hot water flushing at temperatures above 80 °C can also remove biofilms to a large extent and represents another effective combination of mechanical treatment and thermal disinfection. This has been demonstrated by experiments carried out with biofilm monitors.

5.9 Continuous application of chlorine/chlorine dioxide (0.3 and 0.2 mg L-1 respectively) can cause increased numbers of nonculturable *P. aeruginosa*.

Drinking water disinfection with free chlorine or chlorine dioxide represents a stress factor, reducing the culture-based detection of *P. aeruginosa* in the water phase while the organism

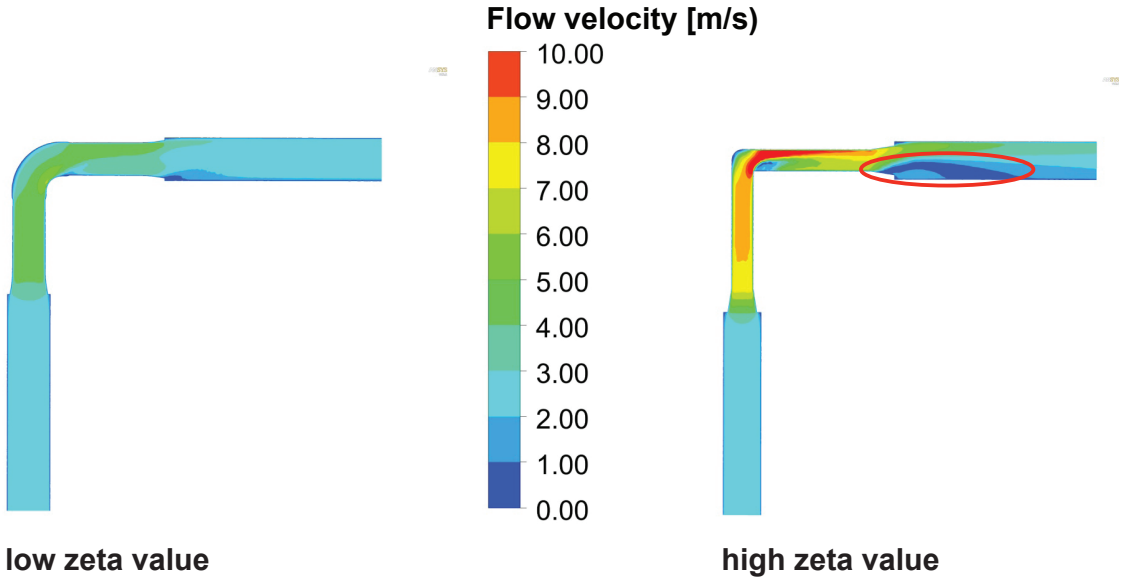
can still be detected by molecular biological methods. Without continuous dosing of the disinfectants, regrowth of the organism was observed only a few weeks after disinfection, and detected both by cultural and culture-independent methods.

The repopulation of *P. aeruginosa* (as a representative of facultative-pathogenic bacteria) could be observed independent of the biofilm age (between 0.1 and 3 years) and temperature (20 °C, 37 °C) in various biofilm models. The target organism could withstand weekly shock treatments (10 mg L-1 chlorine, 6 h), while cultural detection was negative from the first treatment onwards. With qPCR and FISH in parallel, positive signals were found throughout the entire treatment, indicating presence of VBNC state. Thus the elimination of VBNC cells requires much higher disinfectant concentrations and exposure times than the reduction of the culturable state of cells.

5.10 Simulation calculations can allow the location areas with insufficient flow velocities, and are suited to optimize plumbing components in order to avoid problems in operation, cleaning and disinfection.

Flow dynamic simulation of plumbing components and piping sections of drinking water installations allow for analysis of weak points such as areas of stasis, low shear force and flow shade areas in connecting devices with rectangular deflection, in valves and in exit points of T-pieces. Figure 4 shows the pattern of flow distribution inside connection devices. The pattern is caused by the hydraulic situation at low and high pressure loss.

Figure 4: Hygienically unfavorable areas (blue) in connection devices with low (left) and high (right) pressure loss (zeta values)

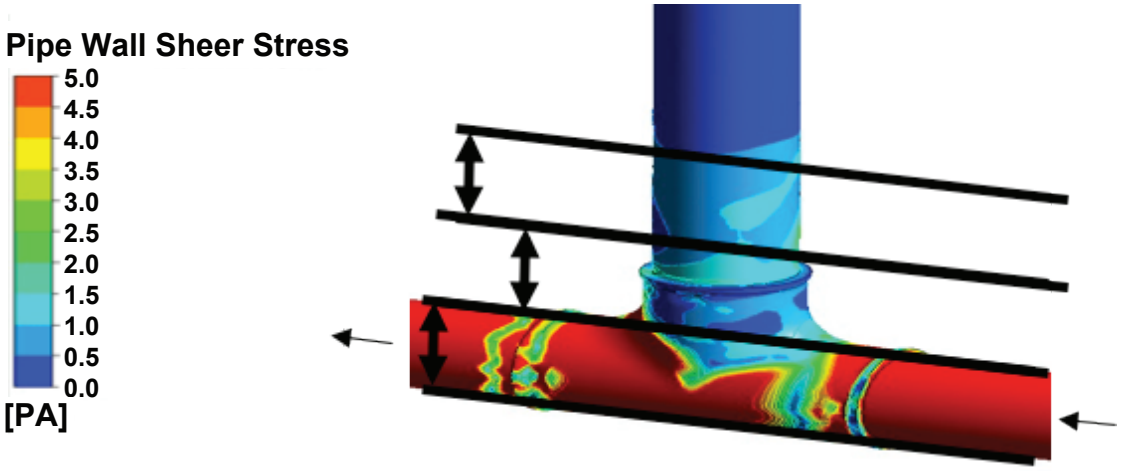


Insufficient flow-through areas in connection devices and other components of the drinking water installation may lead to increased biofilm accumulation and should be minimized by constructive measures. In contamination cases, these areas are not reached by water flushing or flushing with disinfectants. Therefore, only cleaning measures with high flow velocities according to EN 806 or air-water scouring according may be effective; the same is true for disinfection. If necessary, such components have to be disinfected separately or replaced.

5.11 The length of pipe to sparsely used outlets should be no more than 3-fold the inner diameter of the outflow.

To avoid deadlegs and/or blind ends, the water in outflows of T-pieces will be sufficiently exchanged up to a length of approximately 3-fold of the inner diameter of the outflow. Above that length, water exchange will be insufficient (Fig. 5) and hygienic problems expected. This is particularly true for single piping to points of supply of fire hose water outlets, but equally for other outlets.

Figure 5: Water exchange in a T-piece occurs only up to a length of 3-fold inner diameter of the outflow pipe



6. Consequences, laws, norms, technical codes and biofilm management

6.1 Norms and technical codes transfer legal requirements to drinking water installations technically

In norms and technical codes, practical experience in planning, construction, commissioning and operation of drinking water installations are documented. Usually, there is no theoretical explanation provided. The "generally accepted rules of technology" are well known, particularly if their compliance is explicitly defined in laws and decrees. A deliberate deviation has to be justified in cases under legal investigation.

6.2 Norms and technical codes do not usually recognise the relevance of biofilms for hygienic operation of drinking water installations. An exception is the German VDI/DVGW guideline 6023.

Biofilms always exist in water systems, but they are considered unwanted by practitioners because they can have undesired effects on water quality or operation of the drinking water distribution system. This leads to partially unrealistic requirements and measures aiming for complete removal of biofilms. This issue has been addressed in the guideline VDI/DVGW 6023. In the method for assessment of organic plumbing materials according to working sheet DVGW WE 270, instead of "biofilm" the word "microbial colonisation" has been used.

6.3 Biofilm management and consideration of the VBNC state is a prerequisite for hygienic operation of drinking water installations.

Emergence and further presence of biofilms has to be influenced in such a way that no unwanted negative effects on water quality are feared. Biofilm management includes the interaction between biofilm and water and takes into account those parts of the microbial populations in biofilms and water which are not detected by standard cultivation methods (VBNC state). It takes into account that VBNC cells can return to their culturable state and may regain their infectivity.

6.4 Biofilm management has to be implemented into technical codes because it contributes to a better understanding of the hygienic context.

Within biofilm management, microbial analysis has to recognise the occurrence of the VBNC state. Culture-independent methods, even if laborious, have to be involved in analysing suspected contamination cases.

Biofilm management is an important amendment for the German norms DIN EN 806 parts 1-5 and national standard DIN 1988.

The test method(s) for suitability of materials in contact with drinking water according to DVGW working sheet W 270 has to be updated according to the results gained by the "Biofilm Management" research project. The same applies to the European test method "Biomass Production Potential" (BPP; ATP) and the measurement of oxygen demand (MDOD, BS 6920).

Further updating of DVGW working sheet W 551 has to consider establishment of *Legionella* in biofilms and subsequent contamination of drinking water. In this case, the VBNC state can be of high hygienic relevance, particularly when using copper and copper alloys. Hygienic safety cannot be guaranteed by only culture-based methods when considering that legionellae and *P. aeruginosa* may enter a VBNC state when using copper materials.

The technical codes on disinfection (DVGW working sheets W 224, W 225, W 229, W 291 and W 557) have to include biofilm management and consider the VBNC state.

If an autochthonic biofilm is destroyed (after disinfection), a rapid and strong regrowth of *P. aeruginosa* can occur even if only very few cells have survived disinfection of a drinking water system. Particularly vulnerable to contamination by *P. aeruginosa* are newly installed and first operated drinking water systems because they do not yet bear an autochthonic biofilm. The natural biofilm population may influence the extent and multiplication of *P. aeruginosa* and is considered in the concept of biofilm management.

6.5 In laws and decrees on drinking water quality, the parameter of *P. aeruginosa* concentration should be introduced at a technical threshold level of < 1 cfu per 100 mL.
P. aeruginosa can establish, persist and even multiply in biofilms. Routine surveillance of this facultative pathogenic microorganism can significantly contribute to distribution of hygienically safe drinking water. If metabolic activity is found despite lack of growth as determined by culture-based methods, this is an indication of the VBNC state.

7. Glossary

5 °C rule

In a drinking water installation with a water volume above 3 liters between drinking water heater and tap outlet, a circulation system must be installed. The circulation system has to be sized and operated in a way that the temperature difference of the circulating water between heater and tap is less than 5 °Celsius.

AOC

Assimilable organic carbon (AOC) is a fraction of the total dissolved organic carbon (DOC). This comprises low molecular weight, readily degradable substances, e.g. organic acids or amino acids. The AOC therefore serves as an indicator for the regrowth potential of water. Drinking water with an AOC concentration of <5 µg L-1 acetate equivalents of organic carbon and a DOC concentration of <0.6 mg L-1 is considered nutrient-poor.

BEAS-2B

BEAS-2B is a denomination for human bronchial epithelial cells. The bronchial epithelium serves as a barrier against pathogenic bacteria and can eliminate them by specific immune response (e.g. cytokines). As *P. aeruginosa* is known to be a cause of pneumonia in cystic fibrosis, BEAS-2B cells were employed in this project.

Biofilm

The term "biofilm" comprises all accumulations of microorganisms at interfaces (frequently solid/liquid). In a biofilm, the organisms are embedded in a self-produced matrix of extracellular polymeric substances (EPS).

Biofilm populations, biocoenosis

The microbial communities in a biofilm are termed "biocoenosis". They consist of microbial populations of various species. The most important representatives in drinking water biofilm populations are bacteria, protozoa (amoebae, flagellates and ciliates) as well as fungi. These various organisms form complex communities which interact in many ways.

Chelator

Chelators are (mostly organic) compounds with two or more free electron pairs, capable to form coordinative bonds with a central (metal) ion. Thus, they have the capability to fix bivalent or polyvalent cations in stable, ring-like complexes (called chelates). Examples are sodium diethyldithiocarbamate (DDTC) or ethylene diamine tetra acetate (EDTA).

Chelators can bind dissolved free ions (e.g. Cu2+) and, thus, inactivate them chemically. This property is frequently used with complex ions in order to mitigate their toxic effect on organisms.

Cleaning

Cleaning is the process of removal of contamination (e.g. abiotic organic and inorganic substances, microorganisms) using water and, where required, additives such as detergents or enzymes, or by mechanic aids and procedures. Cleaning may not include killing or inactivation of microorganisms. Cleaning efficacy is not quantified or standardised.

Colony forming units (CFU)

The number of visible colonies which emerge from a defined sampling volume at standardized nutrient conditions, incubation temperature and time, on an agar medium. This is a method for determination of culturable heterotrophic microorganisms ("heterotrophic plate count", HPC). Concentrations are expressed as cfu per volume or area. The determination of colony numbers, as indicator parameter according to the drinking water regulation, in 1 mL of water serves the detection of certain, hygienically relevant microorganisms on a relatively nutrient-rich medium. For acquisition of a larger spectrum of heterotrophic bacteria in water and biofilm samples, frequently a nutrient-poor medium (R2A medium), longer incubation time (e.g. 7 days) and lower temperatures (e.g. 20 °C) are employed.

Cytotoxicity

Cytotoxicity is the property of a substance (e.g. bacterial toxin, antibodies, medications) to cause damage in cells and tissue. This damage can also be caused during an immune reaction, e.g. by cytotoxic T cells, macrophages or neutrophile granulocytes.

DAPI

DAPI (4', 6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used extensively in fluorescence microscopy. As DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells, though it passes through.

Disinfection

Disinfection is a process by which the number of pathogenic microorganisms is reduced by killing, inactivation or removal by a standardised, quantifiable mechanism of action with the aim to render a surface or liquid into a state by which it cannot represent any danger of infection. Usually, a disinfecting effect is defined by a removal of 5 logs (bacteria), 4 logs (mycobacteria, yeasts, fungi, viruses) or 3 logs (spores).

DOC

DOC (dissolved organic carbon) comprises all organic carbon compounds in solution.

Drinking water and heated drinking water

Drinking water is water suited for human usage German Drinking Water Regulation § 3, 1a with the properties of quality according to DIN 2000 "guidelines for central drinking water supply" and to DIN 2001 "drinking water supply from small plants and from portable units"). Its quality is regulated by the Drinking Water Regulation. Drinking water should have a temperature of < 25 °C.

Heated drinking water is drinking water from calorifiers which is used for human consumption, particularly for drinking and preparation of foodstuff. Usually, it is provided by the same mounting as cold drinking water to the consumer. The temperature range for heated drinking water is between 25 °C and 85 °C.

Drinking water installation

The drinking water installation comprises all piping, mountings, valves and devices which are between the point of building entrance from the public water supply and the consumer/user at the point of use of drinking water (German Drinking Water Regulation § 3, 3).

DVC (direct viable count)

The DVC method is based on the use of an antibiotic (e.g. pipimedinic acid) which is a gyrase inhibitor and prevents cell division upon growth. This way, growing cells can be distinguished from non-growing cells. Cell division is interpreted as a life sign and can be observed without cultivation and belongs to the culture-independent viability methods.

Exceeding the technical threshold level

If the technical threshold level for legionellae (100 cfu per 100 mL) is exceeded, the owner of the drinking water installation has to initiate the following actions:

1. Analyses for detection of the contamination source. These analyses have to include a site survey and an assessment of compliance to the generally accepted codes of practice.

2. Carry out a risk analysis

3. Carry out all measures required according to the generally accepted code of practice.

The recommendations of the German Federal Office for Environment have to be observed (see reference list).

Ethidium bromide

Ethidium bromide is a fluorescent stain which is a substrate for microbial efflux pumps. Efflux pumps serve the rejection of unwanted molecules in a microbial cell. Intact efflux pumps are interpreted as vitality sign. In the test, ethidium bromide is added to a sample and inspected with a fluorescence microscope. Cells not stained are considered as viable because their efflux system is still active and intact.

Facultative pathogenic microorganism

These are microorganisms which cause infections only under specific conditions, e.g. immunosuppression. Examples are *P. aeruginosa, L. pneumophila*. Other specific conditions can be open wounds or perforation of skin barriers, (by catheters or foreign bodies,) the inactivation of the protective microflora by antibiotics, or other alterations of immune defence.

Fluorescence-in-situ-hybridisation (FISH)

FISH is a culture-independent method for detection of specific microorganisms. It is based on the detection of fluorescently labelled oligonucleotides (gene probes) which bind specifically to ribosomal RNA (rRNA) of the cells. The presence of rRNA is interpreted as a possible indication for active metabolism because it is functional for protein production. The labelled cells can be visualized by epifluorescence microscopy. The specifity of the gene probes can be selected from species to domain specific.

Fluorescein diacetate

Fluorescein diacetate (FDA) is cell-permeable esterase substrate that can serve as a viability probe that measures both enzymatic activity, which is require to activate its fluorescence, and cell-membrane integrity, which is required for intracellular retention of their fluorescent product. Upon hydrolysis by intracellular esterases, this ester yields fluorescein.

Genotoxicity

Genotoxicity is the property of a chemical or biological substance to cause alteration or damage of genetic material of a cell.

Hygienic risk

The term "risk" comprises, according to a report of the risk commission (2003) the qualitative and/or quantitative characterization of a damage concerning its occurrence, extent, and scope. The World Health Organization defines risk as the probability of an event which causes damage to an exposed population within a certain period of time under consideration of the extent of damage. The probability that during a certain period of time damage for persons, groups of persons, plants, animals and/or ecology in a specifically exposed region by a specific dose or concentration of a damaging agent depends both on the toxicity and the extent of exposure.

The "hygienic risk" describes the probability to which health damage to individuals or to public health occurs. This probability refers to e.g. infections, disease, death or handicap over a specific period of time. "Health risk" frequently refers to infection risk. However, hygiene as a scientific discipline, addresses more than microbiological risks alone.

Legionellae, L. pneumophila

The term "legionellae" comprises the family Legionellaceae. These Gram-negative, slim rod-shaped aerobic bacteria with complex nutrient requirements bear a single flagellum and, are therefore mobile. Over 50 different species are distinguished according to their morphological, physiological and genetic characteristics. Legionellae prefer humid and warm locations. Natural habitats are water and moist soils. Cooling towers, air conditioners, drinking water installations (particularly showers) with a risk of formation of aerosols, represent an infection risk for humans. *L. pneumophila* is a facultative human pathogen which is responsible for 80-85 % of all legionellosises (most as pneumonia). Although documentation and common use of language of some health offices refers to "*legionella*", drinking water samples are usually investigated specifically for *L. pneumophila*.

Maintenance metabolism

At low nutrient concentrations, cells only use nutrients to stay alive and not to grow. The same stage can occur as a response to stress. This is known as the maintenance metabolism state. It is assumed that many bacteria under natural conditions remain for extended periods of time in maintenance metabolism. This may be one of the reasons that organisms used to low substrate concentrations do not grow under nutrient rich media conditions. The comparatively high substrate concentration is sensed as a stress and can lead to defective regulation of metabolism.

Micronucleus test

The micronucleus test detects DNA damage (genotoxicity) at the chromosomal level. Micronulei emerge if cells are no longer capable to distribute their heritable material equally to both daughter cells, due to chromosome breaks or loss. The chromosome fragments not integrated in the daughter nuclei condense and exist as micronuclei. The verification is performed by microscopy.

MTT assay

The MTT assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular

oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. The proportion of viable cells correlates with the quantity of the reacting dye (DIN EN ISO 10993-5-2009-10).

Nutrient limitation

Nutrient limitation is a situation where the cells do not have sufficient nutrients for growth but only to maintain their metabolism.

Obligate pathogens

Pathogens which cause infectious diseases (e.g. *Vibrio cholera, Salmonella typhi, Shigella* spp.) in healthy persons without specific immunity.

Opportunistic pathogens

Pathogens which only cause infectious diseases if a person has a compromised immune system (e.g. immunosuppression).

PI (Propidium Iodide)

Propidium Iodide (or PI) is an agent binding to DNA and a fluorescent molecule with a molecular mass of 668.4 Da that can be used to stain cells. As it is charged, it is rejected by intact membranes. It only can pass damaged membranes and is visualized by its red fluorescence. Cell membrane integrity is interpreted as a vitality sign. PI is used for the live/dead staining method and belongs to the culture-independent viability methods.

Polymerase chain reaction (PCR)

The polymerase chain reaction is a molecular biological method for multiplication of specific DNA sequences.

Proliferation test (xCELLigence)

The xCELLigence system allows for real-time monitoring of cellular alterations by measuring electrical impedance. Eukaryotic test cells grow on microelectrodes and any alteration of the cells (adhesion, detachment, structural changes) leads to a change of the electrical impedance between the culture medium and the electrodes. A decrease of impedance is interpreted as a sign for toxic effects.

Pseudomonads, P. aeruginosa

Pseudomonads are Gram-negative rod-shaped bacteria with low nutrient requirements which bear a polar flagellum and are motile. Pseudomonads are ubiquitous in soil and water as well as associated with plants, animals and humans. *P. aeruginosa* is a facultative pathogenic aerobic bacterium which belongs to the genus *Pseudomonas* and is frequently involved in hospital infections where it preterentially infects immunosuppressed persons. Characteristic features are the formation of pyocyanin and fluorescein which allow for the distinction of other members of the genus and which belong to the indicators for its presence in culture-based detection. Although many health authorities and practitioners use the collective term "pseudomonads", usually the species *P. aeruginosa* is addressed.

Quantitative PCR (qPCR)

In qPCR, a fluorescence signal is used to determine the quantity of multiplied DNA.

Rhodamine 123

Rhodamine dyes are used extensively in biotechnology applications such as fluorescence microscopy, flow cytometry, or fluorescence correlation spectroscopy. Rhodamine fluorescence can also be used as a measure of membrane polarization in live cell assays with bacteria. This use relies on the fact that rhodamine 123 accumulates in membranes in a manner which is dependent on membrane polarization, and membrane polarization is taken as a viability sign.

Resazurin

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye, itself weakly fluorescent until it is irreversibly reduced to the pink colored and highly red fluorescent resorufin. It is used as an oxidation-reduction indicator in cell viability assays for bacteria and mammalian cells, and for measuring aerobic respiration.

rtPCR

rtPCR stands for reverse transcriptase PCR and is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA. As such, it is suited to detect protein production even under non-growing conditions.

Settlement of microorganisms

In this project, establishment is understood as the process in which hygienically relevant microorganisms (e.g. *P. aeruginosa, L. pneumophila*) inhabit an existing biofilm in a way that they remain temporarily or constantly (persistence).

Technical threshold level for legionellae

In the German Drinking Water Regulation, a technical threshold level of 100 cfu *Legionella* spp. per 100 mL-1 of drinking water is defined. If the technical threshold level is exceeded, hygienic-technical measures for surveillance of a drinking water installation must be initiated in the form of a risk assessment.

Trypanblue test

The trypan blue test serves the determination of vitality of eukaryotic cells. Trypan blue is an anionic azo dye which, due to its charge, only enters cells with compromised membranes and binds to intracellular proteins.

Viable but Nonculturable (VBNC) State

The VBNC state in this summary is defined as a state of those bacteria of hygienic relevance which normally can be cultivated but (transiently) does not grow on the media used for their detection. They do not multiply because they are not in growth metabolism, but in maintenance metabolism. They do not form colonies on agar media although their viability is maintained. VBNC bacteria frequently can be detected by culture independent methods (see table 1). Bacteria in the VBNC state have functional cell membranes, intact DNA and display metabolic and respiratory activity. The VBNC state is induced by unfavorable environmental temperature, nutrient limitation, presence of disinfectants or toxic metal ions. Under more favorable conditions, they can return into the culturable state and regain infectivity.

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