EXECUTIVE SUMMARY:
RESULTS OF THE COLLABORATIVE RESEARCH PROJECT "BIOFILMS IN DRINKING WATER INSTALLATIONS"

VERSION 2.1 - WITH GLOSSARY

Project duration: 01.10.2006-30.04.2010

5 research partners, 17 industry partners

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Drinking water is the most rigorously monitored class of all foodstuffs. In Germany, the quality of drinking water is excellent – but only until the water meter. Beyond the meter, however, a poorly-studied area of occasional hygienic uncertainty begins: the household installation. Therefore, the microbiological quality of potable water installations was the focus of a joint research project carried out by five research institutions and supported by 17 industry partners under the coordination of Prof. Dr. Hans-Curt Flemming (IWW Mülheim and University of Duisburg-Essen). The study was funded by the German Ministry for Education and Research. The results of this study have attracted a great deal of attention from the press and public.

A nationwide survey of local health authorities revealed that still less than half of the public buildings which were supposed to be monitored have actually been inspected in the five years since the inspection had become mandatory by the new drinking water regulation. This is not due to inactivity of the authorities but rather to being overburdened and understaffed. Furthermore, the methods applied were very diverse and did not always yield comparable results due to different sampling procedures. But even under these conditions, statistical evaluation of more than 20.000 measurements showed that 12 % of the warm water samples contained the facultative pathogenic bacteria *Legionella* and 3 % of about 3,500 samples contained *Pseudomonas* spp. Both were found in cold water much more frequently than expected. It remains unequivocal that strict compliance with the generally accepted codes of practice will minimize the risk of microbial contaminations significantly. But it is similar to traffic: if everybody would stick to the rules, there would be no accidents. One thing the inspections revealed was that the rules were frequently not observed. Although this has not yet led to epidemics, the loss of life quality and working time by illness due to drinking water contamination should not be underestimated. A national survey of community acquired legionellosis (CAL) in Germany demonstrated that there are up to 20.000 – 30.000 CAL; only 2 % of them are notified even if there is a mandatory obligation to notify each legionellosis.

What is the cause of drinking water contamination? Drinking water is not sterile and does not have to be so. The successful strategy of water works is based on nutrient limitation and, thus, prevents microbial growth and produces "biologically stable drinking water." This is a way to avoid chlorination. However, if drinking water bacteria meet materials which release nutrients, they settle, form biofilms and grow. Usual suspects in such cases are synthetic polymers, which are not approved for use in drinking water. For example, shower tubes or even small rubber fittings can become a paradise for bacteria. In such biofilms, facultative pathogens can settle and even proliferate if the conditions are favorable. They are partially released into the water and represent

a hygienic risk. This was investigated and verified in testing systems run under conditions close to actual drinking water systems. Furthermore, it was shown that countermeasures, such as application of disinfectants, could improve the situation but did not lead to essential sanitation. Therefore, the quality of materials in drinking water installations is crucial. Many available materials—in particular those on the lower end of the market—are leading directly to enhanced biofilm formation. One problem is that in household installations, the use of approved materials is not mandatory.

In the framework of these investigations, another problem surfaced: the methods for the determination of microorganisms. The gold standard for the control of microbial quality remains the determination of colony forming units. However, with that method the only organisms found are those which can multiply on the media employed. If bacteria are stressed, (e.g., by disinfectants, UV irradiation or heat), usually less than 100 % are killed; instead a part of the population may enter a transiently non-cultivable state. Then, they are by no means dead, but they disappear from the radar of standard monitoring procedures. Once recovered, they can proliferate and even become infectious again, which was clearly demonstrated in the research project. This phenomenon is likely the cause for difficult practical cases in which sanitation is insufficient, takes a long time, and the contamination re-emerges. Methods for detecting "dormant" organisms have been included and evaluated in the project.

The crucial questions are now: under what circumstances does the transition into the dormant state occur and when and why do the non-culturable organisms become resuscitated? Such processes effectively nullify standard analytical methods, which otherwise have proven successful for more than 100 years in controlling the big waterborne plagues like cholera, typhoid fever and shigellosis. Today's challenge is not these waterborne diseases but waterborne diseases caused by facultative pathogens which have their reservoir in water and are part of the autochthonous aquatic microflora. In problematic situations, however, where non-culturable microbial populations are suspected, it is urgently recommended to include culture-independent molecular biological methods. While molecular techniques could be used to demonstrate improved sanitation, several fundamental questions remain unanswered. In order to make the results of the project accessible to a wider audience, the most important results have been summarized in easily understandable statements.

The scientific summary of the project has been published as chapter 3 of the book **Microbial growth in drinking water distribution systems and tap water installations**, *edited by D. van der Kooij and P. van der Wielen, IWA Publishing, London 2012: Flemming, H.-C., Bendinger, B., Exner, M., Gebel, J., Kistemann, T., Schaule, G., Szewzyk, U., Wingender, J.: "The last meters before the tap: where drinking water quality is at risk" Available on request from Hans-Curt Flemming.*

1. The differences between public networks and drinking water installations

There are significant and important differences between drinking water installations in buildings as opposed to the public drinking water distribution network. Some characteristics of drinking water installations are:

- The range of water temperatures is higher than in the distribution network. Drinking-water systems in buildings usually comprise hot and cold water. It is not uncommon that even the cold water temperature reaches 20 °C and more, particularly if the drinking water pipe is insufficiently insulated, located close to that of warm water, or if pipes run through heated rooms;
- The retention time of the water can be quite long (days to weeks) with stagnation periods, in particular during holidays or other periods of low consumption.
- Drinking water installations tend to include dead ends and areas with particularly low flow through or irregular flow regimes.
- Water outlets such as taps can be contaminated (especially by Pseudomonads) and can act as sources for retrograde contamination.
- The range of materials employed in drinking water installations is partially different from that of drinking water distribution networks (e.g., synthetic polymers, copper, stainless steel, elastomers); possibly, they may be unapproved for use in drinking water. Occasionally, materials are employed which support microbial growth and, thus, biofilm formation.
- The surface-volume ratio is much higher, providing a large surface area ready to be colonized. Thus, biofilms play a more important role in drinking water installations.
- Auxiliary equipment can be part of the installation such as softeners, filters, phosphate dosage devices or others. It has been shown that such equipment raises the risk of elevated microbial numbers in the water
- The range of hygienically relevant organisms is different: in temperate climates, *Legionella pneumophila*, *Pseudomonas aeruginosa* and non-tuberculous mycobacteria are organisms that play a minor role in public water distribution systems compared to drinking water installations in buildings.
- Surveillance is more difficult and less frequent than in public networks.
- In hospitals and other large buildings, frequently an unclear situation of piping system is encountered due to continuous alteration measures.
- The design of plumbing systems in buildings is highly variable and influenced by the diversity of water uses and building types (e.g., schools, hotels, residential buildings, sport facilities, and office blocks as well as hospitals, nursing homes and other health-care facilities).

These factors may enhance biofilm formation and elevate the risk of water contamination in drinking water installations. Most epidemics related to these points are reported, so far, for hospitals. Here, water systems have been identified as reservoirs and sources of contamination by *L. pneumophila* and *P. aeruginosa*.

2. All surfaces in a drinking water installation which are in contact with water are colonized by biofilms

It is long known that in drinking water distribution systems, all surfaces in contact with water can be colonized by microorganisms. The term "biofilm" comprises all microbial aggregates. In biofilms, microorganisms are embedded in a matrix of extracellular polymeric substances (EPS). Microorganisms can establish synergistic consortia, in which they can degrade complex substrates. In this matrix, the organisms can tolerate much higher concentrations of disinfectants than in the planktonic phase. Cell-cell communication is favored in biofilms as well as genetic exchange.

Certain types of plastic and elastomeric materials can promote biofilm formation due to the release of biodegradable compounds such as monomers or additives like plasticizers, anti-oxidants, providing favorable nutrient conditions for microorganisms. It has been estimated that about 95 % of all microbial cells present in drinking water distribution systems exist as biofilms on pipe surfaces, i.e., directly located at the leaching interface—only 5 % occur in the water phase. Similarly, in a domestic hot water system, most of the culturable bacteria (72 %) were found to be surface-associated. Drinking water biofilms are formed predominantly by microorganisms of the autochthonous aquatic microflora without any relevance to human health. However, biofilms have the potential to harbor microbial pathogens, which can harm human health, especially in immunocompromised or otherwise predisposed people. Once integrated in a drinking water biofilm, these organisms can tolerate external stresses, such as the action of disinfectants, much better and can thus persist and possibly multiply inside the biofilm. Contamination of drinking water occurs when opportunistic pathogens are released from a biofilm as a consequence of physical disturbance or active detachment of infectious cells, which then pose a potential threat to human health.

3. Plumbing materials and drinking water are the "usual suspects" as potential sources for enhanced biofilm formation

Even oligotrophic, biologically stable drinking water still contains viable microorganisms. If they come into contact with nutrients, they will multiply. Frequently, freshly installed plumbing materials in drinking water installations serve as nutrient sources because they may leach biodegradable additives such as plasticizers, antioxidants, remaining monomers or separation chemicals. In addition, pipes and other materials may have been contaminated by colonizing microorganisms and leachable nutrients during production, processing or installation. The materials considered in this project are listed in Table 1. Under conditions that are commonly encountered in practice, biofilms usually develop in 1-2 weeks on newly installed materials. After 6-10 weeks of operation, the biofilms typically enter a quasi-stationary phase where cellular multiplication and growth have stabilized or have begun to decline. This process has been observed for all water compositions and temperatures as well as on all investigated materials, including copper. Biofilm formation on the synthetic rubber material ethylene-propylen-dien-monomer (EPDM) was particularly strong and even visible with the naked eye. Such a level of biofilm contamination neither met the national requirements, according to DVGW Work Sheet 270, nor the KTW recommendations. Thus, EPDM was included as a "worst-case" situation, but it is, nevertheless, still used occasionally.

Table 1: Plumbing materials investigated in the project

MATERIAL	USAGE	APPLICATION	COMPLIANCE TO DVGW W 270[5]	KTW CATEGORY[7]
Copper	Metal pipe	Drinking water installation	–[6]	–
HD-PE-Xb[1]	Composite pipe	Drinking water installation	Yes	A[8]
HD-PE-Xc[2]	Polymer pipe	Drinking water installation	Yes	A
EPDM with certificate[3]	Reinforced hoses	Connection to faucets (before 2007) and devices e.g., (dish) washing machines	Yes	C[9]
EPDM without certificate[4]	Reinforced hoses	Fittings and connections in drinking water installations before 1995	No	C

1. HD-PE-Xb: high density silan-cross-linked polyethylene
2. HD-PE-Xc: high density electron-ray cross-linked polyethylene
3. Ethylen-propylen-dien-monomer which has passed the admission by DVGW W 270
4. Ethylen-propylen-dien-monomer which has not passed the admission by DVGW W 270
5. DVGW worksheet W 270: German standard procedure for admission of polymeric materials in drinking water systems (Anonymous 2007).

6. Not applicable
7. German Commission for drinking water. In order to assess the health risk of organic materials in contact with drinking water, different categories were defined (KTW-Empfehlung 1977)
8. Category A applies for pipes with a surface to volume ratio of 1:1 cm^2 ml^{-1}. After a triple migration test for 72 h each, among other parameters to be tested, the TOC content of test water should not exceed 2.5 mg m^{-2} day^{-1}.
9. Category C applies for pieces of equipment and rigid jointing sealants with a surface to volume ratio of 1:6 cm^2 ml^{-1}. After a triple migration test for 72 h each, among other parameters to be tested, the TOC content of test water should not exceed 15.0 mg per m^{-2} day^{-1}.

Of course, the composition of the drinking water also influences biofilm development by supplying nutrients. A combination of poor or inappropriate material quality and unfavorable drinking water composition (e.g., 12 mg L^{-1} nitrate and 1 mg L^{-1} phosphate) led to strong biofilm development.

4. The composition of the biofilm populations varies due to various influences

The microbial composition of biofilms in drinking water installations depends upon a range of influencing factors such as materials, composition of drinking water, temperature, presence of disinfectants etc. Among the most critical factors are, apart from the materials, auxiliary materials, e.g., for fitting, as well as materials used for repairing of existing installations.

In response to the nature and quantity of the nutrients, biofilm populations develop which differ in composition and diversity. Plumbing materials which leach organic substances not only support biofilm growth in terms of biomass, but they also expand the spectrum of biofilm organisms which may otherwise not dominate drinking water biofilms. This can include an increase of the risk for their establishment in such biofilms and possible contamination of the drinking water.

5. Thermo-oxidative measures for system disinfection promote ageing of materials

A new aspect of the project was the comparison of biofilm formation on fresh and aged materials. The relevant factors are schematically shown in Figure 1.

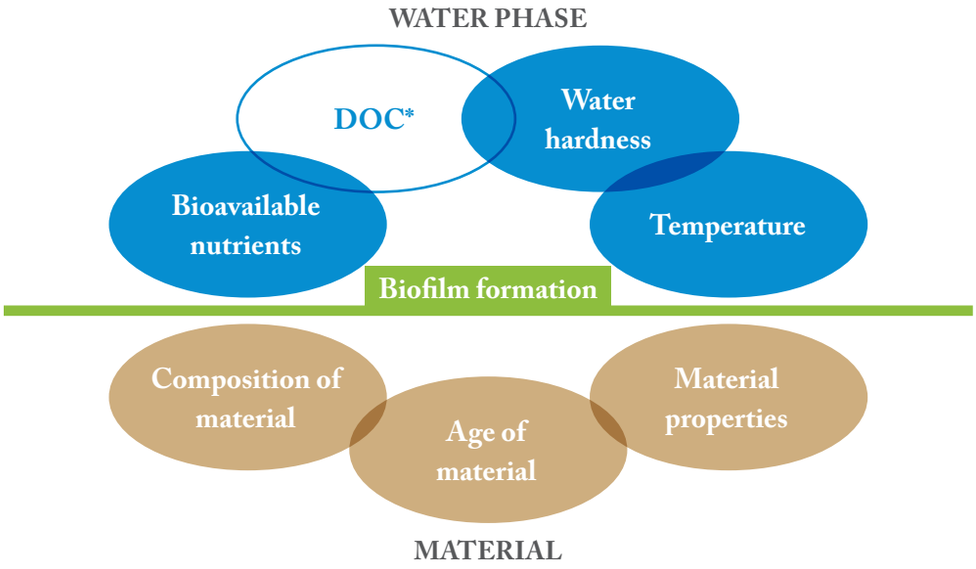


Figure 1: Factors influencing biofilm formation

In order to investigate the influence of material age, artificial ageing was performed according to the standard test methods for evaluating the oxidative resistance of polyethylene (PE) pipe to chlorinated water (ASTM 2008). The hypothesis was that ageing processes may influence the release of bioavailable substances from PE-Xc and EPDM materials, as it is known, from the water phase. It has been shown earlier that oxidizing agents, such as chlorine, may increase the AOC** and enhance regrowth potential. Artificial ageing was performed, e.g.: by exposure of the materials to a pressure of 4 bar at 40 °C in presence of 2.5 mg L^{-1} sodium hypochlorite or 4 mg L^{-1} chlorine dioxide, respectively, for a period of 4 weeks. Chlorine dioxide showed the highest effect on the materials under comparable conditions. It was obvious that ageing procedures may lead to the formation of cracks and crevices in all synthetic materials, but it could not be shown that these cracks are preferred settling areas.

**DOC: dissolved organic carbon in water.*

***AOC: assimilable organic carbon in water. Determined on the basis of growth of defined bacteria in a sample of that water.*

6. Alterations of materials due to ageing are influencing biofilm formation

Biofilm formation was assessed in a multifactorial approach in which the most relevant parameters for biofilm growth were included.

The results of the multi-parameter experiments showed that all chosen factors influence biofilm formation, but they do so to a different extent:

- The amount of nutrients represents the highest single influencing factor;
- If materials leached biodegradable substances, the DOC of water was of minor relevance;
- In situations with materials leaching low amounts of nutrients, the nutrient content of the water played a more important role;
- Temperature played a stronger role under copiotrophic conditions but to a lesser extent under oligotrophic conditions;
- Material age also significantly influenced biofilm formation with aged materials having lower biofilm formation than fresh materials;
- Water hardness showed a low influence on the biofilm formation potential

After prolonged ageing, cracks formed (Figure 2).

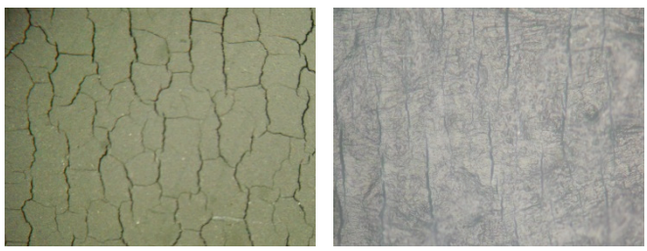
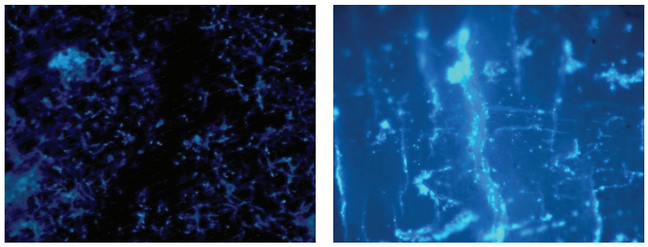


Figure 2 (top):
Micrograph of aged EPDM and HD-PEXb.



Aged EPDM *Aged HD-PEXb*

Figure 2 (bottom):
Epifluorescences microscopical pictures of colonized surfaces; bacteria well visible as blue flurorescent rods.

The formation of cracks tended to shorten the functional lifetime of the material. Furthermore, cracks may represent an indirect hygienic risk because bacteria settling in cracks could be more difficult to remove and could be inactivated by disinfectants.

7. Any preventive disinfection of drinking water installations has to be considered critically and needs an indication

Preventive disinfection is, in Germany, regarded as contradictory to the rule of minimization and can only be recommended in well justified, selected cases. Disinfection measures require clear indications. In the first place, drinking water should be of unobjectionable quality and the system should be in a faultless state, according to the generally accepted rules of practice—or it has to be transformed into that state.

The results of the research project have confirmed, without ambiguity, the importance of adhering to the generally accepted rules of practice. They reflect the average consumer behavior and offer a high degree of safety in the use of drinking water. Departures from the rules of practice increase the risk of a hygienic impairment of drinking water. Such risks often cannot be mitigated by preventive disinfection or other measures, e.g., water treatment. Such measures should only be taken after thorough assessment and under continuous surveillance.

8. Assessment of biofilm growth is dependent on the method applied

Various methods for quantification of microorganisms lead to various results. In drinking water surveillance, culture-dependent methods are used exclusively. The determination of colony forming units (cfu, either as colony counts, according to the German Drinking Water Ordinance, or as HPC on R2A medium) only reflects the number of organisms able to proliferate under the given conditions. It is well known that microorganisms which do not grow (i.e., are not in growth metabolism) are not necessarily dead. In fact, they can live for long periods purely on maintenance metabolism. This applies to the majority of drinking water organisms. They enter this state frequently as a stress response (e.g., nutrient depletion, disinfectants, irradiation, metal stress, etc.) and can return into the culturable state upon abolishment of the stress. In such cases, their reappearance in a drinking water system is not due a new contamination but due to the resuscitation of already present organisms. By determination of the total cell number (TCN) by DNA-specific fluorescent dyes and enumeration by microscopy, the maximum cell number can be determined. However, this method does not allow distinguishing between living and dead or inactive cells.

In all investigations, TCN was significantly higher than any numbers determined by culture-dependent methods. Depending on temperature, biofilm age and water quality, the results

obtained as cfu or HPC varied. Generally, EPDM materials were more densely colonized than copper, HD-PEXc or HD-PEXb. The proportion of culturable bacteria on the TCN decreased with biofilm age.

As one of the consequences of this project, it is recommended for critical cases to determine not only the cfu but also the TCN. The ratio of colony numbers to TCN provides some information about the physiological state of a microbial population: a high proportion of colony numbers may suggest favorable nutritive conditions, which is an indicator of possible contamination by biodegradable substances and, in turn, of less biologically stable drinking water.

9. *Legionella pneumophila* and *Pseudomonas aeruginosa* can become incorporated into existing drinking water biofilms and can contaminate drinking water

If facultative pathogenic bacteria enter drinking water systems, they can be incorporated into existing biofilms, regardless of material quality, material age, water quality and temperature. Once established, such organisms can persist over weeks to months in cold and warm drinking water. Under favorable conditions, they can even multiply. Since they can be released from the biofilm into the drinking water, biofilms represent a source of contamination and, consequently, a potential source of infection. Facultative pathogenic bacteria of clinical relevance detected in drinking water biofilms include *L. pneumophila*, *P. aeruginosa*, and *Enterococcus feacalis* which have the most important epidemiological significance for public health.

Results obtained in a semi-industrial pilot plant showed that *L. pneumophila* became established on any material and in any water quality in drinking water biofilms, particularly at elevated temperatures. Proliferation was favored if amoebae were available as hosts for intracellular multiplication and if actively growing biofilms were present. Under such conditions, it should be expected that legionellae are released from biofilms, contaminating the drinking water. The concentration of *L. pneumophila* in drinking water samples (according to DIN EN ISO 19458) can be above the technical measure value of 100 cfu/100 mL, according to the drinking water ordinance 2011 and to the DVGW Work Sheet 551. In biofilms which developed in cold drinking water (12-15 °C), *L. pneumophila* could be sporadically detected by standard methods (ISO 11731). The concentration of *L. pneumophila* in biofilms depends upon the colonization density of the biofilms. Materials with strong biofilm development and many amoebae (e.g., EPDM without recommendation according to DVGW Working Sheet 270) offer a potential for very high concentrations of *L. pneumophila* in water, especially if temperatures are higher than 20° C in cold water.

Temperature is another crucial factor for proliferation of *L. pneumophila*. Therefore, compliance to the codes of practice, as defined in the DVGW Working Sheet 551 for warm water installations, has to be thoroughly achieved. However, the project has confirmed that cold water installations can also be contaminated with *L. pneumophila*; therefore, such systems should also be sanitized by methods suitable for eradicating *L. pneumophila* contamination.

P. aeruginosa can also be incorporated into biofilms on all new and aged materials and can equally contaminate the water by release from these biofilms. The duration of persistence as determined by cultivation methods can vary between the materials.

A new insight provided by the project is that both *L. pneumophila* and *P. aeruginosa* can occur in biofilms on plumbing materials of drinking water installations also in a "Viable-But-Non-Culturable" (VBNC) state. In this state, the bacteria are no more culturable on common laboratory media designated for their detection, although they are still viable. Indications for the VBNC state of bacteria can be gained, e.g., by culture-independent methods, e.g., by using fluorescence-in-situ-hybridization (FISH) employing genus- or species-specific gene probes, or by PCR-based methods. However, it is acknowledged that a FISH-positive signal does not ultimately prove viability because it cannot be excluded that ribosomal nucleic acids are preserved after cell death. The determination of cell death is surprisingly difficult and exclusively depending upon the method applied for verification.

The concentration of *P. aeruginosa* on new and aged materials, as determined by FISH, was significantly higher than as determined by cultivation. FISH-positive cells cannot be declared dead. The same was true for *L. pneumophila* incorporated into drinking water biofilms. While the concentration of culturable cells varied widely, the concentration of FISH-positive target organisms remained relatively constant during the entire period of the experiments. This allows the conclusion that the overall concentration of the target organisms in the biofilms remained more or less constant while the proportion of culturable cells changed. Obviously, the physiological state of the bacteria in the biofilm can be influenced by a variety of complex processes. Among these, interactions with other bacteria and with protozoa, as well as changes in temperature and nutrient concentration, may be important. This may plausibly explain the sometimes erratic findings in routine and control analyses in practice.

10. Surveillance – the twilight zone

A nationwide survey in Germany showed that only half of the public buildings which had to be controlled have actually been sampled. This was mainly due to over-commitment and understaffing of the health authorities which have to carry out the surveillance. The methods applied for the investigations differ widely and do not always lead to comparable results. But even if this is taken into account, the data allow for some clear conclusions.

For the survey, a total of 4,600 public buildings that have been inspected by German health authorities with 30,000 water samples during 7 years (2003-2009) were available. Furthermore, 16,000 data points in over 1,000 buildings concerning the specific technical drinking water installation construction could be collected. A statistical evaluation of the data showed an exceedance of the technical action value for *Legionella* (100 cfu in 100 mL) in 13 % of the samples and 3 % for *Pseudomonas* spp. (exceedance of the guideline value of 0 cfu per 100 mL as recommended by the Federal Environmental Agency and, since 2011, in the federal drinking water ordinance). Although *Legionella* exceeded the threshold value mainly in the warm water systems, which was not very surprising, the organism was also found in 5 % of the cold water samples. This demonstrates that *Legionella pneumophila* can find suitable survival and persistence conditions in cold water systems. Recently, high *Legionella* numbers (≥ 1,000 cfu/100 mL) were reported in cold water samples at temperatures as low as 11 °C in health-care facilities. This underlines the importance of assessing cold water plumbing systems as potential contamination sources of *Legionella*. Pseudomonads (not further differentiated) could be found in cold and warm water systems to approximately equal percentage. The most relevant results on exceedance of limit values are summarized in table 2.

The generally accepted technical rules of practice were often not observed, such as regular maintenance of the drinking water installation (35 %, n=498), un-dismantled dead legs (65 %, n=327), or flushing after extended interruption of operation, e.g., after summer holidays in schools (56 %, n=290). The statistical evaluation revealed that a daily temperature rise of the warm water reservoirs to at least 60 °C reduced the relative risk for positive reports of *Legionella pneumophila* by about approx. 30 %. Any additional device for water treatment—be it softeners, filters, phosphate dosage or others—will at least double the risk, mainly because of lacking maintenance or other non-compliance to technical guidelines of good practice.. The survey showed clearly that the following points should be harmonized:

- Prioritization of institutions to be surveyed;
- Extent and intensity of inspections;
- Harmonization of methods;
- Documentation and archiving of data;
- Expertise of personnel.

MICROBIOLOGICAL PARAMETERS	SAMPLES	EXCEEDANCE ABSOL.	%
Legionella spp.	22,786	2,908	12.8
Colony count 36 °C	10,928	380	3.5
Colony count 20 °C/22 °C	10,869	129	1.8
Coliform bacteria	8,652	152	1.8
Escherichia coli	8,330	25	0.3
Pseudomonas spp.	3,468	102	2.3

Table 2: Exceedance of guideline values for microbiological and chemical parameters of drinking water installations (Völker et al., 2010)

Furthermore, the data showed that determination of heterotrophic plate counts (HPC) only, according to the analytical methods defined by the Council Directive 98/83/EC and the German Drinking Water Ordinance 2001, is not fully sufficient to assess the hygienic status of drinking water; no correlation could be found between HPC numbers and occurrence of *Legionella* after evaluation of more than 20,200 data points.

The proportion of *Legionella* and pseudomonads in objectionable samples was much higher than that of *E. coli*. In addition, no significant correlation could be found between the presence of *E. coli* and coliform bacteria or *Legionella* or pseudomonads.

Especially in cases of doubt with recurring contaminations, it is strongly recommended to refer to advanced methods for determination and verification of *Legionella*, such as FISH or PCR-based methods. Thus, hidden contamination sources with cells that survive disinfection treatment within the system can be detected. For differentiation between local and systemic contamination sources, it is recommended to employ Pulse-Field Gel Electrophoresis (genetic fingerprinting) of bacterial isolates.

11. The determination of culturable bacteria alone is not always sufficient to ascertain the hygienic quality of drinking water

The standard cultivation methods allow for simple and cost-effective detection of hygienically relevant bacteria (e.g., *E. coli, P. aeruginosa*, *L. pneumophila*). Although these methods have been employed for the analysis of drinking water very successfully, they still have some weaknesses. One of them is that they only detect the "tip of the iceberg" as they do not detect VBNC organisms, which are by no means dead. As indicated earlier, entering and leaving the VBNC state may be the reason for occasional fluctuations of those organisms. The transition between both states is reversible and not yet fully understood, but temperatures, nutrient availability, and presence of disinfectants seem to play important roles. In critical cases, the combined application of conventional cultivation and culture-independent molecular biological methods is strongly suggested.

On this background, the following recommendations for the assessment of the hygienic quality of drinking water can be derived:

- In cases of doubt, not only colony numbers but also total cell numbers should be determined. A high proportion of cfu in TCN indicates good nutrient conditions and, thus, a decrease of the "biological stability" of the drinking water.
- For purposes of identifying sources of contamination or infection, or for verification of sanitation measures, supplementation of conventional methods by advanced, culture-independent methods should be considered.
- For the localization of contamination sources, genotyping Pulse-Field Gel Electrophoresis (PFGE, "genetic fingerprinting") should be applied.

12. Standard microbial parameters provide no indication about the presence of *legionellae* or *P. aeruginosa*

The nationwide survey of official routine monitoring has shown that analysis of the parameters, according to the German Drinking Water Ordinance, is not suited to reliably detect contaminations by the facultative pathogens *L. pneumophila* and *P. aeruginosa*. Evaluation of over 100,000 data points has shown that the proportion of *legionellae* and *P. aeruginosa* was far above that of *E. coli* and coliforms. No reliable correlation between the detection of the latter with the first could be established.

13. In presence of metal ions, detection of opportunistic pathogens by culture-dependent methods can be negative although they may be present in a viable state

The presence of metal ions (e.g., Cu^{2+}) is one of the factors which can inhibit growth of microorganisms in drinking water and drinking water biofilms. This is probably the reason why such contradictory findings about biofilms on copper are reported in the literature, if the numbers are based on culture-dependent methods. It was shown that bacteria (in this case: *Pseudomonas aeruginosa*) exposed to copper concentrations common to those occurring in plumbing systems can enter a VBNC state from which they can return under favorable conditions. How fast these transitions happen and the mechanisms on which they are based are still unclear and the subject of current research of the consortium.

14. Effective cleaning is a prerequisite for successful disinfection

Cleaning is defined as removal of contaminations, deposits and other undesirable substances from drinking water installations. Cleaning allows restoration of hydraulic flow conditions and protects the quality of drinking water flowing through pipes. Contaminants can contain substances which directly impair drinking water quality but which can also cause microorganisms to grow on wet surfaces and then contaminate the water. All loose deposits have to be removed during cleaning. On no account should they become re-deposited in other areas since this would lead to further contamination of the drinking water. Solidly attached inorganic deposits can serve as protective layers and act as corrosion inhibitors. On the other hand, their rough surfaces facilitate adhesion of microorganisms.

Removal of deposits reduces the habitat availability for microorganisms and optimizes operating conditions for drinking water installations.

15. Disinfection is not cleaning

Implicitly, it is often assumed that disinfection also means removal of biomass. This has been investigated in the project, using a model system based on biofilms growing on the inner surface of silicon tubes. This material strongly supports biofilm growth by leaching of biodegradable additives, thus, serving as a "worst case" model, as biofilms in such systems are particularly difficult to remove. Chemicals and methods successful in this system can safely be assumed to be successful in practice.

It was shown that for disinfectants in concentrations as permitted by Section 11 of the German Drinking Water Ordinance it took at least 70 days until the colony counts of biofilms were below detection limit. In the same time, the decrease of total cell numbers (biomass) was negligible. Substantially higher concentrations can only be applied for disinfection during system sanitation. Chlorine, chlorine dioxide, hydrogen peroxide in combination with silver ions and fruity acids were applied. All applied disinfection methods led to a decrease of colony counts below detection limit. However, the reduction of the TCN was mostly insufficient with the exception of ozone (0.1 mg L^{-1}) and chlorine (25 mg L^{-1}). Again, it was verified that disinfection does not replace cleaning, and remaining biomass has to be considered as nutrient for bacteria entering the system subsequently. Cleaning by air-water flushing can be advantageous.

A mobile device was designed, termed the "Hygiene Monitor," which basically consists of an apparatus with silicon tubing (or other tubing materials) which can be implemented in water systems in order to assess the biofilm formation potential of waters. It represents the "worst case" situation and cleaning/disinfection success in this device is considered to be sufficient for extrapolation to a drinking water installation. This system has been shown to be quite useful, especially in situations where recurring growth may occur, e.g., in hospitals.

16. Disinfection can lead to population shifts and favor fast-growing species

The long-term effects of disinfection on biofilm populations were investigated. It was evident that regeneration of the biofilm occurred immediately after termination of the disinfection process, either by resuscitation of stressed cells or by growth of remaining bacteria or by re-colonization from the water phase. The populations which established themselves after disinfection differed significantly in terms of composition and diversity from those prior to disinfection. Depending on the nature and concentration of the disinfectant, a selection pressure was generated which led to the population shift.

A reason for that shift may be that dead biomass was not removed and, thus, served as an easily accessible nutrient source for subsequent bacteria. This highlights the risk that, in response to disinfection, fast growing organisms (which frequently comprise hygienically relevant bacteria) may develop rapidly.

17. A transition from VBNC into culturable state of *P. aeruginosa* possibly can be influenced by disinfection procedures

On all materials investigated and at various water qualities, several weeks after an artificial contamination with *P. aeruginosa*, this organism still was present in the biofilm. However, it could only be found by FISH, but not by culture-dependent methods. This is an indication for the transition into the VBNC state. If such biofilms were treated with chlorine dioxide up to 20 mg L^{-1}, *P. aeruginosa* could be occasionally found in the culturable state even after 24 h or 3 weeks after disinfection, both in biofilm and water. FISH signals remained at the same level as before disinfection. This can either be interpreted as a resuscitation of *P. aeruginosa* by disinfection or as rapid regrowth from colony counts below detection. The latter, however, is quite improbable after 24 h. Here, further research demand is obvious and currently in progress in the consortium.

18. *P. aeruginosa* and *L. pneumophila* can survive cleaning and disinfection

The enduring removal of pathogens from a drinking water installation is only possible with high effort. If a compact biofilm develops on plumbing material, facultative pathogens can survive combined cleaning and disinfection in a biofilm because biofilm removal is so difficult. Surviving facultative pathogens can proliferate in the biofilms and re-contaminate the drinking water. The detection of survivors is, as indicated earlier, dependent on the method and advanced methods are recommended in those cases.

Materials in compliance with the accepted technical rules (KTW guideline, DVGW Worksheet W 270) do not effectively prevent contamination of biofilms with facultative pathogens, but they assist in keeping biofilms thin and patchy and are a good starting point for successful cleaning and disinfection.

19. Reference to technical rules and statuary provisions

The German Drinking Water Ordinance is the foundation for providing safe drinking water, presumably of faultless quality, and refers manifold to the generally accepted codes of practice. Although the generally accepted standards of good practice are an undetermined legal concept and are not defined in laws, they still have an enormous relevance for defining the duties of care and public safety. It is assumed that there would be only a slight risk of microbial or chemical contamination of a drinking water distribution system and, thus, of the drinking water itself if those standards are observed during design, construction, installation and operation of drinking water installations.

Due to the rapid pace of technological development of new materials, components and processes, particularly in the water industry, regulations need continuous updating in response to new developments and scientific findings.

Without any doubt, consistent and correct implementation of the generally accepted standards of good practice will minimize the risk of microbial contamination significantly. However, the situation can be compared to traffic: if everybody follows the rules, the risk of accidents is minimized. In this project, among others, some aspects of non-compliance have been identified and investigated. This is valuable for understanding and responding to such aspects.

Conclusions

Drinking water installations represent hygienically sensitive components in the drinking water supply chain, which is regulated, but insufficiently controlled. In order to effectively use the prevention potential that is inherent in the framework of regulations, it appears particularly important to pay thorough attention to the selection and testing of plumbing materials.

The methods used for monitoring of hygienic quality of materials as well as drinking water play a crucial role for risk assessment. It is recommended to employ, in addition to conventional culture-dependent methods, also the now available advanced culture-independent methods, particularly in cases in which detection of the actual biomass and hygienically relevant microorganisms is mandatory. The transition of facultative pathogens into the VBNC state and their return to the viable state may be the explanation for persistent cases of contamination and regrowth as occasionally observed in practice.

Furthermore, significant research is needed to better understand the conditions under which bacteria enter and leave the VBNC state. First results indicate that they can regain their infectivity after resuscitation.

From the results of the nationwide survey, the question arises as to how advanced testing methods can be used to identify and localize microbial contamination in drinking water installations. The development of a reproducible, standardized and evaluated method for rapid, safe and cost-effective detection of microbial contamination in such installations appears urgently necessary. Requirements stipulating the selection and number of representative sampling points must be standardized and should include sampling frequency, parameter selection and an evaluation of the practical applicability using innovative microbiological detection methods.

Particularly in problematic practical cases, the potential of advanced molecular biological methods should be utilized to compliment the well-proven culture-based standard methods!

Glossary

Biofilm

The term "biofilm" describes any accumulation of microorganisms adhering to interfaces (frequently: solid/liquid). The organisms in a biofilm are embedded in a matrix of extracellular polymeric substances (EPS).

Biofilm-populations/biocoenosis

The biofilm biocoenosis comprises all populations. The most important representatives in drinking water biofilms are bacteria, protozoa (amoebae, flagellates and ciliates) as well as fungi. These different organisms form complex communities (biocoenoses) which interact in a variety of ways.

Disinfection

Disinfection is a process whereby the number of microorganisms capable of proliferation is reduced by destruction or rendering them inert through application of an agent with a specified, standardized and quantifiable effect with the aim or restoring an object/area/medium to a state that no longer carries any risk of infection. The purpose of disinfection is the defined reduction of pathogenic or facultative pathogenic microorganisms but not the elimination of environmental microorganisms which pose no threat to human health.

Facultative pathogens

Facultative pathogens are those which require specific conditions for causing infectious diseases, including suppression of the immune system (e.g., *P. aeruginosa, L. pneumophila, Klebsiella* spp., *Enterobacter* spp.). Examples for specific conditions: access to areas of the body which are normally sterile (e.g., by catheters or foreign bodies), the destruction of the physiological microflora by antibiotics, wounds, or changes in physiological defense mechanisms.

Fluorescence-in-situ-hybridization (FISH)

FISH is a culture-independent method of detecting microorganisms which uses fluorescent oligonucleotides (gene probes) that specifically bind to the ribosomal RNA of cells. Once marked, the cells can then be visualized using a fluorescence microscope. Gene probes can be specific for species, families or domains.

Hygienic risk

The term "hygienic risk" describes the probability that the health of both individuals and the general public will be adversely affected. In this context the probability of an adverse outcome relates to infection, illness, death or disability occurring within a specific period of time.

In the narrower sense, the term "hygienic risk" frequently refers to the risk of infection, although the discipline of hygiene is also concerned with other risks.

Colony count

The colony count is the number of visible colonies that develop in or on nutrient agar based on a clearly defined sample size, nutrient supply, and incubation temperature, and over a specific period of time. It is a method for determining the concentration of culturable heterotrophic microorganisms ("heterotrophic plate count"-method, HPC-method). The concentration is expressed in terms of colony-forming units (CFUs), based on volume or area. The determination of the colony count as an indicator parameter, according to the German Drinking Water Ordinance, in 1 mL of water to be tested, provides recording specific hygienically relevant microorganisms on a relatively nutrient-rich medium. For determination of a wider spectrum of heterotrophic bacteria in water and biofilm samples, frequently a nutrient-poor medium (R2A medium) is employed, as well as longer incubation times (e.g., 7 days) and lower incubation temperatures (e.g., 20 °C).

Legionellae, Legionella pneumophila

The term *legionellae* describes the genus *Legionella*. These gram-negative, slim or coccoid aerobic bacteria with complex nutrient requirements have monopolar flagella and are motile. There are more than 50 different species, distinguished by different morphological, physiological or genetic characteristics. *Legionellae* thrive in damp and preferably warm environments and occur naturally in waters and moist soil. Cooling towers, air conditioning units, drinking water installations etc. can emit aerosols and, thus, represent a risk of infection by inhalation.

L. pneumophila is a facultative human pathogen belonging to the genus *Legionella* which is responsible for 80-85 % of all community acquired legionelloses (mostly a form of pneumonia). Hospital acquired legionellosises are also caused by other *legionella* species. Although the blanket term "*legionellae*" is widely used by some public health offices, drinking water samples are often tested specifically for *L. pneumophila.*

Obligate pathogens

Obligate pathogens are microorganisms which cause infectious diseases almost exclusively as a result of severely compromised immunity (e.g., in the event of severe immunosuppression).

Polymerase chain reaction

Polymerase chain reaction is a molecular biological method of replicating specific DNA sections.

Pseudomonads, Pseudomonas aeruginosa

In microbiological terms, "Pseudomonads" generally describes all gram-negative, rod-shaped bacteria with low nutrient requirements which are polar-flagellated and, thus, mobile. Pseudomonads are ubiquitous both in soil and water and are associated with plants, animals and humans.

Pseudomonas aeruginosa is a member of the genus *Pseudomonas* and a facultative human pathogenic bacterium. It is aerobic and frequently causes nosocomial (i.e., hospital-acquired) infections and predominantly infects immunosuppressed patients. It produced pyocyanin and fluorescein which distinguishes it from other members of the genus and which can be detected by culture methods. Although the blanket term "pseudomonads" is widely used by some public health offices, drinking water samples are often tested specifically for the species *P. aeruginosa*.

Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel Electrophoresis is a technique for separating fragments of DNA from the whole genome. This generates a specific genetic fingerprint (genotyping) which can be used to identify the source of a contamination.

Quantitative PCR (qPCR)

In qPCR, the multiplied quantity of DNA is determined by a fluorescent reporter signal which can give a quantitative assumption of the amount of microorganisms.

Relative risk

Relative risk (RR) is the ratio of the probability of an event occurring in the exposed group versus a non-exposed group. $RR = Risk_{exposed}/Risk_{non\text{-}exposed}$

Cleaning

Cleaning refers to the process of removing impurities (e.g., abiotic substances, organic material or microorganisms), using water and possibly additives enhancing the cleaning effect (e.g., detergents or enzymes) for mechanical or combined mechanical-physical additives or processes. Cleaning does not intend the killing or inactivation of microorganisms. Cleaning performance has yet to be quantified or otherwise standardized.

Drinking water and heated drinking water

Drinking is defined as water intended for human consumption which complies with the standards of DIN 2000 (Central Drinking Water supply – Guidelines regarding the requirements for drinking water from small plants and mobile units). Its quality is regulated by the Drinking Water Ordinance. Drinking water should have a temperature of less than 25 °C.

Heated drinking water is drinking water from heating units which is employed for human consumption, particularly for drinking and for preparation of food. Normally, it is delivered through the same plumbing system as (cold) drinking water. The temperature range of drinking water is between 25 and 85 °C.

Drinking water installation

The domestic plumbing system (here: called "drinking water installation") is the total of the pipework, fittings and appliances that are installed between the point at which the water intended for human consumption leaves the tap and the point where water is delivered to the consumer from a water supply installation (Drinking Water Ordinance § 3 section 3).

Material ageing

Every material changes in its properties (such as structure or chemical composition) by thermal, mechanical or chemical stress. Ageing in plastics may refer to the depletion of additives or breaking of some polymer chains.

VBNC ("Viable-But-Non-Culturable")

VBNC is a state of bacteria in which they cannot be cultivated on conventional nutrient media although they are still viable. They have turned from growth into maintenance metabolism only and, thus, don´t multiply any more. Therefore, they do not form colonies on agar media. VBNC bacteria can frequently be detected by culture-independent (mostly molecular biological or biochemical) methods. Bacteria in the VBNC state have intact cell membranes, intact DNA, and still show metabolic and respiratory activity. Entering the VBNC state is a stress response, generated by unfavorable environmental conditions such as nutrient limitation, or stress by disinfectants or toxic metal ions. The VBNC state is reversible. Upon abolishment of the stress, the bacteria can resuscitate, return into the culturable state and, in the case of pathogens, may become infectious again.

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