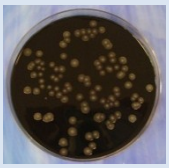
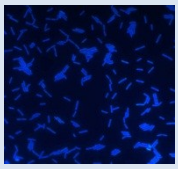
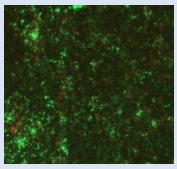
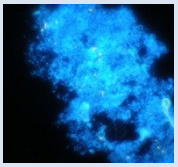
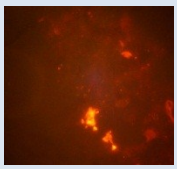
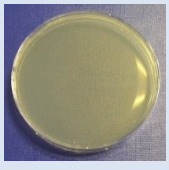
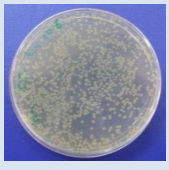




Integration of hygienically relevant bacteria in drinking water biofilms grown on domestic plumbing materials

Integration of hygienically relevant bacteria in drinking water biofilms grown on domestic plumbing materials

Dissertation

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Tag der Disputation: 16.05.2011

Gutachter:	Prof. Dr. Hans-Curt Flemming
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Vorsitzender:	Prof. Dr. Mathias Ulbricht

To my parents and Thomas

“Messieurs, c’est les microbes qui auront le dernier mot.”

Louis Pasteur

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Glossary

AOC	assimilable organic carbon
APS	ammoniumpersulfate
ATP	adenosine triphosphate
BPP	biofilm production potential
CFDA	carboxyfluorescein diacetate
cfu	colony forming units
ClO_2	chlorine dioxide
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DDTC	sodium diethyldithiocarbamate trihydrate
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DVC	direct viable count
DVGW	Deutscher Verein des Gas- und Wasserfaches (German Gas and Water Association)
DW	drinking water
EDTA	ethylene diamine tetra-acetic acid
EPDM	ethylene propylene diene monomer
EPM	ethylene propylene monomer
EPS	extracellular polymeric substances
FDA	fluorescein diacetate
FISH	fluorescence in situ hybridisation
HD-PE	high-density polyethylene
HPC	heterotrophic plate count
LB	Lenox Broth
MDOD	mean dissolved oxygen demand

NaOCl	sodium hypochlorite
NBR	nitrile butadiene rubber
PAS	Page's Amoeba Saline
PB	polybtylene
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	polyethylene
PE-X	cross-linked polyethylene
PE-Xb	silane cross-linked polyethylene
PE-Xc	electron-ray cross-linked polyethylene
PFGE	pulsed-field gel electrophoresis
PI	propidium iodide
PMA	propidium monoazide
PNA	peptide nucleic acid
PP	polypropylene
PVC	polyvinylchloride
PVCc	chlorinated polyvinylchloride
PVCu	unplasticised polyvinylchloride
PYG	proteose peptone yeast extract glucose broth
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SSCP	single strand conformation polymorphism
TEMED	N,N,N',N'-Tetramethylethylenediamine
TOC	total organic carbon
T-RFLP	terminal restriction fragment length polymorphism
TrinkwV	Trinkwasserverordnung (German Drinking Water Ordinance)
UBA	Umweltbundesamt (Federal Environmental Agency)
VBNC	viable but non-culturable
YEB	yeast extract broth

Abstract

Biofilms in domestic plumbing systems can represent a reservoir for potentially pathogenic bacteria including *Pseudomonas aeruginosa*, *Legionella pneumophila* and coliform bacteria. The selection of materials utilised in domestic plumbing as well as their exposure to disinfectant stress ("ageing") may affect the extent of biofilm formation, the composition of biofilm populations and the incorporation and persistence of hygienically relevant bacteria in drinking water biofilms. The presence of protozoa may additionally influence the persistence and multiplication of potentially pathogenic bacteria.

Drinking water biofilms were grown on coupons of plumbing materials including ethylene-propylene-diene-monomer (EPDM) rubber, silane cross-linked polyethylene (PE-Xb), electron-ray cross-linked PE (PE-Xc) and copper under constant flow-through of cold tap water at ambient temperature (19.0 °C ± 3.1 °C). The materials were tested both untreated and after treatment with sodium hypochlorite (5 ppm, 3 bar, 40 °C, 4 weeks) or chlorine dioxide (5 ppm, 3 bar, 40 °C, 4 weeks) in case of EPDM, PE-X b and c or after exposure to unchlorinated drinking water for ≥ 6 months in case of copper. After 14 days, the biofilms were spiked with *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Enterobacter nimipressuralis* (10^6 cells/mL each). The test bacteria were environmental isolates from contamination cases in drinking water systems. After static incubation for 24 h, water flow was resumed and continued for four weeks. Total cell count and heterotrophic plate count (HPC) of biofilms were monitored, and the population diversity of was determined using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* were quantified, using standard culture-based methods or culture-independent fluorescence in situ hybridisation (FISH). 14 day-old biofilms grown on untreated EPDM, PE-Xb, PE-X c and copper were analysed for the

presence of total protozoa and the amoebal genera *Acanthamoeba* and *Hartmannella* using FISH.

After 14 days total cell counts and HPC values were highest on EPDM followed by the plastic materials and copper. The diversity of biofilm populations was higher in biofilms grown on synthetic materials (EPDM, PE-Xb, PE-Xc) compared to biofilms grown on copper. Amoebae were present in drinking water biofilms grown on all domestic plumbing materials tested, with *Acanthamoebae* and *Hartmannella* being the prevalent genera. Material ageing did not significantly influence biofilm formation and biofilm population diversity. After inoculation, *P. aeruginosa* persisted for 28 days in biofilms on EPDM, PE-Xb and PE-Xc, but was unable to colonise copper biofilms. *L. pneumophila* persisted in biofilms on any of the materials for 28 days. *E. nimipressuralis* was not detected in any of the biofilms. The aged materials did not show significant differences compared to untreated materials regarding the incorporation of *P. aeruginosa* and *L. pneumophila* into biofilms. Application of FISH showed that *P. aeruginosa* and *L. pneumophila* often persisted in higher concentrations than detected by culture-based methods indicating that part of the *P. aeruginosa* and *L. pneumophila* populations entered a viable but non-culturable (VBNC) state, in which they were not detectable with standard culture methods.

Additional investigations on *P. aeruginosa* pure cultures showed that copper can be one of the stress factors inducing the VBNC state in drinking water and drinking water biofilms of domestic plumbing systems. Planktonic and biofilm-associated *P. aeruginosa* in the VBNC state became culturable again upon the release of copper stress by incubation in the presence of the chelator diethyldithiocarbamate (DDTC).

The results show that biofilm formation measured as total cell count and HPC as well as biofilm population diversity are material dependent, but not influenced by material ageing. *P. aeruginosa* and *L. pneumophila* are able to incorporate into and persist in drinking water biofilms grown on materials relevant in domestic plumbing. Material ageing did not have an influence on pathogen persistence. The detection of amoebae in all drinking water biofilms suggests that these organisms can interact and probably serve as a host for hygienically relevant bacteria, at least for *L. pneumophila*. The concentrations of *P. aeruginosa* and *L. pneumophila* in drinking water biofilms may be underestimated by conventional culture-based methods. Hygienically relevant bacteria that are not culturable may still be viable and of hygienic relevance as they can retain their virulence or regain it upon resuscitation.

Chapter 1
Introduction

1.1. Biofilms in drinking water systems

In drinking water systems, virtually any surface in contact with water will be colonised by microorganisms (Wingender and Flemming, 2004). The tendency of bacteria to attach to surfaces has been shown early in the twentieth century in studies performed by Henrici (1933) and Zobell (1943), but the term biofilm was not established until the 1970s (Costerton et al., 1978). Since that time the ubiquity and significance of biofilms in various sectors including almost any natural environment, but also household, medical, industrial and technical systems, has become increasingly clear (Hall-Stoodley et al., 2004; Hall-Stoodley and Stoodley, 2009). Donlan and Costerton (2002) described a biofilm as "a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of EPS (extracellular polymeric substances) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton, 2002). EPS consisting of polysaccharides, proteins, DNA and lipids in varying ratios contribute to the mechanical stability of microbial biofilms and play an important role in the organisation of the biofilm community (Flemming and Wingender, 2010). The EPS matrix provides shelter from environmental stress such as high salinity, extreme pH, UV radiation and desiccation and thus permits survival under hostile conditions (Flemming and Wingender, 2010). Moreover, microorganisms in a biofilm are less susceptible to biocides including disinfection measures used in drinking water treatment (Gagnon et al., 2005; Loret et al., 2005; Schulte et al., 2005).

The formation of biofilms can be divided into several distinct stages even though the particular development processes may vary depending on the microbial species involved and the prevailing environmental conditions (Stoodley et al., 2002; Hall-Stoodley et al., 2004). Steps in biofilm formation include the initial attachment of cells to a surface, subsequent multiplication of the cells resulting in microcolonies, followed by the continuous proliferation of attached bacteria leading to the establishment of a mature biofilm, and the passive detachment or active release of single cells or aggregates of cells into the surrounding environment (Fig. 1.1; Stoodley et al., 2002; Dunne, 2002).

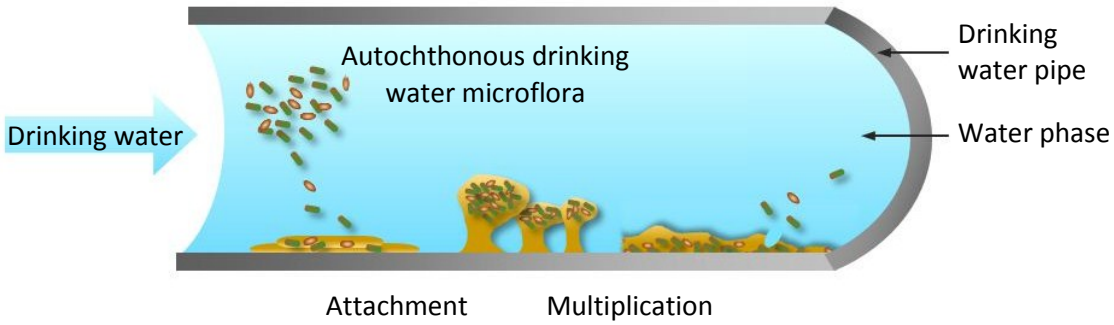

Figure 1.1: Biofilm formation and development in a drinking water pipe.

Depending on the nature of the surface being colonised, on the organisms involved and on the physico-chemical characteristics of the environment (temperature, pH, hydraulic regime, availability of nutrients), biofilms with distinct architectures develop (Melo and Bott, 1997; Manuel et al., 2007; Shin et al. 2007). Despite the fact that drinking water systems provide relatively unfavourable conditions, i.e. low nutrient levels, low temperatures, high shear forces and disinfectant residuals, materials exposed to drinking water systems are rapidly colonised reaching maximal cell counts within weeks. It has been estimated that 95 % of the overall bacterial biomass in drinking water distribution systems can be located in biofilms on surfaces, while only 5 % occur in the water phase (Flemming et al., 2002); in a domestic hot water system, 72 % of the culturable bacteria were found to be surface-associated (Bagh et al., 2004). Colonisation of the internal surfaces of drinking water systems mostly occurs thin and patchy in the form of single cells or microcolonies (Ridgway and Olson, 1981; Pedersen, 1990; Servais, 1995; Martiny et al., 2003; Wingender and Flemming, 2004) but occasionally also dense multi-layer biofilms have been detected (Grubert et al., 1992; Kilb et al., 2003). The extent of biofilm formation in drinking water systems can be assessed either directly by scanning electron microscopy (Lee

et al., 2011; Lenz et al., 2010; Wingender et al., 2003) or by determination of total cell counts and culturable microorganisms of biofilms (Kilb et al., 2003; Wingender et al., 2003; Bressler et al., 2009; Benölken et al., 2010). In several months to years old biofilms on pipe surfaces or coupons exposed to drinking water total cell counts of 10^4 to 10^8 cells per cm^2 were detected (Block et al., 1993; Servais et al., 1995; Kalmbach et al., 1997; Wingender and Flemming, 2004; Långmark et al., 2005a). Concentrations of culturable heterotrophic plate count (HPC) bacteria in established biofilms were found to vary between approximately 10^1 to 10^6 colony-forming units (cfu) per cm^2 (LeChevallier et al., 1987; Block et al., 1993; Wingender and Flemming, 2004; Långmark et al., 2005a). In oligotrophic water environments such as drinking water systems the number culturable bacteria can be several orders of magnitude lower than the total cell numbers. Usually, the fraction of culturable bacteria represent between approximately 0.01 % and a few percent of the total cell counts (Kalmbach et al., 1997; Wingender and Flemming, 2004).

Biofilms are present on all surfaces exposed to water during the different steps in drinking water production and supply including abstraction from source waters, treatment (e.g. coagulation, filtration), disinfection, distribution (water mains, plumbing systems in buildings, etc.) and drinking water storage (Fig. 1.2). Filters, distribution pipes, storage tanks, tap fittings and shower heads provide suitable surfaces for microbial colonisation (LeChevallier et al., 1987; Percival et al., 1998; Zacheus et al., 2001; Kilb et al., 2003; Emtiazi et al., 2004; Lehtola et al., 2004b; Wingender and Flemming 2004; September et al., 2007; Feazel et al., 2009; Eboigbodin et al., 2008).

By the time the water reaches the consumer's tap, its quality may differ from the quality at the time of treatment in the waterworks (Pepper et al., 2004). Apart from aesthetic problems such as changes in odour, taste, colour and turbidity of the water caused by drinking water biofilms (Kerr et al., 2003), it has become obvious that microorganisms with pathogenic properties can persist and multiply in biofilms of drinking water systems. Biofilms have the ability to function as an environmental reservoir for pathogenic microorganisms and thus are potential sources of contamination (Fig. 1.3). They present a health risk when pathogens are released from the biofilms and are transmitted to susceptible human hosts upon exposure to contaminated water. Several strict or opportunistic pathogens including bacteria, viruses and protozoa have been shown to survive in biofilms isolated from drinking water systems (Långmark et al., 2005b; Szabo et al., 2006; Khan, 2006; Lehtola et al., 2007; Gião et al., 2008; Helmi et al., 2008; Bonadonna et al., 2009). Once integrated into an established biofilm, these organisms may find favourable conditions for the

survival in a drinking water system. The biofilm provides nutrients and protection from external stresses, e.g., the action of disinfectants. Biofilm formation is especially critical in plumbing systems of hospitals and other health-care facilities, where biofilm-derived pathogens can contribute to water-associated nosocomial infections (Exner et al., 2005).

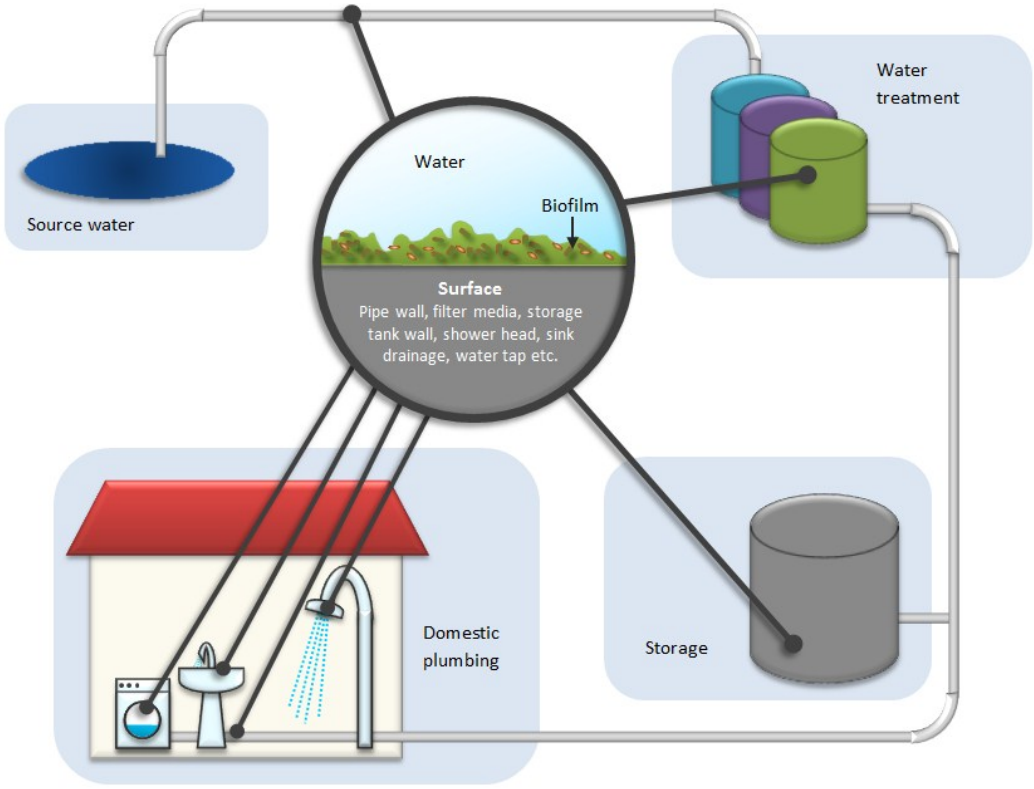

Figure 1.2: Biofilm formation during the different steps of drinking water production and distribution (modified according to Camper and Dirckx, 1999).

Most research on biofilms in drinking water systems has focused on materials of the distribution system, not including domestic plumbing systems in public and private buildings. In Germany, water suppliers have to ensure that chemically and microbially unobjectionable water reaches the water meter of a building. From this point, the owner of the building is responsible for the water quality (TrinkwV, 2001). According to the World Health Organization (WHO) and the European Community directive, drinking water should meet the quality requirements at the point of consumption (WHO, 2008; The Council of the European Union directive, 1998). A high water quality has to be maintained throughout the distribution system, including passage through domestic plumbing. Therefore it is important to investigate and understand biofilms in both distribution mains and domestic plumbing. Conditions prevailing in domestic plumbing systems might differ significantly from those in

water mains. The inner diameter of pipes is much smaller resulting in a larger ratio of surface to water volume. As a consequence of longer periods of stagnation and insufficient insulation of the pipes, the water temperature increases favouring microbial growth. In domestic plumbing, the choice of construction materials is less regulated than in the distribution system leading to a large variety of materials utilised. Some of these materials might leach nutrients and thus support massive biofilm growth (Rogers et al., 1994a; Flemming et al., 2002; Bressler et al., 2009). In many cases domestic plumbing systems are not constructed and operated according to the generally accepted rules of technology impairing the hygienic safety of drinking water (Grummt, 2007; Schauer et al., 2008; Kistemann et al., 2010). The microbiological, physical and chemical control of domestic plumbing systems by local public health authorities in Germany is still incomplete and inconsistent in Germany (Völker et al., 2010).

Biofilm development in drinking water systems has been analysed in field studies (Niquette et al., 2000; Kilb et al., 2003; Wingender et al., 2003; Emtiazi et al., 2004; Wingender and Flemming, 2004; Bonadonna et al., 2009), but most information has been derived from pilot systems and laboratory models where conditions can be easily regulated. The simplest model for testing biofilms in a continuous system is using a perfused tube for growing biofilms (Exner et al., 1987). Another simple and effective way for growing biofilms is the exposure of coupons in a continuously perfused reactor (Bressler et al., 2009). More complex reactor systems for growing biofilms under defined conditions include the RotoTorque™ (rotating annular reactor; Characklis, 1990), the Robbins Device (Ruseska et al., 1982) and the Propella™ Reactor (Parent et al., 1996; Appenzeller et al., 2001). In several investigations laboratory-scale distribution systems have been a useful tool to study biofilm formation in close to practice conditions (Benölken et al., 2010, Lehtola et al., 2005, 2004a; Loret et al., 2005). Although all these systems have shortcomings and do not completely represent conditions within real pipe networks they have been successfully used to study various aspects of biofilms, such as structural developments (Zhang et al., 1994), biofilm community composition (Manz et al., 1993), the effect of biocides (Stewart et al., 1994) and the integration and survival of microorganisms in established biofilms (Lehtola et al., 2006a, b; Helmi et al., 2008; Bressler et al., 2009).

Bacterial diversity in drinking water distribution biofilms usually differs depending on the drinking water distribution system. For instance, drinking water biofilms from the Berlin, Mainz, Stockholm or Montreal distribution systems were characterised by a high number of β-proteobacteria (Kalmbach et al., 1997; Schwartz et al., 1998; Szewzyk et al., 2000; Batté et al., 2004), while others were dominated by α-

proteobacteria (Williams et al., 2004; Schmeisser et al., 2003). Genera that were isolated from drinking water biofilms include *Pseudomonas*, *Acidovorax*, *Bacillus*, *Micrococcus*, *Staphylococcus*, *Chryseobacterium*, *Arthrobacter* and *Flavobacterium* (Emtiazi et al., 2004; Shin et al., 2007). Among the proteobacteria, the proportion of α-, β- and γ-subclasses were reported to depend on pipe material (Kalmbach et al., 1997; Schwartz et al., 1998; Norton and LeChevallier, 2000), biofilm age (Martiny et al., 2003) and disinfection practice (Mathieu et al., 2009; Roeder et al., 2010). In addition to that, characteristic changes in the community composition of drinking water biofilms through potable water treatment and distribution can occur (Norton and LeChevallier, 2000; Emtiazi et al., 2004). Biofilm species composition can be analysed by various molecular approaches including FISH (Manz et al., 1993, 1995; Schwartz et al., 2003; Wellinghausen et al., 2005), 16S rDNA clone libraries (Schmeisser et al., 2003; Feazel et al., 2009) single strand conformation polymorphism (SSCP; Eichler et al., 2006) or PCR-DGGE (Emtiazi et al., 2004; Hoefel et al., 2005; Roeder et al., 2010).

1.2. Hygienically relevant microorganisms in drinking water systems

Drinking water biofilms are formed predominantly by microorganisms of the autochthonous aquatic microflora without any relevance to human health. There is no clear evidence that heterotrophic bacteria in drinking water constitute a public health risk (Exner et al., 2003).

However, drinking water biofilms have the potential to harbour opportunistic pathogens that are able to incorporate into drinking water biofilms and persist or even multiply there (Fig. 1.3).

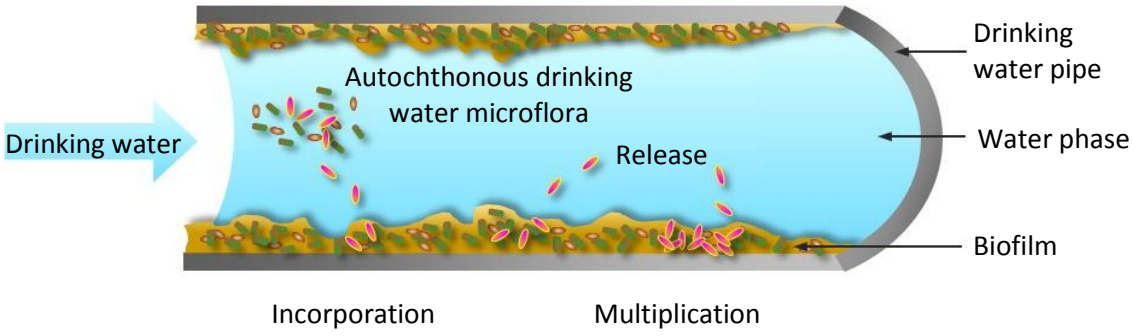

Figure 1.3: Incorporation, persistence, multiplication and release of potentially pathogenic bacteria in drinking water biofilms.

The release of the pathogens from biofilms and contamination of the water phase can pose a threat to human health, especially in immunocompromised people. Although opportunistic pathogens such as *P. aeruginosa*, *Legionella* spp., *Serratia* spp., *Klebsiella* spp. and *Flavobacterium* spp. are ubiquitous in the environment, they are normally absent from treated potable water in the distribution system. But they can occasionally survive drinking water production in very low numbers or enter the distribution system during construction work or through system defects (Craun and Calderon, 2001). Several strict or opportunistic pathogens are able to survive in biofilms isolated from drinking water systems. These include *Legionella pneumophila* (Långmark et al., 2005b; Bonadonna et al., 2009), *Mycobacterium avium*, *Escherichia coli* (Lehtola et al., 2007), *Helicobacter pylori* (Gião et al., 2008), *Salmonella* Typhimurium (Armon et al., 1997), *Klebsiella pneumoniae* (Szabo et al., 2006), *Campylobacter jejuni* (Lehtola et al., 2006a) and sporadically *Pseudomonas aeruginosa* (Kilb et al., 2003), but also viruses such as canine calicivirus (Lehtola et al., 2007) and poliovirus (Helmi et al., 2008) as well as patho genic protozoa such as *Acanthamoeba* spp. (Khan, 2006), *Cryptosporidium parvum* and *Giardia lamblia* (Helmi et al., 2008). Although the tendency of opportunistic pathogens to multiply in biofilms of water mains seems to be limited (Flemming et al., 2002), the special conditions in domestic plumbing systems (increased surface to volume ratio, higher temperatures, prolonged periods of stagnation, less regulated choice of materials) might promote the incorporation, survival and multiplication of these pathogens in drinking water biofilms. In a systematic and nationwide survey of locally available data supplied by national health authorities in Germany, Völker et al. (2010) showed that *Legionella* spp. and *Pseudomonas* spp. can be detected in quantitatively relevant concentrations in domestic plumbing systems, posing a potential health risk to the consumer. Even if routine microbiological monitoring is an essential component of water supply management, it may not be sufficient to protect public health from opportunistic pathogens. The classical microbiological parameters of the German Drinking Water Ordinance (colony count, coliform bacteria, *E. coli*) do not indicate the occurrence of opportunistic pathogens such as *P. aeruginosa* and *L. pneumophila* (Exner et al., 2009).

1.2.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic, non-spore-forming rod-shaped bacterium (Madigan et al., 2003; Botzenhardt and Döring, 1993) that is increasingly recognised as an opportunistic emerging pathogen of high clinical relevance (Szew-

zyk et al., 2000; Reuter et al., 2002; Anaissie et al., 2002; Exner et al., 2007; Trautmann et al., 2009). *P. aeruginosa* is characterised by its simple nutritional requirements, its metabolic versatility and its tolerance to various physical conditions (Boyle et al., 1991; Botzenhardt and Döring, 1993). It is commonly found in soil, surface water, on surfaces of plants and animals as well as in marine and coastal habitats (Mena and Gerba, 2009; Wingender et al., 2009). Almost any man-made environment can be colonised by *P. aeruginosa*. It has been isolated from domestic plumbing systems (Leoni et al., 2005; Schauer et al., 2008), from drinking water distribution systems (Kilb et al., 2003), from technical water systems (Grobe et al., 1995), from swimming pools (Papadopoulou et al., 2008; Guida et al., 2009) and from numerous clinical settings (Muscarella, 2004).

P. aeruginosa is a typical biofilm organism, which is able to form primary colonies on all types of materials. Pure culture biofilms of *P. aeruginosa* have been grown on diverse materials including stainless steel, Teflon, rubber and glass (Bourion and Cerf, 1996; Cochran et al., 2000) and on a number of abiotic surfaces such as urinary catheter materials (Nickel et al., 1985), dialysis membranes (Hoyle et al., 1992) and contact lenses (Fletcher et al., 1993). *P. aeruginosa* is capable of forming multispecies biofilms together with other microorganisms (Stewart et al., 1997; Al-Bakri et al., 2004), in some cases being one of the dominant organisms (Shin et al., 2007; Andersson et al., 2008). In addition to that, *P. aeruginosa* is able to incorporate into existing biofilms (Wingender et al., 2002; Bressler et al., 2009).

P. aeruginosa is the causative agent of a wide variety of local and systemic infections such as skin infections, urinary tract infections, infections of the respiratory tract, infections of burn wounds and corneal disease and it accounts for a significant portion of nosocomial infections (Anaissie et al., 2002; Trautmann et al., 2009). *P. aeruginosa* poses a special threat to immunocompromised individuals, such as cancer patients, aids patients, transplant patients, cystic fibrosis patients, young children and elderly persons. The main route of infection is the contact of damaged tissue (skin or mucous membranes) with contaminated water. Occasionally, a transmission via inhalation of aerosols (toilet, shower, air condition and inhalation devices) can occur. The use of contaminated water for storage and cleaning of contact lenses can lead to the colonisation of contact lenses with *P. aeruginosa* resulting in infections of the eye. Ingestion of contaminated drinking water rarely is a route of *P. aeruginosa* infection (Wingender et al., 2009).

Infections caused by *P. aeruginosa* are particularly problematic, because the organism is able to develop resistance to multiple classes of antimicrobial agents (Lister et al., 2009). The control of *P. aeruginosa* contaminations in drinking water systems

is hindered by the fact that *P. aeruginosa* living in biofilms is able to tolerate several disinfectants including an iodophor disinfectant, a phenolic detergent, a quaternary ammonium, 70% ethanol, 2% glutaraldehyde, 2% formaldehyde, sodium hypochlorite (10-15 ppm free chlorine; Vess et al., 1993). Especially mucoid strains of *P. aeruginosa* were shown to survive at chlorine concentrations commonly used for the disinfection of drinking water, swimming pools and other water systems (Grobe et al., 2001).

According to a risk assessment performed by Hardalo and Edberg (1997), the risk of a water-associated *P. aeruginosa* infection for the general population is limited and a complete elimination of this bacterium from drinking water is neither practical nor necessary. The WHO classified the health significance of *P. aeruginosa* in water supplies as moderate and its relative infectivity as low. Nevertheless the hygienic relevance of *P. aeruginosa* for sensitive settings such as health care facilities and nursing homes is beyond question and measures for the prevention and elimination of *P. aeruginosa* are reasonable here (WHO, 2008). In Germany, a routine monitoring of drinking water for *P. aeruginosa* is not mandatory. The Federal Environmental Agency defined an action value of 0 cfu/100 mL for *P. aeruginosa* in drinking water from domestic plumbing systems of hospital and other care facilities (UBA, 2005).

In studies dealing with drinking water distribution systems, *P. aeruginosa* was not or only sporadically detected (Kilb et al., 2003; Lee and Kim, 2003; Wingender and Flemming, 2004; Emtiazi et al., 2004; September et al., 2007). Compared to water supply mains, *P. aeruginosa* occurs more frequently in domestic plumbing systems of public and private buildings (Wingender et al., 2009). In a survey estimating the microbiological situation of domestic plumbing systems in Germany, the water of 8 % of the sampled domestic plumbing systems was positive for *P. aeruginosa* at concentrations below 10 cfu/100 mL; contaminations were never systemic and solely occurred in plumbings of public buildings, not in private households (Wricke et al., 2007). Völker et al. (2010) conducted a systematic nationwide analysis of data on plumbing systems of German public buildings collected by local public health authorities. In 1.9 % of all cold water samples and 2.3 % of all warm water samples *Pseudomonas* spp. were detected in 100 mL of drinking water (Völker et al., 2010). A persistent *P. aeruginosa* contamination was observed in a big complex of buildings in Germany comprising several residence units as well as commercial premises and medical practices. *P. aeruginosa* concentrations could be reduced neither by flushing nor by ClO_2 treatment. Only the remedy of structurally engineered deficiencies and disinfection with H_2O_2 lead to a sanitation of the buildings (Schauer et al., 2008). In a study on hot water domestic plumbing systems in the city of Bologna,

Italy, *P. aeruginosa* was occasionally found in water collected from private apartments (in 7.1 % of apartments with independent heating and in 9.7 % of apartments with centralised heating) and from hot water domestic plumbing systems of hotels (in 27.7 % of the plumbing systems sampled and in 10.9 % of all samples). The colonisation was limited to single outlets rather than involving the whole system (Leoni et al., 2005).

P. aeruginosa contaminations of water in domestic plumbing systems are often associated with construction work (new construction of buildings, installation of new pipes etc.; Michel et al., 1995; Hambsch et al., 2004; Exner et al., 2008).

The presence of *P. aeruginosa* in hospital water supplies is a well-known risk-factor for nosocomial pneumonia, septicaemia, wound infections and urinary tract infections; up to 50 % of all hospital-aquired *P. aeruginosa* infections are associated with the occurrence of the bacterium in drinking water (Trautmann et al., 2009; Wingender et al., 2009; Exner et al., 2007; Ferroni et al., 1998; Botzenhart and Döring, 1993). Apparently, the occurrence of *P. aeruginosa* in tap water is related to its ability to colonise biofilms in plumbing fixtures (i.e. faucets, showerheads etc.) rather than to its presence in the distribution system or treated water (Mena and Gerba, 2009). Although many nosocomial outbreaks of *P. aeruginosa* were linked to contaminated drinking water (Grundmann et al., 1993; Ferroni et al., 1998; Reuter et al., 2002; Trautmann et al., 2006), contamination probably occurred from colonisation of the tap hardware including faucets, sinks and shower heads and introduction into the water when these devices are used (Döring et al., 1991, 1996). After a *P. aeruginosa* contamination of drinking water of a hospital plumbing system, *P. aeruginosa* was isolated from biofilms grown on different parts of a booster station (Schaule et al., 2010). In laboratory experiments it has been shown that *P. aeruginosa* is able to colonise established drinking water biofilms grown on domestic plumbing materials such as ethylene propylene rubber and to persist there for several weeks (Bressler et al., 2009).

1.2.2. *Legionella pneumophila*

Legionella pneumophila is the medically most important bacterium within the genus *Legionella* and accounts for 90 % of the reported Legionellosis cases comprising a severe and possibly fatal pneumonia called Legionnaires' disease and a milder, influenza-like disease referred to as Pontiac fever (Steinert, 2002; Devos et al., 2005; Declerck, 2010). *L. pneumophila* is a gram-negative, rod-shaped, heterotrophic bacterium and requires certain nutrients including iron salts and amino acids such as L-cysteine, which are used as carbon, nitrogen and energy sources (Fields et al., 2002).

L. pneumophila is ubiquitously present in freshwater environments such as lakes and rivers, being able to tolerate a wide range of physico-chemical parameters. It can survive at temperatures ranging from 5 °C to 63 °C and pH values of 5.0 to 9.2. *L. pneumophila* multiplies at temperatures ranging from 20 °C to 45 °C. The occurrence of *L. pneumophila* in natural aquatic environments enables the bacterium to enter man-made water systems providing ideal habitats for their survival and multiplication (Steinert, 2002; Devos et al., 2005; Declerck, 2010). *L. pneumophila* has been isolated from domestic plumbing systems of hospitals (Petti et al., 2004; Leoni et al., 2005), hotels (Bonetta et al., 2010; Leoni et al., 2005) and private homes (Stout et al., 1992; Leoni et al., 2005; Mathys et al., 2008), from water distribution systems of cruise ships and ferries (Goutziana et al., 2008), from dental-unit waters (Atlas et al., 1995), and from various clinical settings (Qasem et al., 2008; Anbumani et al., 2009). Especially warm water systems such as domestic hot water systems (Bollin et al., 1985; Mathys et al., 2008; Bonetta et al., 2010) and cooling towers (García-Fulgueiras et al., 2003) favour growth of *L. pneumophila*.

The universal persistence of *L. pneumophila* is promoted by the ability to adapt to a variety of different ecological niches. However, survival and growth of *L. pneumophila* is significantly determined by its microbial cohabitants. Their influence can be either antagonistic or growth promoting. In microbial communities shared by *L. pneumophila*, access to nutrients is provided by heterotrophic bacteria and by photosynthetic algae and bacteria (Declerck, 2010). Several gram-negative bacteria including *P. aeruginosa* and *Pseudomonas fluorescens*, as well as *Burkholderia cepacia*, *Aeromonas hydrophila* and *Stenotrophomonas maltophilia* isolated from chlorinated drinking water were found to inhibit *L. pneumophila* growth and biofilm formation, whereas *Acinetobacter lwoffii* enhanced growth of *L. pneumophila* biofilms (Toze et al., 1990; Guerrieri et al., 2006). Under certain conditions *L. pneumophila* is also able to consume and grow on dead bacteria such as *Pseudomonas putida* and *E. coli* (Temmerman et al., 2006).

Despite of its ability to interact with other bacteria in various ways, free-living amoebae and other protozoa are still considered the key factor of the survival and multiplication of *L. pneumophila* in the environment (Lau and Ashbolt, 2009). In 1980, Rowbotham first described intracellular multiplication of *L. pneumophila* within amoebae (Rowbotham, 1980). To date, *L. pneumophila* has been shown to replicate in 14 species of free-living amoebae including *Acanthamoeba* spp., *Naegleria* spp. and *Hartmannella* spp., in two species of ciliated protozoa (*Cyclidium* spp. and *Tetrahymena pyriformis*) and in the slime mold *Dictyostelium discoideum* (Fields et al., 2002). *L. pneumophila* is ingested by protozoa through phagocytosis and

overcomes the host's phagolysosomal degradative pathway by blocking the maturation of their phagosome into a lysosome. *L. pneumophila* resides in a membrane bound vacuole recruiting host cell organelles such as mitochondria and the rough endoplasmatic reticulum (Harb and Abu Kwaik, 2000). During its stay in the intracellular environment, peptides and proteins degraded by the infected host can be utilized (Lau and Ashbolt, 2009). After massive replication of *L. pneumophila* the host cell is lysed due to the secretion of cytotoxins (Harb and Abu Kwaik, 2000; Fields et al., 2002). The uptake and replication of *L. pneumophila* in protozoan hosts can be influenced by the presence of non-*Legionella* bacteria (Declerck et al., 2005).

Protozoa do not only supply nutrients, they also provide protection from hostile environmental conditions. Especially inside cysts formed by some protozoa *L. pneumophila* is able to survive high temperatures (Storey et al., 2004), disinfection procedures (Barker et al., 1992; Loret et al., 2008) and drying (Steinert et al., 2002; Donlan et al., 2005). After intracellular replication in protozoa, *L. pneumophila* exhibits increased virulence and invasiveness to mammalian monocytes (Brieland et al., 1997; Cirillo et al., 1997, 1999). Protozoa also play an important role in the distribution of *L. pneumophila* to new environments; infected protozoa can actively leave a biofilm when conditions become unfavourable and they have also been shown to release vesicles of respirable size (< 5 µm in diameter), each containing 20 - 200 *L. pneumophila* cells. Such vesicles can get incorporated into aerosols leading to the transportation of numerous *L. pneumophila* over distances of several kilometres (Berk et al., 1998).

In oligotrophic environments such as drinking water systems the survival and multiplication of *L. pneumophila* depends on the presence of other microorganisms providing the essential demand of amino acids and organic carbon. Thus, aquatic multispecies biofilms are ideal ecological niches for *L. pneumophila*. Instead of attaching to a surface and developing a biofilm, the pathogen integrates into established biofilms as a secondary coloniser. Access to nutrients is provided either directly by other microorganisms living in the biofilm, such as photosynthetic algae and bacteria, heterotrophic bacteria and protozoa or indirectly by decaying organic matter (Declerck, 2010). It has been demonstrated in practical situations and in model systems that *L. pneumophila* is rapidly incorporated into established biofilm communities (Rogers and Keevil, 1992; Murga et al., 2001; Vervaeren et al., 2006; Declerck et al., 2007b).

Although several investigations found that *L. pneumophila* only persisted in biofilm communities and did not multiply in the absence of amoebae (Murga et al., 2001; Kuiper et al., 2004; Declerck et al., 2009), the discussion whether biofilm-associated

L. pneumophila really require protozoan hosts for their multiplication or whether they are able to replicate independently in the community is still ongoing. Surman et al. (2002) and Temmerman et al. (2006) reported that intracellular growth is not essential for the multiplication of *L. pneumophila* within a mixed microbial consortium. Declerck et al. (2009) proposed that biofilm-associated *L. pneumophila* may use amoebae solely as a host for replication and spend the major part of its lifespan as an extracellular bacterium.

Biofilm-associated *L. pneumophila* can be released from the biofilm and contaminate the adjacent water phase (Berk et al., 1998; Storey et al., 2004; Liu et al., 2006). Outbreaks of Legionnaires' disease occurred in offices, hotels, hospitals and cruise ships have often been traced to environmental water sources (Atlas, 1999). Infections occur predominantly by the inhalation of contaminated aerosols which can be produced by taps, shower heads, whirlpools, spas, dental devices, air conditioning systems, cooling towers, fountains and respiratory therapy equipment. Natural environments are rarely associated with *L. pneumophila* infections (Fliermanns, 1996).

According to the WHO, the health significance of *Legionella* spp. in water supplies is high and its relative infectivity is moderate (WHO, 2008). Therefore the implementation of water safety plans for the control of *Legionella* is strongly recommended, especially for medical facilities, hotels and public buildings (WHO, 2007). As the detection and elimination of *L. pneumophila* from drinking water systems may be difficult, the main focus is on the prevention of *Legionella* contaminations of drinking water systems. The adherence to state-of-the-art technologies preventing conditions that favour the multiplication of *Legionella* in water systems (water temperatures between 25 and 45 °C, long periods of stagnation, the presence of biofilms and sediments) are usually the most efficient way of minimizing and controlling *Legionella* contamination of domestic plumbing systems (Exner et al., 2009).

In Germany, several guidelines for the prevention, detection, control and elimination of *Legionella* spp. and *L. pneumophila* exist (ISO 11731; UBA, 2000; UBA 2006; DIN 1988; VDI 6023; DVGW W 551; DVGW W 553). As a consequence of the coming into force of the European Council Directive 98/83/EC many European countries including Germany, France, the Netherlands and the United Kingdom implemented guideline and target values for *Legionella* (WHO, 2007). The German Drinking Water Ordinance regulates the periodic monitoring of domestic warm water systems for *Legionella* spp. (TrinkwV, 2001). The threshold ("Maßnahmewert") is 100 cfu/100 mL, for health facilities at high risk a target value of 0 cfu/100 mL is set (DVGW, 2004; UBA, 2006).

Although *L. pneumophila* is often not detected in samples from drinking water dis-

tribution systems during routine monitoring using cultural methods (Emtiazi et al., 2004; Pryor et al., 2004; Wingender and Flemming, 2004), it may survive drinking water production and distribution and colonise domestic plumbing systems. Hospital plumbing systems provide an important reservoir of *L. pneumophila* and potential source for nosocomial pneumonia (Exner et al., 2007; Garcia-Nuñez et al., 2008), but there is evidence of a widespread distribution of *L. pneumophila* in plumbing systems of hotels and private households (Leoni et al., 2005; Mathys et al., 2008).

In a study on the occurrence of *Legionella* spp. in hot water systems of single-family residences in Germany, 10.6 % of the water samples were positive for *Legionella* spp., of which 93.9 % were identified as *L. pneumophila* (Mathys et al., 2008). An investigation of drinking water from domestic warm water plumbing systems of German households *Legionella* spp. were detected in 22 % of the samples with 12 % of these samples containing ≥ 100 cfu/100mL (Wricke et al., 2007). Völker et al. (2010) assessed the extent of microbial contamination of domestic plumbing systems of public buildings in Germany in a systematic nationwide survey of data collected by local public health authorities. In 12.8 % of samples from warm water systems the action value for *Legionella* spp. (100 cfu/100mL) was exceeded (Völker et al., 2010). During a survey of the hot water systems of private homes in Italy, *Legionella* spp. were detected in 22.6 % of the water samples, 75.3 % of the isolates being *L. pneumophila* (Borella et al., 2005). Stout et al. (1992) found *L. pneumophila* in hot water tanks, kitchen and bathroom taps, shower heads and bath tub outlets of private homes in Pittsburgh. In a study on hot water domestic plumbing systems in the city of Bologna, Italy, *L. pneumophila* was most frequently detected in water sampled from plumbing systems of hospitals (in 100 % of the plumbing systems sampled and in 93.7 % of all samples) followed by hotels (in 63.6 % of the plumbing systems sampled and in 60.9 % of all samples). In water collected from private apartments the occurrence of *L. pneumophila* strongly depended on the heating system; 41.9 % of the water from apartments with centralised heating and only 3.6 % of the water from apartments with independent heating were positive for *L. pneumophila* (Leoni et al., 2005).

Cold water is not often sampled for *Legionella* spp. as this organism is usually associated with warm water (Völker et al., 2010). In a survey of the microbiological characteristics of domestic plumbing systems of German public buildings based on data collected by local public health authorities, 5.4 % of all cold water samples exceeded the action value (100 cfu/100 mL) for *Legionella* spp. (Völker et al., 2010). In an investigation on the occurrence and identity of *Legionella* spp. in Dutch cold tap water, 3.9 % of the water samples were positive for *L. pneumophila* by either culture or

PCR (Diederen et al., 2007). Petti et al. (2004) found 4.2 % of cold water samples taken from the plumbing system of a dental hospital to be positive for *Legionella* spp., *L. pneumophila* was the most frequently recovered species (Petti et al., 2004).
Investigations in model drinking water systems and in laboratory tests have shown that *L. pneumophila* colonises established biofilms and is able to persist and even multiply there (Murga et al., 2001; Långmark et al., 2005b; Liu et al., 2006; Lehtola et al., 2007; Gião et al., 2009; Declerck et al., 2009). Factors influencing the survival and growth of *L. pneumophila* in a drinking water biofilms include temperature (Rogers et al., 1994a; Gião et al., 2009b), flow regime and the presence of disinfectants as well as protozoan hosts. *L. pneumophila* readily colonises biofilms at temperatures ranging from 20 °C to 50 °C, but not at 60 °C (Rogers et al., 1994a). Gião et al. (2009b) suggests that lower temperatures (15 °C) support the integration of *L. pneumophila* into established biofilms. Even though it is often recommended to avoid stagnation in water pipes to prevent *Legionella* contamination (WHO, 2007), Liu et al., (2006) found significantly higher colony counts of *L. pneumophila* in pipes with turbulent flow compared to pipes with laminar flow and stagnant water. *L. pneumophila* survived in biofilms for more than 2 weeks even under high-shear turbulent flow conditions (Lehtola et al., 2007).
In addition to that, certain materials have clearly been demonstrated to promote *L. pneumophila* growth in biofilms (Colbourne and Ashworth, 1986; Rogers et al., 1994b; van Kooij et al., 2002; van der Kooij et al., 2005).

1.2.3. Coliform bacteria

Coliform bacteria include a wide range of aerobic and facultatively anaerobic, Gram-negative, non-spore-forming bacteria. The term coliform bacteria is the collective name of members of the *Enterobacteriaceae* that are capable of the fermentation of lactose and the production of acid or aldehyde within 24 h at 35 °C – 37 °C. The group of coliform bacteria comprises both fecal and environmental species including genera such as *Escherichia*, *Citrobacter*, *Klebsiella* and *Enterobacter*. Some of these bacteria are common inhabitants of the intestinal tract of humans and warm-blooded animals and thus excreted in their feces, but many coliform bacteria are heterotrophic and able to multiply in water and soil environments. Observations from practical situations and experimental systems indicate that coliform bacteria are able to survive and replicate under low-nutrient and low-temperature conditions (LeChevallier et al., 1987; Camper et al., 1991; Fass et al., 1997).
Coliform bacteria, particularly *Enterobacter*, *Citrobacter*, *Klebsiella* and *Serratia* species are classified as facultative pathogens. Especially in health care facilities they

can induce a number of infections in predisposed and immunocompromised individuals. They can cause wound infections, catheter-associated infections, pneumonia and septicemia (Stevens et al., 2003; Feuerpfeil et al., 2009).

The group of coliform bacteria has traditionally been used as an indicator of a fecal or another exogenous contamination of drinking water. They should be absent from finished potable water and the presence of coliform bacteria indicates deficient water treatment, inadequate disinfection or accidental contamination of the drinking water network through cross connections or other system defects. Occasional coliform contaminations of drinking water have been described in several studies. In a survey of domestic plumbing systems in Germany, coliform bacteria have been found in 3 % of the water samples (Wricke et al., 2007). September et al. (2007) detected fecal coliforms in 7.7 % of tap water samples from different areas on South Africa with the dominant species being *Enterobacter* and *Klebsiella*. The investigation of 31 drinking water distribution systems in North America showed 27.8 % of the samples to be positive for coliform bacteria. The presence of these bacteria was associated with several factors such as quality or type of raw water, temperature, type and concentration of disinfectant, AOC level, corrosion control and operational characteristics of the treatment process and the distribution system (LeChevallier et al., 1996). Deposits and sediments in storage tanks or pipes have been shown to promote the survival of coliform bacteria (Zacheus et al., 2001; Korth et al., 2008). In a systematic survey of data collected by German local public health authorities, Völker et al. (2010) found that 1.9 % of all cold water samples and 1.6 % of all warm water samples taken from domestic plumbing systems of public buildings exceeded the threshold for coliform bacteria (0 cfu/100mL).

The role of biofilms in the persistence of coliform bacteria in drinking water systems as well as in the contamination of drinking water is unclear. LeChevallier et al. (1987) did not find any coliform bacteria in samples taken from cement lined pipes of a drinking water distribution system, only one coupon taken from a cast iron pipe contained 5.1 cfu/5 cm² coliform bacteria (LeChevallier et al., 1987). Bonadonna et al. (2009) found coliform bacteria (1 - 3 cfu/100mL) in 4 of 19 biofilm samples from plumbing systems of sport facilities, but not in the corresponding water samples (Bonadonna et al., 2009). Wingender and Flemming (2004) rarely detected coliform bacteria in biofilm samples from drinking water distribution pipes in Germany. In an investigation on water mains in France, 11 % of the water samples were positive for coliform bacteria, whereas only 3.5 % of the biofilms contained detectable coliform bacteria (Batté et al., 2006). Kilb et al. (2003) demonstrated that coliform bacteria can integrate, accumulate and multiply in biofilms grown on rub-

ber-coated valves in a drinking water distribution system resulting in the biofilms acting as a point source for recurring coliform detection in water.

The ability of coliforms, including *C. freundii*, *K. pneumoniae* and *K. oxytoca*, to incorporate into pre-existing mixed-population biofilms grown under drinking-water conditions has been described in several laboratory studies (Robinson et al., 1995; Camper et al., 1996; Packer et al., 1997; Szabo et al., 2006). But many of the results indicate that these bacteria do not tend to permanently colonise distribution biofilms (McMath et al., 1999, Szabo et al., 2006; Lehtola et al., 2007). Coliform bacteria injected into a pilot drinking water distribution system could not be recovered from the water downstream of inoculation. In a situation simulating dead-end conditions coliforms could be detected in low concentrations for up to 7 weeks, but were not found in the autochthonous biofilm grown on the pipewall after flushing the pipe (McMath et al., 1999). *Klebsiella pneumoniae* remained in biofilms on corroded iron pipes for 2 – 9 days depending on the concentration in the inoculum, persistent colonisation of the surfaces was not observed (Szabo et al., 2006). *E. coli* in drinking water biofilms grown under high-shear turbulent-flow conditions were detectable by culture for only 4 days in biofilms and 8 days in water (Lehtola et al., 2007). These field and laboratory studies demonstrate that coliforms have the potential to form biofilms, integrate into biofilms and to multiply within biofilms, although a persistent colonisation does not occur. Release of coliform bacteria from biofilms into the bulk water may be one of the causes of sporadic or intermittent events of coliform occurrence identified during routine drinking water analysis. As it is difficult to differentiate between bacteria grown in and sheared from a biofilm and those present as a result of deficient water treatment or contamination, the indicator function of coliform bacteria is impaired by their possible persistence in drinking water biofilms (Kilb et al., 2003; Stevens et al., 2003).

1.2.4. Free-living amoebae

The term amoeba comprises a heterogeneous group of diverse unicellular eukaryotes that share common morphological and behavioural characteristics (Winiecka-Krusnel and Linder, 2001; Khan et al., 2006). Infections caused by free-living amoebae that are common to soil and aquatic environments are relatively rare, but from a public health perspective their role as potential reservoirs for pathogenic bacteria has gained more importance and relevance (Marciano-Cabral and Cabral, 2003; Khan, 2006).

Amoebae possess a vesicular nucleus and are able to form retractable cytoplasmic protrusions called pseudopodia that are used for locomotion and feeding (Visves-

vara et al., 2007). More than 11,300 amoebal species have been identified up to now (Loret and Greub, 2010). The organisms' life cycle comprises two distinct stages: a motile, metabolically active trophozoite stage in which the amoebae are capable of multiplication, and a dormant cyst stage. Cysts are dehydrated structures with a double wall composed of cellulose, proteins and lipids in which the organisms are resistant to desiccation, disinfection and extremes of temperature (Loret and Greub, 2010; Thomas et al., 2009).

In their vegetative form, amoebae actively feed on bacteria, algae, small organic particles and dissolved nutrients. Food uptake occurs either via pinocytosis, the ingestion of solutes, or via phagocytosis, a process describing the engulfment and subsequent digestion of larger particles (Greub and Raoult, 2004; Khan, 2006).

Amoebae can be found in virtually any kind of aquatic or moist environment, even in those representing adverse physical and/or chemical conditions. They have been isolated from lakes, ponds, rivers and seawater (Ettinger et al., 2003; Marciano-Cabral and Cabral, 2003), from various sites in drinking water treatment plants (Thomas et al., 2008; Corsaro et al., 2010), from drinking water distribution systems (Hoffmann and Michel, 2001, 2003) and from domestic plumbing systems of private homes and hospitals (Michel et al., 1995; Kilvington et al., 2004; Thomas et al., 2004, 2006; Shoff et al. 2008; Marciano-Cabral et al., 2010).

In water, species exhibiting a flagellar stage (i.e. *Naegleria* spp.) are able to swim, but generally amoebae attach to submerged surfaces or particulate suspended matter in order to feed on the biofilms growing thereon (Greub and Raoult, 2004). Wherever bacteria colonise surfaces in an aquatic environment, their protistan predators rapidly follow (Parry et al., 2001) as the high concentration of bacteria immobilised to a surface in a biofilm constitutes an excellent nutrient source for amoebae (Huws et al., 2005). In drinking water systems, free-living amoebae share a habitat with opportunistic pathogens such as *P. aeruginosa* and *L. pneumophila*. The close proximity of the organisms in the biofilm favours the contact and thus the interaction between bacteria and their possible amoebal hosts. It is well established that protozoan grazing is a key factor in regulating biomass concentration, biofilm composition and dynamics and in the recycling of nutrients in aquatic food chains (Azam et al., 1983; Pedersen, 1990; Barker and Brown, 1994; Kalmbach et al., 1997). Although it is often stated that the biofilm mode of growth prevents bacteria from predation (Costerton et al., 1987), it has been shown that protozoa can decrease biofilm thickness by 60 % (Huws et al., 2005) or even completely remove a biofilm (Sherr et al., 1983). The occurrence of amoebae has been associated with the presence of biofilms in granular activated carbon filters and sand filters of drinking wa-

ter treatment plants (Thomas et al., 2008), in drinking water distribution systems (Corsaro et al., 2010), in domestic plumbing systems (Kilvington et al., 2004), in dental water units (Barbeau and Buhler, 2001) and on contact lenses (Simmons et al., 1998).

Conventional grazing activity of amoebae involves the uptake of bacteria by phagocytosis into a membrane-bound vacuole called phagosome and the subsequent fusion of the phagosome with the lysosome resulting in the digestion of the bacteria. However, some bacteria have developed resistance to this process. According to a definition by Greub and Raoult "amoeba-resistant bacteria" are "bacteria that have evolved to resist destruction by free-living amoebae" (Greub and Raoult, 2004). This phenomenon has been described in detail for *L. pneumophila* (1.2.2.). Ingestion of *L. pneumophila* by an amoeba results in enhanced proliferation of the bacteria inside its host with subsequent release of high concentrations of *L. pneumophila* into the environment (Harb and Abu Kwaik, 2000; Molmeret et al., 2005). Amoebae thus constitute a reservoir for *L. pneumophila* and other *Legionella* spp. (Rowbotham, 1980; Holden et al., 1984; Berk et al., 1998; Kuiper et al., 2004; Hwang et al., 2006; Bouyer et al., 2007). Significant correlations between the presence of *L. pneumophila* and free-living amoebae have been observed in samples taken from environmental surface waters (Declerck et al., 2007), drinking water treatment plants (Loret and Greub, 2010), hospital water systems (Thomas et al., 2006), eye-wash stations (Paszko-Kolva et al., 1991) and cooling units of power plants (Declerck et al., 2007).

Other bacteria including *Klebisella pneumonia*, *Vibrio cholera*, *Pantoea agglomerans* and several *Aeromonas* spp. are able to establish a stable symbiotic relation with their amoebal host after being phagocytised (Abd et al., 2006; Pagnier et al., 2008). These bacteria benefit from the nutrients and protection provided by amoebae without lysing their host (Barker and Brown, 1994; Molmeret et al., 2005).

In addition to that, there are certain bacteria that avoid engulfment by free-living amoebae by simply killing their eukaryotic predators. *P. aeruginosa* was shown to inhibit growth of *A. castellanii*, especially in rich medium and in the presence of a high bacterium-to-amoeba ratio (Wang and Ahearn, 1997). Effects of *P. aeruginosa* on amoebae include necrosis and apoptosis (Abd et al., 2008) as well as reduced locomotion of amoebae resulting in a decelerated ingestion process (Pickup et al., 2007). The amoebicidal activity of *P. aeruginosa* is toxin mediated (Qureshi et al., 1993) and can involve binding of *P. aeruginosa* to the amoebal membrane and the action of one or more *P. aeruginosa* exoproducts (Pukatzki et al., 2002; Abd et al., 2008; Matz et al., 2008). The extent of resistance of *P. aeruginosa* to amoebal predation varies with different strains and strongly depends on bacterial density (Weitere

et al., 2005; Wang and Ahearn, 1997). In some cases intracellular infection of amoebae with *P. aeruginosa* has been observed (Michel et al., 1995). Cengiz et al. (2000) even reported a supporting effect of *P. aeruginosa* on *A. castellanii* survival in different contact lens solutions which was attributed to a protective layer of bacteria formed around the amoebae that prevented the biocides from disrupting the amoebal cell membrane.

In contrast to enteric amoebae such as *Entamoeba histolytica*, infections caused by free-living amoebae are rare. Some species of *Acanthamoeba* and *Naegleria* are opportunistic pathogens causing life-threatening and often fatal infections of the central nervous system in humans referred to as granulomatous amoebic encephalitis (with *Acanthamoeba* spp. as causative agents) or primary amoebic meningoencephalitis (infections caused by *Naegleria fowleri*). In addition to that, *Acanthamoeba* spp. are related to amoebic keratitis, a painful ocular infection that may result in the loss of sight (Khan, 2006).

The hygienic relevance of free-living amoebae is to a great extent related to their ability to serve as reservoirs for potentially pathogenic bacteria such as *L. pneumophila*. Amoebae are highly resistant against a number of biocides including various contact lens solutions (Cengiz et al., 2000; Beattie et al., 2003), benzisothiazolone (Barker et al., 1992), chlorine and chlorine dioxide (Loret et al., 2008) as they are able to encyst upon exposure to biocides (Thomas et al., 2004). The double cyst wall constitutes a physical barrier against most disinfectants. Survival within protozoa can provide bacteria with increased resistance to various biocides including chlorine (Loret et al., 2005; Loret and Greub, 2010). This poses a clear challenge in eradicating bacterial pathogens from public water supplies (Khan, 2006).

Growth of hygienically relevant bacteria such as *L. pneumophila* and *P. aeruginosa* within or in the presence of amoebae significantly enhanced the resistance of the bacteria to silver and copper (Hwang et al., 2006), to monochloramine and free chlorine in the (Donlan et al., 2005) and to various biocides used for cooling tower disinfection (Barker et al., 1992; Berk et al., 1998). The survival of pathogens in amoebal cysts might be the origin of the rapid re-colonisation of domestic water systems by the pathogens immediately after cessation of disinfection (Thomas et al., 2004). Amoebae were also involved in the resuscitation of bacteria from a viable but non-culturable state (Steinert et al., 1997; Hwang et al., 2006).

Control of amoebae, especially those of the genus *Acanthamoeba* cannot be achieved by the disinfection practices currently applied in drinking water production and distribution (Loret and Greub, 2010). Therefore, Loret et al. (2008) suggest to consider the hazard of amoebae and their interaction with bacteria in any risk

assessment conducted in the framework of a water safety plan and to focus control strategies on physical removal of amoebae and cysts rather than on disinfection.

1.3. Bacteria in the viable but non-culturable state and their detection

According to the widely accepted definition by Oliver (2005) bacteria in the viable but non-culturable (VBNC) state fail to grow on routine bacteriological media on which they would normally grow and form colonies, but they are still alive and demonstrate low levels of metabolic activity. The list of bacteria that are capable of entering this state as a means of survival include several strict or opportunistic pathogens with relevance to drinking water. Among these are *L. pneumophila*, *P. aeruginosa*, *E. coli*, several *Enterobacter* spp., *Campylobacter jejuni* and *Helicobacter pylori* (Oliver, 2005, 2010).

Bacteria may enter a VBNC state as a response to adverse environmental conditions such as nutrient depletion, osmotic stress, incubation outside the range of optimum growth temperature as well as the presence of biocides or toxic metal ions (Oliver, 2005). Laboratory experiments have shown that bacteria undergo transition into the VBNC state under oligotrophic conditions as they prevail in drinking water distribution systems. *L. pneumophila* enters a VBNC state within a few days to weeks of incubation in sterile drinking water (Hussong et al., 1987; Steinert et al., 1997) or in synthetic drinking water (Hwang et al., 2006). A treatment of *L. pneumophila* with chlorine (0.5 mg/L for 24 h), hypochlorite (100 ppm for 2 min) or with heat (70 °C for 30 min) also results in the transition of *L. pneumophila* to the VBNC state (Dusserre et al., 2008; Bej et al., 1991; Allegra et al., 2008). These results indicate that common disinfection measures such as treatment with chemicals or heat can cause bacteria to enter the VBNC state. Stress conditions induced by metal ions such as copper was shown to result in a VBNC state in *P. aeruginosa* (Dwidjosiswojo et al., 2010), in plant pathogens (Ordax et al., 2006; del Campo et al., 2009) and in *E. coli* (Grey and Steck, 2001). In mixed communities, the mutual effects of different microorganisms may also lead to the VBNC state in some bacteria (Rahman et al., 2008).

There are several studies demonstrating the presence of non-culturable *Pseudomonas* spp., *L. pneumophila*, *C. jejuni* and *Mycobacterium avium* in water distribution systems (Diederen et al., 2007) or domestic plumbing of hospitals (Wellinghausen et al., 2001; Felföldi et al., 2009) as well as in drinking water biofilms (Lehtola et al., 2006a; Långmark et al., 2005b; Lehtola et al., 2006a, b, 2007; Gião et al., 2009a, 2009b).

If the VBNC state is considered to be a survival strategy of bacteria, it has to include the capability of a bacterium to increase metabolic activity and to regain culturability (Whiteside and Oliver, 1997). The process of resuscitation upon the release of stress factors has been demonstrated in various studies (Kell et al., 1998). For *L. pneumophila* the importance of amoebae for the retrieving of culturability has been shown (Steinert et al., 1997; Hwang et al., 2006; Allegra et al., 2008). Resuscitation has been extensively studied for *Vibrio vulnificus* (Oliver, 1995). The question, whether the culturable cells detected following the removal of the inducing stress factor are a result of true resuscitation or of regrowth of a few residual culturable cells has long been discussed (Whiteside and Oliver, 1997; Kell et al., 1998; Oliver, 2000, 2005). As most investigations on the induction of and the resuscitation from the VBNC state have been performed on planktonic cells (Steinert et al., 1997; Whiteside and Oliver, 1997), it is largely unknown if these processes can also occur in biofilm environments.

During the transition from a culturable to a non-culturable state, bacteria undergo significant morphological and physiological changes. These include a reduced cell size ("dwarfing"; Federighi et al., 1998), modifications in the composition of the cytoplasmic membrane (Signoretto et al., 2000) as well as reductions in nutrient transport and respiration rates (Porter et al., 1995). The expression of a variety of genes including those for 16S rRNA synthesis is continued (Yaron and Matthews, 2002) and ATP levels remain relatively high in VBNC cells (Federighi et al., 1998).

Generally, VBNC bacteria maintain certain physiological attributes that indicate viability. Viability markers of VBNC cells may be respiratory activity, cytoplasmic membrane integrity, the capacity to metabolise nutrients or the presence of ribosomes (Fig. 1.4). There is a number of cytological methods to estimate bacterial viability making use of these markers (Kell et al., 1998; Keer and Birch, 2003). Membrane integrity analysis is based on the capacity of the cells to exclude fluorescent dyes, such as propidium iodide (PI) or propidium monoazide (PMA; Nocker et al., 2007). Actively respiring bacteria can be determined by the intracellular hydrolysis of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and subsequent detection of the CTC-formazan crystals produced by respiring bacteria using epifluorescence microscopy (Rodriguez et al., 1992). Fluorogenic esters such as fluorescein diacetate (FDA) or carboxyfluorescein diacetate (CFDA) can be used to assess intracellular enzyme activity.

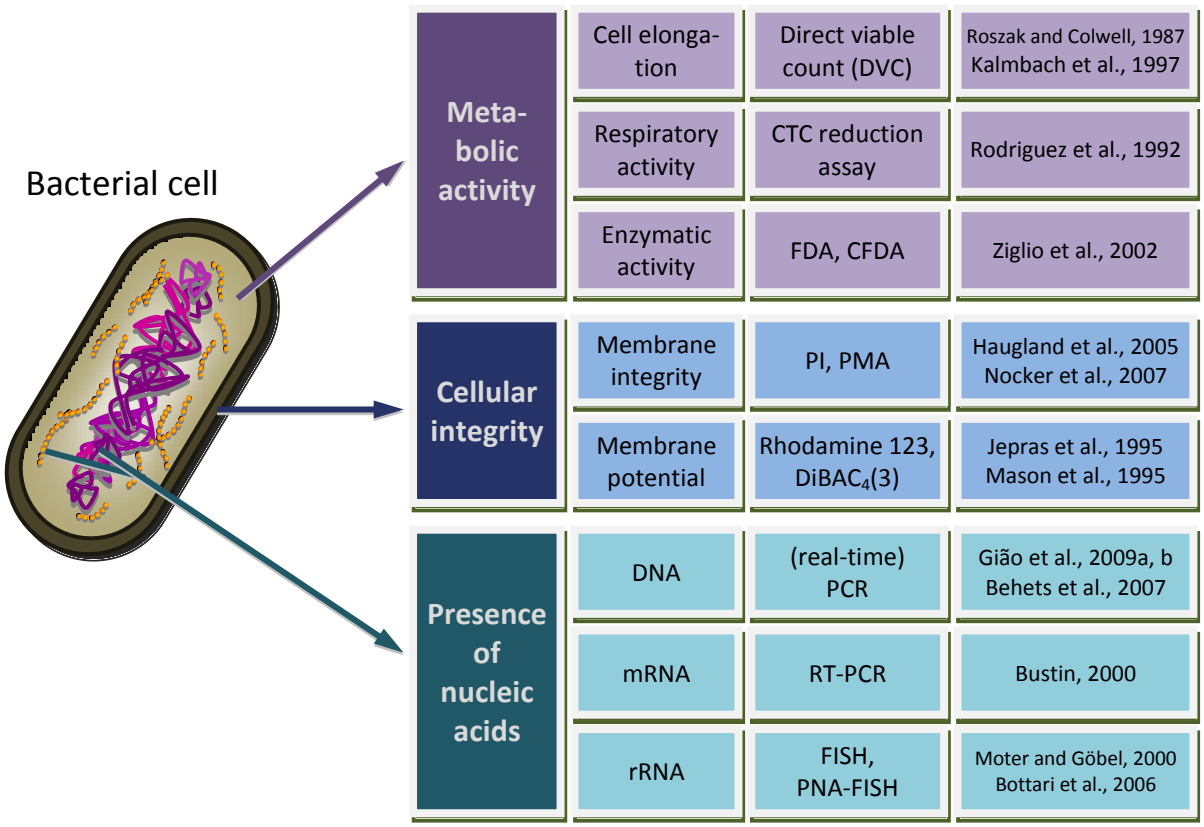

Figure 1.4: Approaches and methods used for the assessment of bacterial viability (adapted from Keer and Birch, 2003)

By coupling these methods with flow cytometry, it is possible to enumerate a large number of bacteria in a very short time and thus obtain more precise values (Ziglio et al., 2002). The potential capacity of bacteria to metabolize nutrients can be determined using a method known as direct viable count (DVC). The method is based on the use of gyrase inhibitors such as pipemidic acid or nalidixic acid that prevent cell division resulting in elongated cells which can be enumerated by means of epifluorescence microscopy (Roszak and Colwell, 1987; Kalmbach et al., 1997).

The above mentioned methods are generally used for the assessment of the physiological state of either a mixed bacterial population as a whole or of specific bacteria in pure culture. For the specific detection and/or quantification of hygienically relevant bacteria such as *L. pneumophila* and *P. aeruginosa* in water or biofilm samples from distribution systems culture-independent methods based on PCR or FISH are frequently used.

The polymerase chain reaction is a technique for the amplification of a specific DNA sequence by repeated cycles of synthesis. PCR can be used to amplify very small quantities of DNA present in a sample. Using appropriate primers, one can identify a single microbial cell in a sample even if large numbers of other species are pre-

sent. DNA amplicons can be used for further cloning or sequencing. Real-time PCR permits the analysis of the products while the reaction is actually in progress by using various fluorescent dyes that react with the amplified product. This also allows for a quantification of DNA on the basis of an increase in fluorescence signal during the PCR. Several primer and probe systems have been described for the detection of *Legionella* spp. and *L. pneumophila* (Wellinghausen et al., 2001; Declerck et al., 2007), but also for *P. aeruginosa* (Qin et al., 2003) and for amoebae (Khan et al., 2001). However, PCR-based methods do not give any information on the viability of bacteria or on the developmental stage of amoebae. Additionally, the techniques are vulnerable to inhibitory compounds like humic acids or polysaccharides and the amplification of released naked DNA or DNA from dead cells may lead to false-positive results and thus to an overestimation of the infectious risk (Wellinghausen et al., 2001; Lehtola et al., 2007; Felföldi et al., 2009).

Fluorescence in situ hybridisation (FISH) detects nucleic acid sequences by a fluorescently labelled probe that hybridizes specifically to its complementary target sequence within the intact cell. Identification of individual microbial cells with rRNA-targeted oligonucleotide probes is based on the high cellular content of ribosomes and thus rRNA molecules, which can be found in all viable organisms (Bottari et al., 2006). FISH allows the detection and quantification of culturable and non-culturable cells and can therefore help in understanding complex microbial communities (Moter and Göbel, 2000). For instance, FISH can be used for the visualisation of bacteria inside their amoebal hosts (Declerck et al., 2009). Recently, FISH methods have been developed using fluorescently labelled peptide nucleic acid (PNA) probes targeting specific rRNA gene sequences. PNA molecules are uncharged DNA analogues that penetrate the cells more easily and bind to nucleic acids much stronger than oligonucleotide probes (Bottari et al., 2006).

The detection of opportunistic pathogenic bacteria in drinking water is usually performed by methods based on the culturability of the organisms on defined selective media under given conditions in the laboratory. As VBNC bacteria do not form colonies under these conditions, standard methods may lead to an inaccurate estimation of the real infectious risk. From a public health perspective this is of great importance, because pathogenic bacteria in the VBNC state may retain their virulence (Hussong et al., 1987; Rahman et al., 1996). Additionally, a change in environmental conditions, e. g. an increase in temperature, a boost in nutrient supply or the release of stress factors (disinfectants, toxic metal ions) can lead to the resuscitation of VBNC bacteria from a non-culturable state back to a state in which they are able to grow on routine media. This process might explain phenomena such as rapid re-

colonisation of water systems by the pathogens immediately after cessation of a disinfection or recurring incidents of contamination. Opportunistic pathogens in the VBNC state can initiate infection when they undergo transition back to the culturable state. Thus, they constitute an infectious potential when present in biofilms of man-made water systems (Oliver, 2010). Improvements in detection, especially in sensitive areas or in the detection of sources of infection, could be achieved by complementation of the standard culture-based methods with molecular tools such as PCR and FISH (Grobe et al., 2010). These tools have been successfully applied for the detection and quantification of hygienically relevant bacteria and amoebae in both laboratory experiments and environmental samples (Stothard et al., 1999; Grimm et al., 2001; Khan et al., 2001; Schroeder et al., 2001; Kuiper et al., 2006; Declerck et al., 2007; Declerck et al., 2009; Feazel et al., 2009). With these methods bacteria were in most cases detected in higher concentrations and/or over longer periods of time compared to conventional culture-based detection methods (Steinert et al., 1997; Långmark et al., 2005b; Lehtola et al., 2007; Diederen et al., 2007; Gião et al., 2009a, b; Bonnetta et al., 2010).

1.4. Materials in domestic plumbing systems

The German drinking water ordinance defines a domestic plumbing system as the entity of all pipework, faucets and equipment located between the point of abstraction of water for human consumption and the point of delivery of water from a water supply facility to the consumer (TrinkwV 2001).

Water systems built before the 1950s mainly consisted of cast iron, steel or lead pipes with joints and seals formed exclusively from natural materials such as jute, leather, cork or natural rubber (Colbourne, 1985). Numerous localized incidents of microbial contaminations of drinking water were later found to be associated with the encouraging effect of these materials on the growth of microorganisms including coliform strains. From the 1950s on, materials usage gradually shifted towards synthetic materials for reasons of economy and improved functional properties (Colbourne, 1985; Schönen, 1986).

It is estimated that 12 % of residential homes in the USA use PE-X plumbing material whereas copper is the most commonly used metallic piping material (Durand and Dietrich, 2006). In Germany, domestic plumbing systems are predominated by copper, galvanised iron and plastic materials (Elfström Broo et al., 2001; Kistemann et al., 2010). A systematic and nationwide survey of locally available data on the microbiological, chemical and physical characteristics of domestic plumbing sys-

tems of German public buildings showed that copper is used in 52 % of domestic plumbing systems; further materials used for domestic plumbing were galvanised iron, plastic materials, steel, iron, stainless steel, galvanised steel and lead (Kistemann et al., 2010; Fig. 1.5).

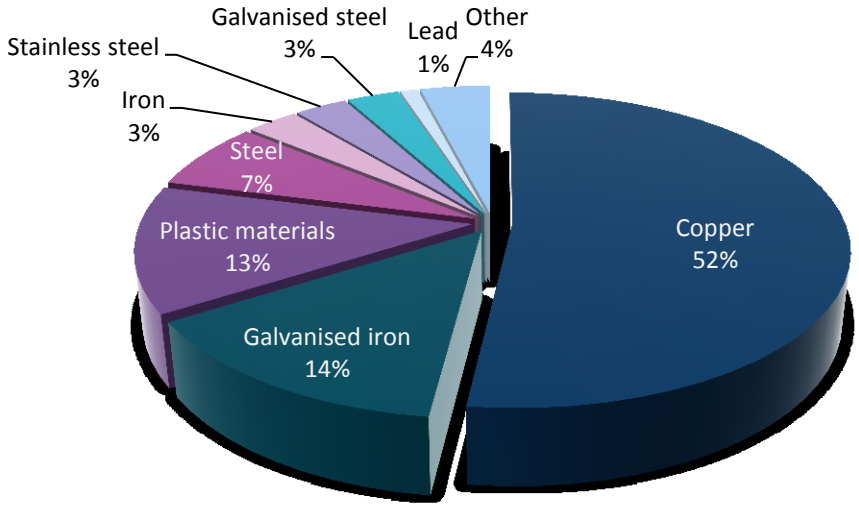

Figure 1.5: Materials utilised in domestic plumbing systems of German public buildings (Kistemann et al., 2010)

PE is produced in an anionic polymerisation of ethylene molecules resulting in semi-crystalline macromolecules with a low degree of branching (Domininghaus et al., 2008). PE is frequently used in domestic plumbing because of its low density in comparison with other plastics, its high ductility, ultimate elongation, resistance to chemicals and its good processability and machining properties (Domininghaus et al., 2008). The linear structure of PE can be modified by spatial cross-linking using chemical methods including by peroxide cross-linking (PE-Xa), silane cross-linking (PE-Xb) or physical techniques such as electron-ray cross-linking (PE-Xc; Fig. 1.6).

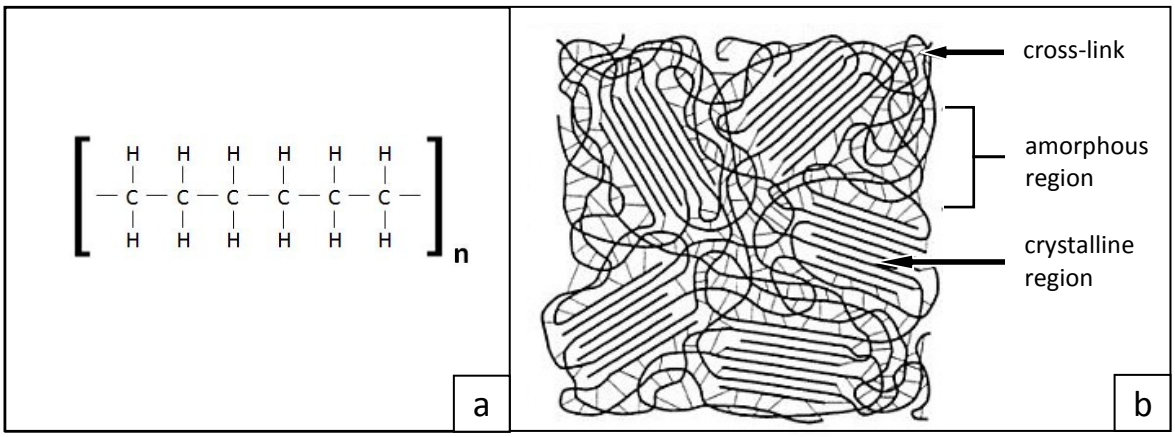

Figure 1.6: (a) General chemical structure of polyethylene and (b) molecular structure of cross-linked polyethylene (Wolters, 2006).

Cross-linking results in an increase in stress rupture strength, abrasion resistance, temperature stability, resistance to chemicals and in a lower susceptibility to tension cracks. (Wolters, 2006).

EPDM is produced in a coordinative, anionic polymerisation of ethylene, propylene and a non-conjugated diene such as hexadiene, dicyclopentadiene or ethylidene norbonene in a solution, suspension or gas phase technique (Röthemeyer and Sommer, 2006; Fig. 1.7;).

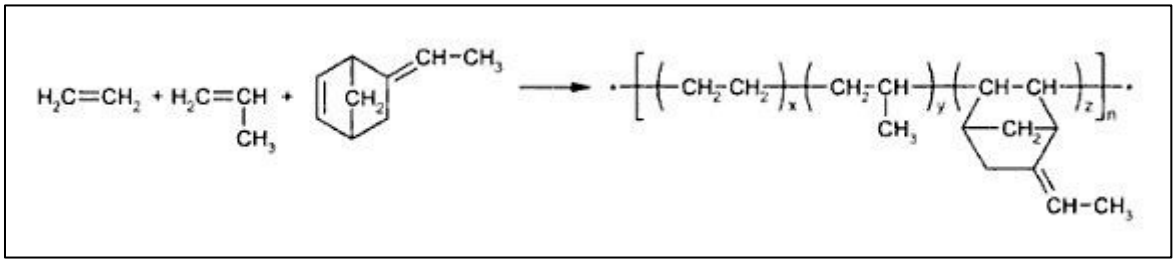

Figure 1.7: Polymerisation of EPDM from ethylene, propylene and ethylidene norbonene (Röthemeyer and Sommer, 2006)

The specific properties of EPDM are determined by the arrangement and sequence length of monomers, by the type and amount of diene used and by its molecular mass and molecular mass distribution (Röthemeyer and Sommer, 2006). Generally, EPDM is characterised by its resistance against UV radiation, heat and ozone, its ageing resistance, weathering resistance and swelling resistance and is used for hoses (showers, washing machines, dishwashers), gaskets and valves in domestic plumbing (Domininghaus et al., 2008).

Copper is a favoured material for domestic plumbing pipes, because of its corrosion resistance, its low heat expansion, high tensile strength and ultimate elongation as well as its good processability (German Copper Institute, 2010). Copper is only to be used in drinking waters with a pH ≥ 7.4 or water with a pH of 7.0 - 7.4 and a total organic carbon (TOC) content of ≤ 1.5 mg/L (DIN EN 1057; DVGW, 2002).

The intensive contact between materials and drinking water in water-bearing systems strongly affects water quality with leaching of material components being the main origin to various problems. The application of metal pipes may result in the exceeding of permitted metal levels in drinking water due to the release of metal ions and corrosion (Elfström Broo et al., 2001; Edwards et al., 2001; Völker et al., 2010) leading to public health and environmental problems. Plastic materials are supplemented with additives such as antioxidants, light stabilisers (e. g. UV absorbers), lubricants, softeners and colorants giving them the desired technical and

physical characteristics (Schlosser et al., 2007). These additives can migrate into the adjacent bulk water and result in objectionable tastes, odours or colour of the water (Elfström Broo et al., 2001; Tomboulian et al., 2001; Brocca et al., 2002; Skjevrak et al., 2003; Durand and Dietrich, 2006; Denberg et al., 2007). In addition to that, the release of organic constituents that can be utilised by microorganisms leads to enhanced microbial growth on surfaces in contact with drinking water (Schönen, 1986; Rogers et al., 1994a, 1994b).

1.4.1. Influence of plumbing materials on biofilm development in domestic plumbing

Microbial growth in drinking water systems is generally limited by the low content of organic carbon in drinking water (LeChevallier et al., 1987; van der Kooij, 1992) which is achieved by a sustainable water treatment. Construction materials utilised in drinking water distribution and domestic plumbing, especially those containing organic substances may represent a major source of organic material for enhanced microbial growth. As a consequence, colony counts of drinking water are much higher at the consumer's tap compared to finished water delivered by the water supply facility (Pepper et al., 2004). Plasticisers and other organic constituents migrating from the materials may be directly utilisable by bacteria present in the water phase. Massive biofilm development with total cell counts was observed on rubber materials including ethylene propylene (diene) monomer (EP(D)M), nitrile butadiene rubber (NBR), silicone and latex compared to glass, stainless steel and copper (Bressler et al., 2009; Kilb et al., 2003; Rogers et al., 1994b; Schönen et al., 1988). Generally, synthetic materials such as PE and PVC were colonised in significantly higher densities than steel and copper (Schwartz et al., 1998; van der Kooij et al., 2005). Other studies reported biofilm formation on copper and stainless steel to be delayed (Lehtola et al., 2004b) or similar compared to that on PVC, PE, (Zacheus et al., 2000; Wingender and Flemming, 2004). Table 1.1 shows a selection of literature data on drinking water biofilm formation on different materials.

Table 1.1: Selection of literature data on drinking water biofilm formation on different materials.

Material	Experimental system	Biofilm age	T Water (°C)	Total cell count (cells/cm²)	HPC (cfu/cm²)	Reference
EPDM, PE-Xb, PE-Xc, Copper	Pilot scale DPS [a)],	39 weeks	12, 37	10^6 - 10^8	n. sp.	Benölken et al., 2010
EPDM	glass flow-through reactor connected to DPS [a)]	14 d	15.8 – 23.0	1.2 x 10^9	1.0 x 10^8	Bressler et al., 2009
PVC, Stain-less steel, galvanized steel, cast iron	Acryl pipe reactor fed with tap water	167 d	13.0 - 24.7	n. dt.	2 x 10^1 - 8 x 10^4	Shin et al., 2007
Cast iron, PVC, stain-less steel	Coupons exposed to by-pass of DWDS [b)]	1 - 6 months	12 - 21	10^5 - 10^8	10^2 - 10^7	Juhna et al., 2007
PVC	Propella reactor, turbulent flow,	1 month	15.0	2.2 x 10^5 - 1.9 x 10^6	1.0 x 10^5 - 2.0 x 10^5	Lehtola et al., 2007;
PVC, PE-X, HD-PE, PP	Batch reactor, Propella reactor, flow cell recator	55 d	18.5 - 22.3	~7 x 10^6 - 9 x 10^8	~8 x 10^5 - 2 x 10^7	Manuel et al., 2007
PE	pilot scale DWDS [b)]	94 d	5.2 – 8.2	1.4 x 10^4 – 2.3 x 10^5	n. dt.	Långmark et al., 2005b
Copper, stainless steel, PE-X	Model DPS, simu-lating domestic warm water use	829 d	37	n. dt.	2,51 x 10^4 1,58 x 10^4 5,01 x 10^4	van der Kooij et al., 2005
Copper, PE	pilot scale DWDS [b)]	200 - 300 d	14.7 – 19.8	~4 x 10^5 - 1.8 x 10^6	~1 x 10^4 - 3.0 x 10^6	Lehtola et al., 2004a
PVC	biofilm collector pipes installed in DWDS [b)]	280 d	6.6 – 15.4	10^5 - 10^7	10^4 - 10^7	Lehtola et al., 2004b
Cast iron, cement, gal-vanised steel, PVC	pipes cut from DWDS [b)]	2 - 99 years	11.7	3.0 x 10^5 - 2.8 x 10^8	1.2 x 10^1 - 1.7 x 10^5	Wingender and Flem-ming, 2004
EPDM, NBR	Rubber-coated valves removed from DWDS [b)]	3 weeks - 4 years	n. dt.	2.7 x 10^6 - 1.8 x 10^9	3.0 x 10^5 - 5.4 x 10^9	Kilb et al., 2003
Copper, V4A-steel, PVC, PE	biofilm reactors installed in DWDS [b)]	2 years	11.6	5.4 x 10^5 - 2.3 x 10^7	1.5 x 10^3 - 1.1 x 10^5	Wingender et al., 2003

Table 1.1 (continued): Selection of literature data on drinking water biofilm formation on different materials.

Material	Experimental system	Biofilm age	T Water (°C)	Total cell count (cells/cm²)	HPC (cfu/cm²)	Reference
MDPE PVC cement	biofilm potential monitor, vertical pipe reactor (flow-through with tap water)	21 d	5.0 – 15.0	n. dt.	$1{,}4 \times 10^{1}$ - $1{,}0 \times 10^{5}$	Hallam et al., 2001
Steel, PVC, PE	Laboratory pipe and slide system	1 week	14.0 – 21.5	~ 10^{5} - 10^{6}	~ 10^{4} - 10^{5}	Zacheus et al., 2000
Copper, steel, PVC, HD-PE	modified Robbins device installed in DWDS [b)]	9 d	n. sp.	$5{,}0 \times 10^{5}$ - $2{,}5 \times 10^{6}$	~1 log unit lower than total cell counts	Schwartz et al., 1998
LD-PE, glass	modified Robbins device connected to water tap	70 d	n. dt.	5.9×10^{5} - 4.2×10^{6}	0.01 % - 0.1 % of total cell count	Kalmbach et al., 1997
copper, PB, PVCc	chemostat fed with sterile tap water inoculated with mixed microbial community	21 d	20, 40, 50, 60	n. dt.	4.47×10^{2} - 3.21×10^{6}	Rogers et al., 1994a
stainless steel, PP, PCVc, PVCu, mild steel, PE, EPM, latex	chemostat fed with sterile tap water inoculated with mixed microbial community	28 d	30	n. dt.	2.13×10^{5} - 5.50×10^{7}	Rogers et al., 1994b
Steel, PVC	biofilm reactors connected to DWDS [b)]	167 d	n. sp.	$4{,}9 \times 10^{6}$	n. dt.	Pedersen, 1990

[a)] DPS - domestic plumbing system [b)] DWDS - drinking water distribution system
n.d. - not detected n.dt.- not determined n.sp. - not specified

Significant adverse health effects have been associated with inadequate plumbing systems within public and private buildings arising from enhanced biofilm formation on certain plumbing materials (WHO, 2008). There are studies indicating a growth-promoting effect of certain materials such as PB, PE, PVC, silicone and rubber on *Legionella* spp. and *L. pneumophila* whereas copper pipes seem to inhibit *Legionella* growth (Schönen et al., 1988; Colbourne and Ashworth 1986; Rogers et al., 1994a; van der Kooij et al., 2005; Mathys et al., 2008).

Table 1.2: Selection of literature data on the occurrence of hygienically relevant bacteria drinking water biofilms on different materials

Material	Experimental system	Inoculation	T Water (°C)	Hygienically relevant microorganisms	Detection method	Concentration	Reference
EPDM, PE-Xb PE-Xc, Copper	Pilot scale DPS[a)]	*P. aeruginosa, L. pneumophila E.nimipressuralis*	12, 37	*P. aeruginosa, L. pneumophila E.nimipressuralis*	Cultivation FISH (n.q.)	10 cfu/cm² 5 x 10^5 cfu/cm² n.d.	Benölken et al., 2010
Copper, stainless steel, zinc-coated steel, PVCc, PB, PE	1 L-glass reactors containing tap water (batch)	*E. coli*	25	*E. coli*	cultivation	3.59 x 10^2, >4 x 10^3, >4 x 10^3, 4 x 10^2, 2.5 x 10^3, 2.4 x 10^3 cfu/cm²	Yu et al., 2010
EPDM	glass flow-through reactor connected to DPS[a)]	*P. aeruginosa*	15.8 – 23.0	*P. aeruginosa*	Cultivation	10^5 cfu/cm²	Bressler et al., 2009
PVC, PE, PP, stainless steel, copper, galva-nized steel	Model cooling tower system, recirculation	*L. pneumophila*	29	*L. pneumophila*	Cultivation	1,02 x 10^3, 1,58 x 10^3, 1,26 x 10^3, 1,51 x 10^3, 1,58 x 10^3, 1,58 x 10^4 cfu/cm²	Türetgen and Cotuk, 2007
PVC	Propella reactor, turbulent flow	*L. pneumophila M. avium E. coli* CaCV[c)]	15.0	*L. pneumophila M. avium E. coli* CaCV[c)]	Cultivation FISH PCR (CaCV[c)])	8.8 x 10^3 cfu; 9.3 x 10^3-cells/cm² 50-155 cfu; 8.0 x 10^4 cells/cm², 10^2 cfu/cm², n.d. after 4d 1.5 x 10^5-1.9 x 10^5 PCR units/cm²	Lehtola et al., 2007;
Cast iron, PVC, stainless steel	Coupons exposed to by-pass of DWDS[b)]	-	12-21	Coliforms *E. coli*	Cultivation FISH	1.1 cfu/cm² in 58 % of samples (n.q.)	Juhna et al., 2007
PE	pilot scale DWDS[b)]	*L. pneumophila*	5.2 – 8.2	*L. pneumophila*	Cultivation FISH	0.1 – 4.84 x 10^3cfu/cm² 2.4 x 10^3 – 32.9 x 10^4 cells/cm²	Långmark et al., 2005b
Copper, stainless steel, PE-X	Model DPS[a)]	mixed microbial community containing *L. pneumophila*	37	*L. pneumophila*	cultivation	4.0 x 10^1, 1.02 x 10^3, 3.70 x 10^3	van der Kooij et al., 2005
Cast iron, cement, galvanised steel, PVC	pipes cut from DWDS[b)]	-	11.7	Coliforms	Cultivation	0.04 cfu/cm² (cement) 0.26 cfu/cm² (PVC)	Wingender and Flemming, 2004

Table 1.2 (continued): Selection of literature data on the occurrence of hygienically relevant bacteria drinking water biofilms on different materials

Material	Experimental system	Inoculation	T Water (°C)	hygienically relevant micro-organisms	Detection method	Concentration	Reference
PVCp, PVCu	Batch test	mixed micro-bial community con-taining *L. pneu-mophila*	37	*L. pneumophila*	cultivation	2,00 x 10^6 7,94 x 10^4	Kuiper et al., 2004
EPDM, NBR	Rubber-coated valves removed from DWDS [b)]	-	n. dt.	Coliforms *P. aeruginosa*	Cultivation	4.7 x 10^3 0.4 cfu/cm² (in 1/13 biofilms)	Kilb et al., 2003
Copper, V4A-steel, PVC, PE	biofilm reac-tors installed in DWDS [b)]	-	11.6	*P. aeruginosa*	Cultivation	41 cfu/cm² (1 biofilm on PE)	Wingender et al., 2003
Copper, steel, PVC, HD-PE	modified Rob-bins device installed in DWDS [b)]	-	n. sp.	*Legionella* spp., fecal streptococ-ci	FISH	up to 7 % of the total count, n.q.	Schwartz et al., 1998
copper, PB, PVCc,	chemostat fed with sterile tap water	mixed microbial community con-taining *L. pneumophila*	20, 40, 50, 60	*L. pneumophila*	cultivation	<10 - 1.67 x 10^3, <10 - 1.12 x 10^5, <10 - 6.84 x 10^4 cfu/cm²	Rogers et al., 1994a
stainless steel, PP, PCVc, PVCu, mild steel, PE, EPM, latex	chemostat fed with sterile tap water	mixed microbial community con-taining *L. pneumophila*	30	*L. pneumophila* *P. aeruginosa*	cultivation	1.03 x 10^4, 2.10 x 10^4, 2.24 x 10^4, 7.75 x 10^3, 2.06 x 10^4, 6.76 x 10^3, 1.44 x 10^5, 2.20 x 10^5 cfu/cm² n.d., 1.9, n.d, n.d., 30, 2.6 x 10^2, n.d., n.d. cfu/cm²	Rogers et al., 1994b

[a)] DPS - domestic plumbing system [b)] DWDS - drinking water distribution system [c)] CaCv - calicivirus
n.d. - not detected n.dt.- not determined n.q. - not quantified

It is not clear whether nutrients released from the substratum directly promote the proliferation of hygienically relevant bacteria or just indirectly improve conditions for their survival by enhancing growth of the general microflora. Table 1.2 gives an overview of several studies investigating the presence of hygienically relevant bacteria in drinking water biofilms on different materials.

Materials used in drinking water distribution do not only affect the extent of microbial growth but also the composition and species diversity of biofilm communities. Investigations using PCR-DGGE showed that biofilms grown on plastic materials like PE and PB had a higher phylotype diversity than those grown on stainless steel and copper. The lowest species richness was observed for chlorinated PVC (Yu et al., 2010). In situ hybridisation with fluorescence labelled, group-specific rRNA targeted oligonucleotide probes revealed that the proportion of α-, β- and γ-subclasses in drinking water biofilms depends on the substrate material (Kalmbach et al., 1997; Schwartz et al., 1998).

The effect of pipe materials on biofilm formation as well as on the persistence of pathogens in biofilms has mainly been studied on materials which make up drinking water distribution systems, while domestic plumbing materials that usually differ from those of distribution systems have been considered less frequently (Eboigbodin et al., 2008; Rogers et al., 1994a, b). There is evidence that the major part of bacteria in drinking water at the point of use originates from the piping and faucets of the domestic plumbing system rather than from source waters or the water mains (Pepper et al., 2004). Thus, in order to provide consumers with inobjectionable potable water at the point of use it is important to take into account the design and installation of domestic plumbing systems, especially the selection of materials used.

1.4.2. Approval for materials in contact with drinking water

In order to elucidate questions arising from incidents of massive microbial growth attributed to materials in contact with drinking water, many investigators performed controlled tests. A variety of test procedures determining if a product will promote significant microbial growth was devised including static tests, tests with regularly exchanged water and tests focusing on surface growth. Some of these methods, i. e. the mean dissolved oxygen difference test of Colbourne and Brown (1979) or the slime production test of Schönen and Schöler (1983) were further developed and adopted as routine test protocols.

Methods for determining the growth-promoting properties of construction products have been developed in several European countries (Tab. 1.3). In the United King-

dom, materials in contact with treated water are tested according to the British standard 6920. The test consists of five individual tests including analyses on odour and flavour, appearance of colorant and turbidity, growth of aquatic microorganisms, extraction of substances harmful to health and extraction of metals. Microbial testing is based on the mean dissolved oxygen difference (MDOD). As the growth of aerobic microorganisms increases, the concentration of oxygen in the test system decreases. This decrease is compared with the control. Materials with an MDOD level above 2.3 mg/L are considered unsuitable for use in contact with potable water (Colbourne and Brown, 1979; Colbourne, 1985; Moorman, 2006).

Table 1.3: European methods for the approval of materials in contact with drinking water

	Parameter for microbial activity	Surface tested (cm^2)	Temperature (°C)	Duration (weeks)	Type of water	Replacement of water
Technical rule W 270 Germany	volume of biomass	800	12 ± 5	12 - 16	drinking water*	continuous flow (20 ± 2 L/h)
Mean dissolved oxygen demand (MDOD) United Kingdom	oxygen demand	150	30 ± 1	7 - 10	drinking water*	twice a week
Biofilm production potential (BPP) Netherlands	ATP	12 x 8	25 ± 1	16	slow sand filtrate	none

*without any substances inhibiting microbial growth

In the Netherlands, the biostability of materials is tested with the biofilm production potential (BPP) test (van der Kooij and Veenendaal, 2001; Smeets et al., 2008). The concentration of active biomass on the surface of material coupons incubated in biologically stable water under static conditions at 25 °C including the concentration of the suspended biomass is measured using ATP analysis. Typical BPP values range from less than 10^2 pg ATP/cm^2 for unplasticised polyvinyl chloride to values above 10^4 pg ATP/cm^2 for certain plastic and rubber materials (van der Kooij, 2003). In addition to that the suitability of products in contact with drinking water is evaluated based on information about product composition and the toxicity of the constituents (van der Kooij and Veenandaal, 2001).

In Germany, the evaluation of materials in contact with drinking water is performed according to technical rule W 270 of the German Gas and Water Association (DVGW).

The method is based on determining the amount of biomass formed on the surface of a material and it distinguishes between surface colonisation and surface growth. Surface colonisation describes the biomass that is formed on material surfaces in contact with water that can be detected using contact cultures, smear tests or microscopy, whereas the biomass consisting of microorganism developing on the surface and the surrounding EPS that can be scraped from a surface is referred to as surface growth. Test surfaces of defined area are exposed to drinking water at a constant flow rate of 20 L/h. After 4, 8, 12 and optionally after 16 weeks test surfaces are removed, the biomass is harvested by scraping and centrifugation and the volume of biomass is determined. Materials that show only surface colonisation or a surface growth ≤ 0.05 ± 0.02 mL/800 cm² comply with the requirements of the standard and can be used in contact with drinking water. Materials showing a surface growth > 0.05 ± 0.02 mL/800 cm² are not suitable for use in contact with potable water. Exceptions exist for materials used for joints and gaskets (DVGW, 2007).
Chemical and aesthetical suitability of plastics and elastomers in contact with drinking water are assessed according to the guidelines of the Federal Environmental Agency (UBA, 2008) or the recommendations of the German commission for drinking water (KTW; Anonymous, 1985), respectively. Copper has to comply with the requirements of DIN EN 1057 (2006) and DVGW technical rule GW 392 (DVGW, 2002) and are tested according to DIN 50931-1 (1999). The material has to contain at least 99.90 % copper and silver and between 0.015 % and 0.04 % phosphorous. As a material for domestic plumbing systems copper is only suitable in drinking waters with a pH ≥ 7.4 or water with a pH of 7.0 - 7.4 and a total organic carbon (TOC) content of ≤ 1.5 mg/L (DIN EN 1057; DVGW, 2002).
On a European level a harmonized and standardised approval system for construction products in contact with drinking water is currently being devised. The Regulators Group composed of representatives from the European Commission, the member states, the European Standardization Organisation and different organisations having a stake in the subject proposed to design this European Acceptance Scheme on the basis of the Dutch approach of the BPP test using ATP as a parameter for active biomass (Elfström Broo et al., 2001; Tsvetanova and Hoekstra, 2009).

1.4.3. Effect of chemical disinfection on domestic plumbing materials

In Germany, a continuous disinfection of drinking water is not desired (“Minimierungsgebot”; TrinkwV 2001; DVGW, 2009). Efforts are made to produce stable drinking water containing a minimal amount of microbially available nutrients. The risk of microbial re-growth can be minimised by planning, construction,

operation and maintainence of drinking water distribution systems as well as domestic plumbing systems according to state-of-the-art technology. These prerequisites allow for the supply of a microbiologically unobjectionable drinking water at the point of use. If thresholds for microbiological parameters of the drinking water ordinance or guideline limits for *Legionella* spp. and *Pseudomonas aeruginosa* are exceeded, disinfection measures become inevitable in order to avert health risks. Cause and location of the contamination have to be identified and a chemical disinfection with sodium hypochlorite (NaOCl), chlorine dioxide (ClO_2) or hydrogen peroxide or a thermal disinfection are carried out in due consideration of the particular water quality and the characteristics of the materials employed in the system (DVGW, 2009). Disinfectants and disinfection measures for continuous disinfection of drinking water are listed in § 11 of the German drinking water ordinance. Discontinuous disinfection of water distribution systems in case of microbial contamination is performed according to DVGW technical rule W 291. Substances and concentrations approved for disinfection in Germany are listed in Table 1.4.

Table 1.4: Substances and techniques used for continuous disinfection of drinking water and discontinuous disinfection of drinking water distribution systems

Substance, agent, technique	Continuous disinfection of drinking water[1]		Discontinuous disinfection of water distribution systems[2]
	Permitted addition	Concentration after termination of treatment	
Calcium hypochlorite	1.2 mg/L free Cl_2	max. 0.3 mg/L free Cl_2 min. 0.1 mg/L free Cl_2	50 mg/L free Cl_2
Chlorine	1.2 mg/L free Cl_2	max. 0.3 mg/L free Cl_2 min. 0.1 mg/L free Cl_2	-
Chlorine dioxide	0.4 mg/L ClO_2	max. 0.2 mg/L ClO_2 min. 0.05 mg/L ClO_2	6 mg/L ClO_2
Hydrogen peroxide	-	-	150 mg/L H_2O_2
Sodium hypochlorite	1.2 mg/L free Cl_2	max. 0.3 mg/L free Cl_2 min. 0.1 mg/L free Cl_2	50 mg/L free Cl_2
Ozone	10 mg/L O_3	≤ 0.05 mg/L O_3	-
Potassium permanganate	-	-	15 mg/L $KMnO_4$
UV-radiation (240 – 290 nm)	≥ 400 Joule/m³	-	-

[1] according to § 11 of the German drinking water ordinance (TrinkwV, 2001)
[2] according to DVGW technical rule W 291

Depending on the quality of the raw water and the performance of drinking water production in a particular region, materials used for the construction of drinking water distribution systems and domestic plumbing systems may be permanently exposed to disinfectants, in most cases chlorine. During sanitation measures concentrations of up to 50 mg/L chlorine or 6 mg/L chlorine dioxide can be applied (DVGW, 2000; Tab. 1.4). The free chlorine released by these disinfectants creates a highly oxidative environment affecting all components of the plumbing system. The structural, physical and chemical integrity of construction materials, especially of synthetic polymers such as plastics or elastomers can be deteriorated and their lifetime may be reduced. This phenomenon is also referred to as ageing (Castagnetti et al., 2008).

Hassinnen et al. (2004) exposed high-density polyethylene (HD-PE) pipes stabilised with a combination of phenolic and phosphate antioxidants to water containing 3 ppm chlorine at a pressure of 1 MPa and temperatures of 95 °C and 105 °C. The antioxidants were rapidly consumed by chlorine resulting in an extensive polymer degradation of the inner wall.

Chung et al. (2003) observed a layer of highly degraded and discoloured material with micro-cracks coalescing into larger cracks on the inner surface of PE-X pipes exposed to chlorine, chloramines and chlorine dioxide at concentrations of 0.1 ppm, 3 ppm and 5 ppm free chlorine and temperatures of 105 °C and 115 °C for up to 423 days. The successive oxidative deterioration of the pipes was described to proceed according to the following mechanism: depletion of stabilizers at the inner pipe surface, oxidation of the inner layer, micro-cracking of the inner layer, crack propagation through the wall with oxidation in advance of the crack front and final rupture of the remaining layer resulting in ultimate failure of the pipe (Chung et al., 2003). Other materials have been found to exhibit similar mechanisms of initial oxidant attack with the mechanism of ultimate failure being material dependent (Vibien et al., 2001). Bigg et al. (2004) analysed failed polybutene (PB) pipes withdrawn from a drinking water distribution system. Failure of the pipes was attributed to a comparable process of oxidative degradation.

The oxidative failure mechanisms of a construction material in the presence of disinfectants are significantly influenced by the characteristics of the potable water (Chung et al., 2003) and the effect is enhanced with increasing temperature (Castagnetti et al., 2008). ClO_2 was shown to be much more aggressive than NaOCl against polyethylene pipes (Castagnetti et al., 2008).

Ageing of EPDM, PE-Xb and PE-Xc in water containing 5 ppm NaOCl or 3 ppm ClO_2 at 40 °C and 3-4 bar resulted in chemical and structural changes of the material sur-

faces as revealed by means of Fourier-transformation infrared spectroscopy, atomic force microscopy and determination of the oxidation induction time (Schaule et al., 2010). Microtome sectioning of EPDM showed that the aged material had a higher surface roughness than the new material; by contact angle measurement it was demonstrated that aged materials were more hydrophilic than new materials (Schaule et al., 2010).

Most of the previous studies on the ageing of plumbing materials have focused on changes in the physical, chemical and structural characteristics of the pipe leading to preterm failure of a plumbing component. The effect of changes in material characteristics on their colonisation by microorganisms, especially by hygienically relevant bacteria in drinking water has not been taken into account yet. There is evidence that surface parameters such as hydrophobic or hydrophilic properties as well as roughness of a surface do influence the attachment of microorganisms (Pedersen et al., 1990; Melo and Bott, 1997; Percival et al., 1998; Shin et al., 2007). Thus, possible alterations of the surface properties of a synthetic plumbing material due to the application of high concentrations of oxidative disinfectants in the framework of a sanitization measure might have an influence on the subsequent (re-)colonisation of the particular surface. Schaule et al. (2010) suggested that crack formation and enhanced release of microbially degradable additives from plumbing materials aged by chemical disinfectants increases the risk of bacterial attachment and proliferation. However, batch experiments on the colonisation of EPDM and PE-Xb aged by exposure to ClO_2 (3 ppm, 40 °C, 4 bar, 4 weeks) showed that the biofilm formation potential measured as total cell count of the aged materials was slightly (< 1 order of magnitude) lower compared to new materials (Schaule et al., 2010).

1.5. Aim of the study

The main aim of this work was to investigate the formation of biofilms on materials relevant to domestic plumbing systems and the incorporation of hygienically relevant bacteria into these biofilms. The research aims were set as follows:

- To quantify biofilm formation on materials that are utilised in domestic plumbing systems, namely ethylene propylene diene monomer (EPDM), two types of cross-linked polyethylene (PE-Xb, PE-Xc) and copper, and to analyse biofilm population diversity of biofilms grown on these materials.
- To determine the influence of possible material alterations as a consequence of chemical disinfection on biofilm formation and on biofilm population diversity.
- To study the fate of *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Enterobacter nimipressuralis* in drinking-water biofilms grown on domestic plumbing materials under laboratory conditions with quantification of the target organism using standard culture-based methods and the culture-independent fluorescence in situ hybridisation (FISH) for recognising organisms that are possibly in the VBNC state.

Chapter 2
Materials

2.1. Microbial test strains

P. aeruginosa AdS was isolated from the residual water within an automatic shut-off valve of a shower in a contaminated domestic plumbing system of a school.
L. pneumophila AdS (serogroup 1) was isolated from a biofilm in the same automatic shut-off valve.
Enterobacter nimipressuralis 9827 clone A was isolated from an elevated tank of a drinking water supply system; the isolate was kindly supplied by Prof. Dr. M. Exner, Institute for Hygiene and Public Health, University of Bonn, Germany.
Cultures of *P. aeruginosa* AdS and *E. nimipressuralis* 9827 clone A were stored on nutrient agar at 4 °C. *L. pneumophila* AdS was stored on BCYEα agar (Oxoid) at 4 °C. For permanent preservation, a cryogenic culture of each of the bacteria was kept on glass beads (Cryobank system, Mast Diagnostica GmbH, Germany) at -70 °C.
A. castellanii ATTC 30234 was obtained from the American Type Culture Collection (LGC Promochem, Germany). The culture was kept in PYG medium at 30 °C. For permanent preservation, a cryogenic culture was prepared in DMSO (dimethyl sulfoxide; Sigma) and kept in cryogenic vials (Nalgene) at -70 °C.

2.2. Growth Media

Preparation of media:

Media were prepared using deionised water and autoclaved at 121 °C for 20 min or alternatively filter sterilized (pore size 0.2 µm). If not mentioned otherwise, the preparation was done according to the manufacturers' instructions. All solid growth media were poured in portions of 25 mL into sterile Petri dishes.

BCYEα agar (OXOID)

Commercial agar plates, ready-to-use.

Composition in g/L (ISO 11731:1998 (E)): Yeast extract 10.0, agar 12.0, activated charcoal 2.0, alpha-ketoglutarate, monopotassium salt 1.0, ACES buffer (N-2-actetamido-2-aminoethanesulfonic acid) 10.0, potassium hydroxide (KOH) 2.8, L-cysteine hydrochloride monohydrate 0.4, iron(III)pyrophosphate 0.25, deionised water ad 1000 mL.

Biolog Universal Growth (BUG) medium (OXOID)

No specification of the medium's composition made by the manufacturer.

GVPC agar (OXOID)

Commercial agar plates, ready-to-use.

Composition in g/L (ISO 11731:1998 (E)): Yeast extract 10.0, agar 12.0, activated charcoal 2.0, alpha-ketoglutarate, monopotassium salt 1.0, ACES buffer (N-2-actetamido-2-aminoethanesulfonic acid) 10.0, potassium hydroxide (KOH) 2.8, L-cysteine hydrochloride monohydrate 0.4, iron(III)pyrophosphate 0.25, ammonium-free glycine 3.0, polymyxin B sulfate 80.000 iu, vancomycin hydrochloride 0.001, cycloheximide 0.08, deionised water ad to 1000 mL.

LB medium (Lenox Broth)

Composition in g/L: Tryptone 10.0, NaCl 5.0, yeast extract 5.0 / pH 7.0 ± 0.2 at 25 °C.

The compounds were dissolved in deionised water, pH was adjusted to 7.0 ± 0.2 and deionised water was added up to 1 L.

Nutrient agar (NA, Merck)

Composition in g/L: Peptone from meat 5.0, meat extract 3.0, agar 12.0 / pH 7.0 ± 0.2 at 25 °C.

20 g of the commercially available granulate were dissolved in 1 L of deionised water.

Peptone yeast extract glucose (PYG) medium (ATCC medium 712)

PYG medium consists of a basal medium, five inorganic salt solutions and a glucose-sodium citrate-solution. All components were prepared and autoclaved or filter-sterilized separately and aseptically mixed to yield PYG medium.

Composition of 1 L PYG medium

Component	mL
Basal medium	900
0.4 M Magnesium sulfate stock solution	10
0.05 M Calcium chloride stock solution	8
0.005 M Ammonium iron(III) sulfate stock solution	10
0.25 M Disodium hydrogen phosphate stock solution	10
0.25 M Potassium dihydrogen phosphate stock solution	10
Glucose-sodium citrate stock solution	50

Basal medium

Composition in g/L: Proteose peptone 22.22, yeast extract 1.11

The compounds were dissolved in 1 L deionised water, pH was adjusted to 6.5 ± 0.2 with 1 M HCl and the medium was autoclaved for 20 min at 121°C.

Stock solutions of inorganic salts

Stock solution	Salt	g/L
0.4 M Magnesium sulfate	$MgSO_4$ x 7 H_2O	98,59
0.05 M Calcium chloride	$CaCl_2$ x 2 H_2O	7,35
0.005 M Ammonium iron(III) sulfate	$Fe(NH_4)_2(SO_4)_2$ x 6 H_2O	1,96
0.25 M Disodium hydrogen phosphate	Na_2HPO_4 x 2 H_2O	44,50
0.25 M Potassium dihydrogen phosphate	KH_2PO_4	43,55

The stock solutions were prepared separately, autoclaved for 20 min at 121°C and stored at room temperature in the dark.

Glucose-sodium citrate stock solution
Composition in g/50 mL: Glucose 18.0, trisodium citrate dihydrate 1.0.
The compounds were dissolved in 50 mL deionised water, pH was adjusted to 6.5 ± 0.2 with 0.2 M HCl and the solution was filter sterilized (0.2 µm). The glucose stock solution was freshly prepared prior to each experiment.

Pseudomonas selective agar (CN agar, Oxoid)
Composition in g/L: Gelatine peptone 16.0, casein hydrolysate 10.0, potassium sulfate 10.0, magnesium chloride 1.4, agar 11.0 / pH 7.1 ± 0.2 at 25 °C.
24.2 g of the commercially available granulate of Pseudomonas agar base were suspended in 500 mL of deionised water and 5 mL of glycerol were added before autoclaving (121 °C, 20 min). The content of one vial of Pseudomonas CN supplement (100 mg cetrimide, 7.5 mg sodium nalidixate; Oxoid) dissolved in 2 mL of a 1:1 (vol/vol) mixture of ethanol and sterile deionised water was added to 500 mL of autoclaved agar base cooled to 50 °C.

R2A liquid medium (Reasoner and Geldreich, 1985)
Composition in g/L: Yeast extract 0.5, Difco proteose peptone no. 3 0.5, casamino acids 0.5, glucose 0.5, soluble starch 0.5, sodium pyruvate 0.3, K_2HPO_4 0.3, $MgSO_4 \times 7\ H_2O$ 0.05. / pH 7.2 ± 0.2 at 25 °C
The compounds were dissolved in 1 L of deionised water and the pH was adjusted using crystalline KH_2PO_4.

R2A medium (Difco)
Composition in g/L: Yeast extract 0.5, Difco proteose peptone no. 3 0.5, casamino acids 0.5, glucose 0.5, soluble starch 0.5, sodium pyruvate 0.3, K_2HPO_4 0.3, $MgSO_4 \times 7\ H_2O$ 0.05, agar 15.0 / pH 7.2 ± 0.2 at 25 °C.
18.2 g of the commercially available granulate were dissolved in 1 L of deionised water.

Yeast extract (0.02 %) (Sigma)
0.02 g yeast extract were dissolved in 100 ml deionised water and filtered through a Filtropur S plus filter (pore size 0.2 µm).

Yeast Extract Broth (Ristroph et al.,1980)

Composition in g/L: Yeast extract 10.00, L-cysteine-hydrochloric monohydrate 0.40, iron(III)pyrophosphate 0.25.

The pH was adjusted to 6.9 ± 0.2 with 1 M KOH and the medium was filter sterilized (0.2 µm).

2.3. Buffers and solutions

2.3.1. Buffers and solutions for general use

0.9 % NaCl-solution

9.0 g NaCl were dissolved in 1 L deionised water. The solution was autoclaved for 20 min at 121 ˚C.

1 % Agarose solution

2 g agarose (Certified™ low melt; Bio-Rad) were dissolved in 200 mL 1x TAE-buffer and stored at 55 ˚C.

Acid buffer (ISO 11731: 1998 (E))

Solution A: 0.2 M HCl

200 mL 1 M HCl in 800 mL of deionised water.

Solution B: 0.2 M KCl

14.9 g KCl were dissolved in 1 L deionised water.

For preparation of the acid buffer 3.9 mL of solution A were mixed with 25 mL of solution B. After adjusting the pH to 2.2 ± 0.2 with 1 M KOH, the buffer was filter sterilized (0.2 µL).

DAPI stock solution (25 µg/ml) in 2 % (v/v) formaldehyde

12.5 mg 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) were dissolved in 27 ml formaldehyde (37 %) and 473 ml deionised water and filtered through a cellulose acetate filter (pore size 0.2 µm).

DNA-Ladder

MassRuler™ DNA Ladder Mix, ready-to-use, range 80-10000 bp (Fermentas, SM0403).

Loading Dye

6 x TriTrack™ DNA Loading Dye (Fermentas, R1161).

Composition: 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.15% orange G, 60% glycerol, 60 mM EDTA.

Ethidium bromide solution

10 mg/mL (Bio-Rad, 161-0433)

McFarland standard (bioMérieux, 70900)

Particle-free deionised water

Deionised water was filtered through a cellulose acetate filter (pore size 0.2 µm) and then autoclaved (20 min, 121 °C).

Phosphate-buffered saline (PBS)

Composition in g/L: NaCl 8.00, KCl 0.20, Na_2HPO_4 x 2 H_2O 1.81, KH_2PO_4 0.24.

The components were dissolved in Rotipuran water (Roth T143.1). The pH was 7.2 ± 0.2. The solution was autoclaved for 20 min at 121 °C.

TAE (Tris/Acetic Acid/EDTA) buffer, 50x

Commercial concentrate (Bio-Rad, 161-0743).

TAE (Tris/Acetic Acid/EDTA) buffer, 1x

Composition in mM: Tris 40, acetic acid 20, EDTA 1 / pH 8.3.

20 mL 50x concentrated TAE-buffer were diluted in 980 mL deionised water.

2.3.2. Solutions for the quantification and identification of amoebae

0.4 % Trypan blue staining solution

0.04 g Trypan blue were dissolved in 10 mL of Prescott and James Saline.

Page's Amoeba Saline (PAS)

Composition in g/L: Na_2HPO_4 x 2 H_2O, 0.178; KH_2PO_4, 0.136; NaCl, 0.12;
$MgSO_4$ x 7 H_2O, 0.004; $CaCl_2$ x 2 H_2O, 0.004.

The components were dissolved in 10 mL deionised water and diluted to 1 L with deionised water. The solution was autoclaved for 20 min at 121 °C.

Prescott and James Saline (PJ Saline; Page and Siemensma, 1991)
Stock solution 1 (composition in g/100 mL): $CaCl_2$ x 2 H_2O 0.433, KCl 0.162
Stock solution 2 (composition in g/100 mL): KH_2PO_4 0.512
Stock solution 3 (composition in g/100 mL): $MgSO_4$ x 7 H_2O 0.280
The stock solutions were autoclaved separately for 20 min at 121 ˚C.
1 mL of each stock solution was added aseptically to 997 mL of autoclaved deionised water.

2.3.3. Buffers, solutions and primers for polymerase chain reaction

dNTP Mix
Commercial ready-to-use reagent mix (5 Prime, 2201200).
10 mM of each dNTP.

MasterMix 2.5x
Commercial ready-to-use reagent mix (5 Prime, 2200100).
Composition: 62.5 U/µL Taq DNA Polymerase, 125 mM KCl, 0.5 % ®-CA630, 75 mM Tris-HCl (pH 8.3), 3.75 mM Mg^{2+} ($Mg(OAc)_2$), , 500 µM dNTP (each), 0.25% Igepal, stabilizers.

Primers

Table 2.1: Primers used in the present study

Primer	Sequence	Reference
fd1	5'-AGA GTT TGA TCC TGG CTC AG-3'	Weisburg et al., 1991
rp2	5'-ACG GCT ACC TTG TTA CGA CTT-3'	Weisburg et al., 1991
27f_GC	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CAG AGT TTG ATC (A/C)TG GCT CAG-3'	Medlin et al., 1988 (underlined); Murray et al., 1996 (GC-clamp)
517r	5'-ATT ACC GCG GCT GCT GG-3'	Murray et al., 1996
Ami6F1	5'-CCA GCT CCA ATA GCG TAT ATT-3'	Thomas et al., 2006
Ami6F2	5'-CCA GCT CCA AGA GTG TAT ATT-3'	Thomas et al., 2006
Ami9R	5'-GTT GAG TCG AAT TAA GCC GC-3'	Thomas et al., 2006

Probes were obtained as lyophilisates from Eurofins MWG Operon (Ebersbach, Germany).

Taq buffer advanced
Commercial ready-to-use reagent mix (5 Prime, 2201240).
10x concentrated with 15 mM Magnesium.

Taq DNA Polymerase (5 U/µL; 5 Prime, 2200000).

Taq Master PCR Enhancer

Commercial ready-to-use reagent mix (5 Prime, 2201250).

5x concentrated.

2.3.4. Buffers, solutions and oligonucleotide probes for fluorescence in situ hybridisation

0.05 % Agarose solution

0.005 g Agarose (Certified™ low melt; Bio-Rad) were dissolved in 10 mL of Rotipuran water.

DAPI solution (1 µg/mL) in PBS

40 µL of the DAPI stock solution were mixed with 960 µL PBS.

0.25 M EDTA (Ethylene diamine tetra-acetic acid)

104.50 g EDTA ($C_{10}H_{12}N_2Na_4O_8$ x $2H_2O$, Sigma) were dissolved in 1L of Rotipuran water. The solution was autoclaved for 20 min at 121 ˚C

Hybridisation buffers

Table 2.2: Hybridisation buffers for oligonucleotide probes targeting bacterial 16S rRNA

	EUB338 / NONEUB338	Psae 16S-182	LEG705	LEGPNE1
5 M NaCl	0.9 M	0.9 M	0.9 M	0.9 M
1 M Tris pH 7.6	20 mM	-	20 mM	20 mM
1 M Tris pH 8.0	-	20 mM	-	-
10% SDS	0.01 %	0.01 %	0.01 %	0.01 %
Formamid	20%	40 %	20 %	25 %

Table 2.3: Hybridisation buffers for oligonucleotide probes targeting eukaryotic 18S rRNA

	EUK516	HART498	GSP
5 M NaCl	0.9 M	0,9 M	0.9 M
1 M Tris pH 7.6	20 mM	20 mM	20 mM
1 M Tris pH 8.0	-	-	-
10% SDS	0.01 %	0,01 %	0.01 %
Formamid	25 %	40 %	-
0.25 M EDTA	-	-	5 mM

For the preparation of hybridisation buffers, Rotipuran water was used.

5 M NaCl-solution

292.2 g NaCl were dissolved in 1 L of Rotipuran water. The solution was autoclaved for 20 min at 121 °C.

Oligonucleotide probes

Table 2.4: Oligonucleotide probes used in the present study

Probe	Sequence	Specific for	Reference
EUB338	5'-GCT GCC TCC CGT AGG AGT-3'	Eubacteria	Ammann et al., 1990
NONEUB338	5'-CGA CGG AGG GCA TCC TCA-3'	negative control	Wallner et al., 1993
Psae16s 182	5'-CCA CTT TCT CCC TCA GGA CG-3'	*P. aeruginosa*	Wellinghausen et al., 2005
LEGPNE1	5'-ATC TGA CCG TCC CAG GTT- 3'	*L. pneumophila*	Grimm et al., 1998
LEG705	5'-CTG GTG TTC CTT CCG ATC- 3'	*Legionella* spp.	Manz et al., 1995
EUK516	5'-ACC AGA CTT GCC CTC C-3'	Eukarya	Ammann et al., 1990
HART498	5'-TCG CGG AGAG GGT GTC GGT-3'	*Hartmanella* spp.	Grimm et al., 2001
GSP	5'-TTC ACG GTA AAC GAT CTG GGC C-3'	*Acanthamoeba* spp.	Stothard et al., 1999

All oligonucleotide probes were Cy3-labelled at the five prime end and HPLC cleaned. Probes were obtained as lyophilisates from Eurofins MWG Operon (Ebersbach, Germany).

Oligonucleotide probe solutions

Lyophilisates were dissolved in water for molecular biology (Roth) to a final concentration of 1 µg/µL. Then stock solutions of the specific probes were prepared by diluting the dissolved lyophilisates with water for molecular biology to a final concentration of 50 µg/µL.
For preparation of the working solutions stock solutions were diluted 1:10 in the appropriate hybridisation buffer.

10 % (w/v) SDS

100.00 g SDS (sodium dodecyl sulfate; Riedel de Haen) were dissolved in Rotipuran water and the solution was filter sterilized (0.2 µm).

1 M Tris-buffer pH 7.6/pH 8.0

121.14 g Tris(hydroxymethyl)-aminomethane (Roth) were dissolved in 1 L of Rotipuran water and the pH was adjusted to pH 7.6 or pH 8.0 ± 0.2 with 7 M HCl. The solution was autoclaved for 20 min at 121 °C.

Washing buffers

Table 2.5: Washing buffers for oligonucleotide probes targeting bacterial 16S rRNA

	EUB338 / NONEUB338	Psae 16S-182	LEG705	LEGPNE1
1 M Tris pH 7.6	20 mM	-	20 mM	20 mM
1 M Tris pH 8.0	-	20 mM	-	-
5 M NaCl	225 mM	56 mM	225 mM	160 mM
0,25 M EDTA	-	5 mM	-	5 mM
10% SDS	0.01 %	0.01 %	0.01 %	0.01 %

Table 2.6: Washing buffers for oligonucleotide probes targeting eucaryotic 18S rRNA

	EUK516	HART498	GSP
1 M Tris pH 7.6	20 mM	20 mM	4 mM
5 M NaCl	160 mM	56 mM	30 mM
0,25 M EDTA	5 mM	5 mM	1 mM
10% SDS	0.01 %	0,01 %	0.01%

For the preparation of washing buffers, Rotipuran water was used.

2.3.5. Buffers and solutions for pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) was performed for the characterisation of *P. aeruginosa* and *L. pneumophila* isolates.

1.2 % Agarose in TBE/Thiourea solution

For one gel (140 mm x 202 mm) used for *P. aeruginosa* PFGE, 1.8 g agarose (Pulsed Field Certified, Bio-Rad) were dissolved in 150 mL 0.5x TBE/Thiourea solution and stored at 55 °C.

1.2 % Agarose in TBE

For one gel (140 mm x 127 mm) used for *L. pneumophila* PFGE, 1.2 g agarose (Pulsed Field Certified, Bio-Rad) were dissolved in 100 mL 0.5x TBE/Thiourea solution and stored at 55 °C.

2 % Agarose solution in SE solution

0.5 g agarose (Certified™ low melt; Bio-Rad) were dissolved in 25 mL SE solution and stored at 50 °C.

ES solution

20.81 g EDTA (final concentration 0.5 M) and 0.5 g N-laurylsarcosine (final concentration 0.5 %; for *P. aeruginosa* PFGE) or 1.0 g N-laurylsarcosine (final concentration 1.0 %; for *L. pneumophila* PFGE) were dissolved in 100 mL deionised water. The pH was adjusted to 7.5 (for *L. pneumophila* PFGE) or 8.0 (for *P. aeruginosa* PFGE) with 25 % HCl and the solution was filter-sterilized (0.2 µm).

Lambda Ladder PFG Marker

50 µg/mL, 50 gel lanes (New England BioLabs, N0340S).

NE buffer 2 10x

Commercial concentrate (New England Biolabs, B7002S).

NE buffer 2 1x

2 mL 10x NE buffer 2 were diluted in 18 mL of deionised water and stored on ice. Composition of 1x NE buffer 2: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol / pH 7.9 at 25 °C.

Protease inhibitor cocktail (PIC)

Commercially available lyophilized powder for use with bacterial cell extracts, (SIGMA, P8465).

Proteinase K solution 20 mg/mL

Commercial ready-to-use solution (New England BioLabs, P8102S).

Restriction buffer

For 10 agarose plugs 3.0 mL 1x NE buffer 2, 30 µL bovine serum albumin (BSA; 100x, 10 mg/mL, New England BioLabs) and 20 µL of the respective restriction enzyme were mixed. The buffer was stored on ice.

Restriction enzymes

SfiI, 20000 U/mL (New England BioLabs, R0123L)
SpeI, 10000 U/mL (New England BioLabs, R0133L)

SE solution

4.383 g NaCl (final concentration 75 mM) and 10.405 g EDTA (final concentration 25 mM) were dissolved in 1 L deionised water. The solution was adjusted to pH 7.5 or 8.0 with 1 M HCl and autoclaved for 20 min at 121 °C.

TBE (Tris/borate/EDTA) buffer 10x

Commercial concentrate (Roth, 3061.2).
Composition: 1.0 M Tris-Borat (pH 8.3), 20.0 mM EDTA.

TBE/thiourea buffer

100 mL 10x TBE buffer (final concentration 0.5x; Roth) were mixed with 1 mL 100 mM thiourea solution and 1.9 L deionised water. The buffer was stored at 4 °C.

Thiourea solution (100 mM)

0.3806 g thiourea (Merck) were dissolved in 50 mL deionised water and autoclaved for 20 min at 121 °C.

TE (Tris/EDTA) buffer

1.2114 g Tris (final concentration 10 mM) and 0.4162 g EDTA (final concentration 1 mM) were dissolved in 1 L deionised water. The solution was adjusted to pH 7.5 or 8.0 with 1 M HCl and autoclaved at for 20 min 121 °C.

2.3.6. Buffers and solutions for denaturing gradient gel electrophoresis

7,5 % acrylamide solution (0 % denaturation)

Composition in mL/100 mL: 40 % acrylamide 18.8, 50x TAE buffer 2.0, Rotipuran water 79.2.
The components were mixed and stored at room temperature in the dark.

10 % Ammoniumpersulfate (APS) solution

0,1 g APS were dissolved in 1 mL Rotipuran water.
The solution was freshly prepared prior to use.

100 % Denaturing solution in 7,5 % acrylamide

Composition/100 mL: 40 % acrylamide 18.8 mL, 50 x TAE-Puffer 2.0 mL, formamide 40.0 mL, urea 42 g, Rotipuran water ad 100 mL.

The components were dissolved in Rotipuran water at 50 °C, cooled to room temperature and filled to 100 mL with Rotipuran water. The solution was at room temperature in the dark for 4 weeks.

Developing solution

For staining of one polyacrylamide gel: 30 g Na_2CO_3, 0.3 mL formamide (37 %), 2 mL $Na_2S_2O_3$ x 5 H_2O solution (1 mg/mL in deionised water), deionised water ad. 500 mL.

Fixing solution

For staining of one polyacrylamide gel: 250 mL methanol (chemically pure), 60 mL acetic acid, 0.3 mL formamide (37 %), deionised water ad. 500 mL.

Impregnation solution

For staining of one polyacrylamide gel: 1 g $AgNO_3$, 0.45 mL formamide (37 %), deionised water ad. 500 mL.

Pretreatment solution

For staining of one polyacrylamide gel 0.1 g $Na_2S_2O_3 \times 5\ H_2O$ were dissolved in 500 mL deionised water.

Stopping solution

For staining of one polyacrylamide gel: 250 mL methanol (chemically pure), 60 mL acetic acid, deionised water ad 500 mL.

2.4. Commercially available kits

api® 20 E (bioMérieux, 20 100)
- API Suspension Medium (bioMérieux, 20 150)
- TDA (bioMérieux, 70 402)
- JAMES (bioMérieux, 70 542)
- VP 1 + VP 2 (bioMérieux, 70 422)
- NIT 1 + NIT 2(bioMérieux, 70 442)
- Zn reagent (bioMérieux, 70 380)
- Mineral oil (bioMérieux, 70 100)

api® 20 NE (bioMérieux, 20 050)
- API NaCL 0.85 % Medium (bioMérieux, 20 070)
- JAMES (bioMérieux, 70 542)
- NIT 1 + NIT 2 (bioMérieux, 70 442)
- Zn reagent (bioMérieux, 70 380)
- Mineral oil (bioMérieux, 70 100)

Biolog GN2 MicroPlate (BIOLOG)
- GN/GPInoculating Fluid (BIOLOG)

Colilert-18 Quanti-Tray®/2000 system (IDEXX)
DNeasy® Blood & Tissue Kit (QIAGEN, 69504)
DNeasy® Plant Mini Kit (QIAGEN, 69104)
Legionella Latex Test (OXOID, DR0800M)
LIVE/DEAD® Bacterial Viability Kit (BacLight™) (Molecular Probes, L7012)
PerfectPrep® Gel Cleanup Kit (Eppendorf, 955 152000)
Quant-iT™ PicoGreen® dsDNA Reagent Kit (Invitrogen, P7589)

2.5. Chemicals

4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma)
40 % Acrylamide/Bis Solution 37.5:1 (Bio-Rad, 161-0148)
Agarose (Certified™ low melt; Bio-Rad, 161-3111)
Agarose (Pulsed Field Certified; Bio-Rad, 162-0137)
Ammonium Persulfate (Bio-Rad, 161-0700)
Bovine serum albumin (BSA), 100x, 10 mg/mL (New England BioLabs, B9001S)
Copper(II)sulfate pentahydrate ($CuSO_4$ x 5 H_2O) (Acros Organics)
Dimethyl sulfoxide (DMSO; Baker)
Ethanol Rotipuran (Roth)
Formaldehyde solution, ~ 36 % in H_2O (Fluka, 47630)
Formamide deionised, ≥ 99.5%, p.a (Roth, P040.1)
Glycerol ($C_3H_8O_3$) (KMF Laborchemie Handels GmbH)
L-cysteine (Fluka; Ultra ≥ 99.0 % RT)
Loading Dye TriTrack (Fermentas)
N,N,N',N'-Tetramethylethylenediamine (TEMED; Bio-Rad, 161-0800)
Paraformaldehyde (Merck, 1.04005)
Rotipuran Water (Roth, p.a., ACS)
SDS (Sodium Dodecyl sulfate; Riedel de Haen)
Silver nitrate GR for analysis (Merck, 1.01512)
Sodium diethyldithiocarbamate trihydrate (DDTC; Fluka)
Thiourea GR for analysis (Merck, 1.07979)
Trypan blue ($C_{34}H_{24}N_6Na_4O_{14}S_4$, Roth)
Urea for microbiology (SIGMA, 51456)
Water for Molecular Biology (Roth, DPEC treated)

2.6. Domestic plumbing materials

As a substratum for growing drinking water biofilms, materials that are commonly employed in domestic plumbing systems in Germany have been chosen (Table 2.7). All materials were utilised in the form of 26 mm x 76 mm coupons In Germany, elastomers and plastomers to be used for drinking water distribution are tested by the German Gas and Water Association (DVGW) and by the German Commission for drinking water (KTW) and categorized according to their quality. The evaluation of the microbial quality of materials in contact with drinking water is performed according to technical rule W 270 of the DVGW. Chemical and aesthetical suitability of plastics and elastomers in contact with drinking water are assessed according to the guidelines of the Federal Environmental Agency (UBA, 2008) or the recommendations of the German commission for drinking water (KTW; Anonymous, 1985), respectively. Metallic materials have to comply with the requirements of DIN 50930-6 (2001) and are tested according to DIN 50931-1 (1999). In this study, different qualities of elastomers and plastomers and copper as a metal reference were investigated.

Table 2.7: Domestic plumbing materials used in the present study

Name	Application	KTW category	Fulfilling DVGW W 270	Release of TOC[1)] [mg TOC m^{-2} d^{-1}]	Slime formation [mL / 800 cm^{-2}]
EPDM 1 Ethylene-propylene-diene-monomer	connection of faucets and gadgets for visible and accessible installations (group I) until 31.12.06, connection of washing machines, dish washers and drum dryers (group II)	C	yes	≤15	< 0.1
EPDM 2 Ethylene-propylene-diene-monomer	connection of faucets and gadgets until 1995	-	no	>125	> 0.1
PE-Xb Silane cross-linked polyethylene	Domestic plumbing systems	A[2)]	yes	≤2.5	< 0.1
PE-Xc Electron ray cross-linked polyethylene	Domestic plumbing systems	A[2)]	yes	≤2.5	< 0.1
Copper	Domestic plumbing systems	-[3)]	-[3)]	-	-

[1)] TOC – total organic carbon
[2)] Plastomers comply with the German „Guideline for the hygienic evaluation of organic materials in contact with drinking water“ („Leitlinie zur hygienischen Beurteilung von organischen Materialien im Kontakt mit Trinkwasser (KTW-Leitlinie)"; UBA, 2008)
[3)] Not required for metals.

In order to investigate the influence of material alterations on biofilm formation, all materials were used in an untreated and aged form (Table 2.8). For ageing, materials were treated either with 5 ppm sodium hypochlorite (NaOCl) at 40 ° C and 3 bar for 2 weeks or with 3 ppm chlorine dioxide (ClO_2) at 40 °C and 4 bar for 4 weeks. Disinfectant-treated materials were kindly supplied by Dr. G. Schaule, IWW water center, Mülheim an der Ruhr, Germany. Copper was aged by exposure in a drinking water distribution system for at least 6 months (Wingender et al., 2004).

Table 2.8: Ageing of domestic plumbing materials

	NaOCl-treated	ClO_2-treated	Aged by exposure to drinking water
EPDM 1	+	+	-
EPDM 2	-	-	-
PE-Xb	+	+	-
PE-Xc	+	-	-
Copper	-	-	+

2.7. Equipment

Analytical scales, BP 210 S, max. 210 g, d=0.1 mg, (Sartorius)

Analytical scales, BP1200, max. 1200 g, d=0.01 g, (Sartorius)

Bactident® Oxidase Test Strips (Merck, 1.13300)

Black polycarbonate membrane filters, pore size 0.2 µm (Millipore)

Cell density meter model 40 (Fisher Scientific)

Cooling centrifuge, Sorvall® RC26PLUS (Sorvall)

DCode™ Universal Mutation Detection System (Bio-Rad)

Desk centrifuge 5415 D (Eppendorf)

Disposable Bottle Top Filter OR, non-fiber releasing membrane, surfactant free cellulose acetate pore size 0.20 µm (NALGENE®)

Disposable cuvettes PMMA semi-micro, 12.5 × 12.5 × 45 mm (BRAND, 7591 15)

Disposable Plug Mold for the preparation of DNA-imbedded agarose plugs (Bio-Rad, 170-3713)

Electrophoresis chamber HE 33 mini horizontal Submarine unit (Amersham Bioscience) with power supply Power Pack P 25 (Biometra)

Epifluorescence microscope: Leitz Laborlux S (Leitz)
- Objectives: PL Fluotar 100x / 1.32 oil
- Eye-piece: 2x Periplan 10x / 18
- UV-unit HBO 50

Fluorimeter SFM 25 (BIO-TEK KONTRON Instrumente)

Fuchs-Rosenthal counting-chamber (Marienfeld)

Gel documentation system Universal Hood II (Bio-Rad)

GelBond® PAG film for polyacrylamide gels, 203 × 260 mm (GE Healthcare, 80-1129-37)

Gradient Delivery System Model 475 (Bio-Rad)

Hybridisation oven (Thermo electron cooperation)

Imaging Densitometer GS-710

Incubator (20 °C): ICE 400-800 (MEMMERT GmbH+Co. KG)

Incubator (30 °C): Kelvitron® t (Heraeus)

Incubator (36 °C): Kelvitron® t (Heraeus)

Light microscope; Leica DM LS, (Leica Microsystems)

Light table Color Control 5000 (BIOTEC-Fischer)

Mastercycler ep gradient S (Eppendorf)

Membrane filters black, MicroPlus-31 ST, 0.45 µm pore size, ∅ 50 mm, mixed cellulose ester (Whatman)

Membrane filters white, 0.45 µm pore size, ∅ 47 mm, mixed cellulose ester (Pall)

Neubauer improved counting chamber (DigitalBio C-Chip)

pH meter WTW (ph 549 ELP) MultiCal®

Phase contrast microscope; Leica DM LS, (Leica Microsystems)

Pulsed field gel electrophoresis CHEF DR® III System (Bio-Rad)
- Electrophoresis Cell (Bio-Rad)
- Variable speed pump (Bio-Rad)
- Cooling module (Bio-Rad)

Quanti-Tray® Sealer Model 2X (IDEXX)

Reaction chambers for Fluorescence in situ hybridisation (Vermicon)

Rocking Platform (BioMetra)

Shaking water bath: (room temperature, 36 °C, 44 °C) GFL 1092 (Gesellschaft für Labor-Technik mbH)

Six-fold stainless-steel vacuum filtration apparatus (Millipore)

Syringe filters, Filtropur S plus 0.2, pore size 0.2 µm (Sarstedt)

Thermo Scientific Diagnostic slides epoxy-coated 8-well 6 mm (Menzel)

Thermomixer comfort (Eppendorf)

Thoma counting chamber (Optik Labor)

Three-fold stainless-steel membrane filtration apparatus (Sartorius)

Tissue culture flask 75 cm^2, polystyrene, sterile PE vented cap, non-pyrogenic according to FDA LAL guidelines, acceptance level < 0.06 EU/mL, non-cytotoxic according to ISO 10993-5 (Sarstedt)

Tissue Culture Plate 6-Well Flat Bottom with lid (Sarstedt)

Tygon® tube, $\varnothing_{in}$ 6.4 mm, $\varnothing_{out}$ 9.6 mm, wall 1.6 mm, (Saint-Gobain Performance Plastics, T3608-23)

UV-light bulb, 254 nm (Faust)

2.8. PC-Software

Chromas Version 2.01 (Technelysium Pty Ltd, Australia)

APILAB Plus V 3.3.3 (bioMérieux)

Biolog MicroLog™ 1, release 4.20 (BIOLOG)

Chapter 3
Methods

3.1. Characterisation of bacterial test strains

For characterising the test strains, pure cultures of *Pseudomonas aeruginosa* AdS and *Enterobacter nimipressuralis* Klon A were grown on nutrient agar at 36 °C for 24 h. *Legionella pneumophila* AdS was cultured on BCYEα agar at 36 °C for 72 h.

3.1.1. Biochemical characterisation

P. aeruginosa and *E. nimipressuralis* were identified using the API 20 NE system and the API 20 E system (bioMérieux), respectively. Test strips were inoculated according to the manufacturer's instruction and incubated at 30 °C for 24 h (API 20 NE) or at 36 °C for 18 to 24 h (API 20 E). Identification was performed using the software APILAB Plus V 3.3.3.

An additional identification of the bacteria was performed using GN MicroPlates™ (BIOLOG). Bacteria were pre-cultured on Biolog Universal Growth (BUG) medium (Oxoid) at 36 °C for 24 h and cell material was suspended in Inoculating Fluid (BIOLOG) to a turbidity according to the turbidity standard G-ENT 63 % (BIOLOG). The bacterial suspension was transferred to a GN2 MicroPlate™ using a multichannel pipette. Plates were incubated at 36 °C for 16 h to 24 h and analysed visually. Purple coloured wells were counted positive. Identification was performed using the software MicroLog™ 1, release 4.20 (BIOLOG).

For *L. pneumophila*, a biochemical profile of substrates utilised was recorded. *L. pneumophila* was pre-cultured on BCYEα agar for 72 h at 36 °C and suspension, inoculation and reading of the plates and identification were performed as

described for *P. aeruginosa* and *E. nimipressuralis* using GN MicroPlates™ and the software MicroLog™ 1, release 4.20 (BIOLOG).

3.1.2. 16S rDNA sequence analysis

3.1.2.1. Isolation of DNA from pure cultures

Cell material from 24 h or 72 h old pre-cultures was suspended in 0.9 % NaCl solution to a concentration of approximately 1.2×10^9 cells/mL according to McFarland standard 4. Cells were harvested by centrifugation (10 min, 5000 x g). For DNA isolation the QIAGEN DNeasy® Blood & Tissue Kit was used following the protocol for DNA isolation from Gram-negative bacteria. For the final elution of the DNA from the spin column 2 x 100 µL elution buffer were used. The DNA solution was stored at -20°C.

3.1.2.2. Amplification of 16S rDNA fragments

For the amplification of bacterial 16S rDNA gene fragments the polymerase chain reaction (PCR) was performed using primer fd1 and rp2 (Weisburg et al., 1991; see Table 2.6).

For each reaction the following components were pipetted into a 0.2 mL PCR tube:

Table 3.1: Components and concentrations of the PCR reaction used for bacterial 16S rDNA amplification

Constituent	Final concentration	Volume in 50 µL
Primer fd1	50 pM	0.5 µL
Primer rp2	50 pM	0.5 µL
MasterMix 2.5x		20 µL
Taq DNA Polymerase	1.25 U	
KCl	50 mM	
Tris-HCl pH 8.3	30 mM	
$Mg(OAc)_2$	1.5 mM (Mg^{2+})	
Igepal®-CA630	0.1%	
dNTP (each)	200 µM	
Stabilizers		
DNA solution (3.1.2.1.)		1 µL
H_2O (molecular biology grade)		28 µL
Total Volume		50 µL

A negative control without DNA template was included in all PCR reactions.

The PCR program parameters were set as follows:

Initial Denaturation	94 °C	120 s
30 Cycles:		
Denaturation	94 °C	60 s
Annealing	59 °C	60 s
Elongation	72 °C	90 s
Final Elongation	72 °C	5 min
Cooling	4 °C	

The presence and size of the amplicons were analysed using agarose gel electrophoresis (see 3.7.5.)

3.1.2.3. Extraction of PCR-products from agarose gels

DNA bands were cut from the agarose gel with a sterile scalpel and transferred to sterile reaction tubes. In order to isolate the DNA from the gel matrix and to remove excess PCR components, the Eppendorf PerfectPrep® Gel Cleanup Kit was used following the manufacturer's instruction. The DNA was eluted with 30 µL molecular biology grade water and stored at -20°C for sequencing (3.1.2.4).

3.1.2.4. DNA sequencing and comparative sequence analysis

Sequencing of cleaned PCR products was carried out by Sequence Laboratories Göttingen GmbH (Göttingen, Germany). 10 µL of DNA with a concentration of 80 ng/µl and 10 µL of the forward primer fd1 with a concentration of 10 pM/µL were sent.
The sequence data was edited and processed using the software Chromas Version 2.01 (Technelysium Pty Ltd, Australia). Microbial species identification was performed using the nucleotide-nucleotide Blast service available at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), where the nucleotide sequences obtained were compared to sequence databases (Altschul et al., 1997). The sequence with the most significant statistical match was considered. According to Angenent et al. (2005) a minimum of 95% sequence homology was set to signify relation on genus level and a sequence match of ≥ 97% to imply species-level relation.

3.2. Sampling of drinking water

The sampling of water was performed according to the standard ISO 19458:2006 (2006). For sampling, sterile (180 °C, 4 h) 1 L glass bottles were used.

Table 3.2 gives an overview of the different drinking waters collected for the preparation of bacteria for the inoculation of drinking water biofilms (3.3.2.) and for copper sensitivity testing (3.4.).

Table 3.2: Overview of drinking water samples

Type of water	Sampling point	Origin of water
Finished water	tap installed after an activated carbon filter	waterworks Styrum-Ost, surface water treatment plant (RWW Rheinisch-Westfälische Wasserwerksgesellschaft)
Drinking water	laboratory tap at the IWW Water Centre (Mülheim/Ruhr), base floor, close proximity to the water meter	mixture of drinking water supplied by the waterworks Styrum-Ost and Styrum-West (RWW Rheinisch-Westfälische Wasserwerksgesellschaft)
Drinking water	laboratory tap at the IWW Water Centre in Mülheim/Ruhr, 1st floor	mixture of drinking water supplied by the waterworks Styrum-Ost and Styrum-West (RWW Rheinisch-Westfälische Wasserwerksgesellschaft)
Drinking water	laboratory tap of the Biofilm Centre in Duisburg (University of Duisburg-Essen)	Stadtwerke Duisburg

Drinking water samples were collected after flushing for 30 min; finished water samples were taken from a continuously running sampling tap.

3.3. Incorporation of hygienically relevant bacteria into drinking water biofilms

3.3.1. Growth of drinking water biofilms

Drinking water biofilms were grown on coupons of domestic plumbing materials (2.7., Fig. 3.1c). Coupons were treated with 70 % (v/v) ethanol for 10 min, washed in deionised water and air-dried for 24 h before use. Up to 32 coupons of the plumbing materials fixed to a stainless steel bar with stainless steel clamps were introduced vertically into in a 200-L stainless steel tank (Fig. 3.1a) connected to a cold water laboratory tap at the IWW Water Centre in Mülheim an der Ruhr. The tank was perfused with drinking water at a flow rate of 20 L/h with the water flowing from bottom to top (complete change of the water volume inside the tank within 10 h). The concentration of assimilable organic carbon in the drinking water was approximately 6 µg C/L.

Alternatively, up to 8 coupons were vertically placed in a 100-mL stainless steel flow-through reactor (Fig. 3.1b) connected to a tap in the same laboratory. Between the coupons there was a distance of 3 mm. The flow rate in the 100-mL stainless steel flow-through reactors was 3 L/h leading to a complete turnover of the water volume inside the reactor within approximately 100 s. For the connection of the stainless steel tank and the flow-through reactors to the tap Tygon® tubing was used.

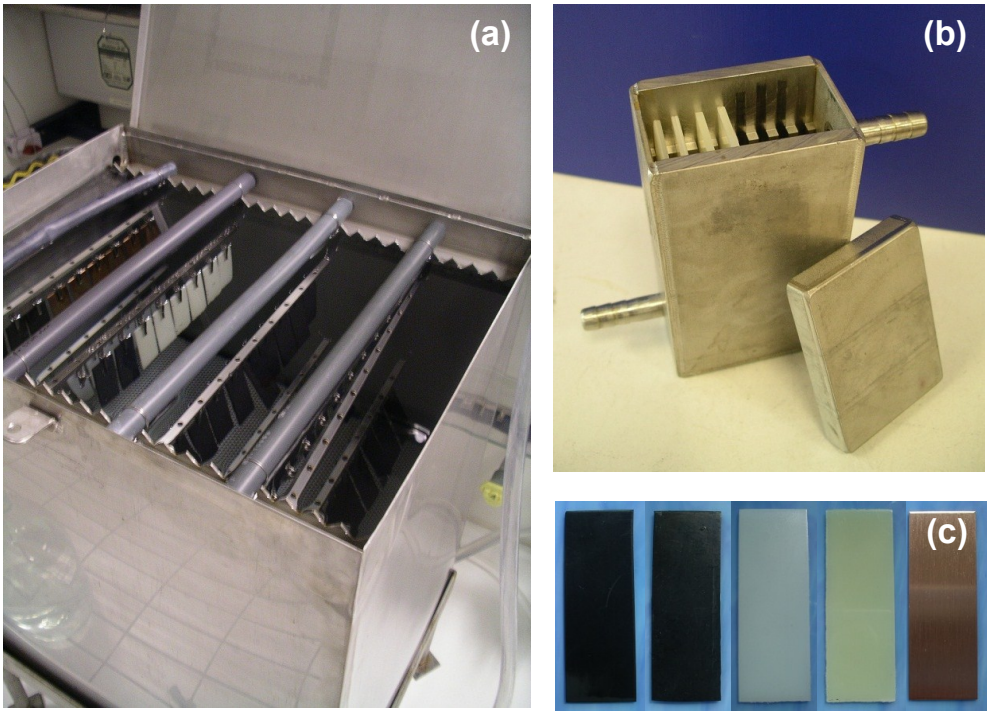

Figure 3.1: Overview of equipment used for biofilm growth. (a) 200-L stainless steel tank with coupons of different domestic plumbing materials, (b) 100-mL stainless steel flow-through reactor with coupons of PE-Xb, (c) coupons (76 mm x 26 mm) of domestic plumbing materials: EPDM 1, EPDM 2, PE-Xb, PE-Xc, copper (left to right; see Tab. 2.7 for further specification)

3.3.2. Preparation of the inoculum

P. aeruginosa and *E. nimipressuralis* were pre-grown on nutrient agar at 36 °C for 24 h. Liquid cultures were obtained by transferring a single colony from the agar plate into 20 mL LB medium in a 100-mL Erlenmeyer flask and incubating in a water bath at room temperature (approximately 23 °C) with agitation (180 rpm) for 24 h. *L. pneumophila* was pre-grown on BCYEα agar at 36 °C for 72 h. A single colony was transferred into 20 mL YEB medium in a 100-mL Erlenmeyer flask and incubated in a water bath at 36 °C and 180 rpm for 24 h. Bacteria from the liquid cultures were harvested by centrifugation (15 min, 1912 x g, 10 °C), washed twice in 20 mL of filter-sterilized tap water from a laboratory tap at the IWW Water Centre in Mülheim an der Ruhr and suspended in 200 mL of filter-sterilized tap water to a concentra-

tion of approximately 1 x 10^6 cells/mL. In order to achieve the appropriate bacterial concentration, cells were counted using a Thoma counting chamber and phase contrast microscopy. The bacterial suspensions were incubated statically at 20 °C (*P. aeruginosa* and *E. nimipressuralis*) or 30 °C (*L. pneumophila*) for 24 h. In order to inoculate drinking water biofilms with a mixture of all three organisms, suspensions of *P. aeruginosa*, *E. nimipressuralis* and *L. pneumophila* with a concentration of 3 x 10^6 cells/mL were prepared as described above and combined after incubation at 20 °C or 30 °C resulting in an inoculum with a concentration of 1 x 10^6 cells/mL of each organism.

3.3.3. Inoculation of drinking water biofilms

Coupons with 14-day-old drinking water biofilms grown in the 200-L stainless steel tank were transferred to 100-mL stainless steel flow-through reactors filled with 80 mL of the inoculum. When biofilms were grown in 100-mL flow-through reactors, the flow-through was stopped and 100 mL of the inoculum were injected at the reactor inlet using a syringe and a sterile injection needle. After static incubation at room temperature for 24 h, the reactors were connected to a cold water laboratory tap and continuously perfused with drinking water for 4 weeks.

3.3.4. Sampling of drinking water biofilms and water phase

For biofilm analysis, the biomass from both sides of two coupons was scraped off, using a sterile rubber scraper and suspended into 20 mL deionised water. The rubber scraper was treated with 99 % ethanol and washed with sterile deionised water twice before use. For biofilm dispersion, the suspensions were vortexed for 2 min.

The water phase was sampled by collecting reactor effluent in sterile 250-mL glass flasks. Serial dilutions of biofilm suspension and water sample were prepared in deionised water.

Biofilms and water phase were sampled before inoculation and 1, 7, 14, 21 and 28 days after inoculation.

During the whole course of the experiment, room temperature, water temperature in the stainless steel tank, in 100-mL flow-through reactors and in the feed water was monitored. The pH of the feeding water was measured at regular intervals.

3.4. Testing of the copper sensitivity of *P. aeruginosa*

3.4.1. Preparation of bacterial suspension

P. aeruginosa AdS was cultured, harvested, washed and resuspended as described above (3.3.2.). Depending on the experiments the washing and resuspension procedures were done with filter sterilized deionised water, drinking water or finished water from the waterworks. A bacterial suspension with a density of 2 x 10^6 cells/mL was prepared.

3.4.2. Exposure of bacteria to copper

To prepare test suspensions, 20 mL bacterial suspension (see 3.4.1) was mixed with either 20 mL deionised water without copper (control), with deionised water supplemented with copper sulphate ($CuSO_4$ x 5 H_2O; final copper concentration 0.001 µM – 1 mM), with drinking water or with finished water in 100-mL Erlenmeyer flasks. The final cell density in the test suspensions was 1 x 10^6 cells/mL.

Furthermore, experiments using the copper chelator sodium diethyldithiocarbamate (DDTC; Harrison et al., 2005) were performed by adding DDTC solution (final concentration 100 µM) to water samples containing copper.

These test suspensions were incubated statically for 24 h at 20 °C. For analysing the effect of copper on the viability and growth of *P. aeruginosa* AdS several tests were performed: total cell count, colony count and viability tests using LIVE/DEAD® kit. As a reference, bacterial test suspensions in deionised water without copper were tested after 0 h and 24 h incubation time.

In order to assess the effect of copper on *P. aeruginosa* over a longer period of time, two bacterial test suspensions in filter sterilized deionised supplemented with copper sulphate were prepared as described above and statically incubated at 20 °C for a maximum of 14 d. At various times (0 h, 24 h, 5 d, 7 d, 14 d), aliquots of 10 mL were removed and the total number of cells (DAPI), the colony numbers (spread plate count, nutrient agar, 36 °C) and viable cells (LIVE/DEAD) were determined. As a reference, a bacterial test suspensions in deionised water without copper was incubated and tested after 0 h, 24 h, 5 d, 7 d and 14 d incubation time.

3.4.3. Resuscitation of copper-stressed planktonic bacteria

A bacterial test suspension of *P. aeruginosa* AdS (cell density ≈ 2 x 10^6 cells/ml) was prepared in deionised water containing 1 µM copper (see 3.4.2) and was incubated for 24 h at 20 °C. The bacterial suspension was diluted (1:1) in deionised water supplemented with DDTC (final concentration 100 µM) and incubated for a maximum of 7 d at 20 °C. At various times (2 h, 24 h, 4 d, 7 d), aliquots of 3 mL were removed to

determine the total cell count (DAPI method) and the colony numbers using the spread plate count method on nutrient agar (36 °C, 2 d) and R2A (20 °C, 7d).

3.4.4. Resuscitation of copper-stressed biofilm bacteria

Drinking water biofilms on coupons of EPDM 1 (2.7) were cultured and inoculated with a suspension of *P. aeruginosa, L. pneumophila* and *E. nimipressuralis* as described above (see 3.3.1. – 3.3.3.). Seven days after inoculation, the biomass of both sides of 8 coupons was scraped off (3.3.4.) and suspended in 80 mL deionised water. For biofilm dispersion, the suspension was vortexed for 2 min. The biofilm suspension was diluted (1:1) in deionised water with or without DDTC (final concentration 100 µM) and incubated statically at 20 °C. After seven days of incubation, total cell count (DAPI method) and colony count (HPC on R2A) as well as the number of bacteria with intact ribosomal RNA using FISH (3.7.1.1) with probe EUB338 were determined. *P. aeruginosa* was quantified using the spread plate method on CN agar and FISH (3.7.1.1). As a control, the same test was performed with a 16-day-old drinking water biofilm not spiked with *P. aeruginosa, L. pneumophila* and *E. nimipressuralis.*

3.4.5. Copper analysis of water and biofilm samples

The copper concentrations of biofilms and water samples were performed at the IWW Water Centre, Mülheim an der Ruhr, according to DIN EN ISO 11885 (2007) using ICP-OES multi-element method.

3.5. Co-Cultivation of *P. aeruginosa*, *L. pneumophila* and *Acanthamoeba* castellanii

P. aeruginosa, L. pneumophila and *Acanthamoeba castellanii* were incubated in Page's amoeba saline (PAS) or proteose peptone yeast extract glucose (PYG) medium either in pure culture, as combinations of two organisms or as a combination of all three organisms. *P. aeruginosa* was pre-cultured on nutrient agar at 36 °C for 24 h. Cell material from the agar plate was suspended in the respective medium or saline and the cell density was adjusted to a final concentration of 1 x 10^6 cells/mL in the main culture. *L. pneumophila* was pre-cultured on BCYEα agar at 36 °C for 72 h, suspended in PYG or PAS and the cell density was adjusted to a final concentration of 1 x 10^6 cells/mL. *A. castellanii* was pre-cultured in 30 mL to 60 mL PYG in a Petri dish (20 cm Ø) at 30 °C for 8-10 days. To harvest *A. castellanii*, the amoebal cells attached to the bottom of the Petri dish were gently scraped off with a sterile cell scraper and the suspension was centrifuged at 500 x g for 8 min and washed in PYG or PAS. The cell density of *A. castellanii* was adjusted to a concentration of 1 x 10^5 cells/mL.

All cultures were prepared in six well plates with a total volume of 12 mL or 9 mL of microbial suspension in each well. The six well plates were incubated statically at 30 °C. Sampling was performed after 0 h, 24 h, 48 h, 72 h and 7 d for the co-cultivations in YEB, after 0 h, 24 h, and 7 d for co-cultivation in PYG, and after 0 h, 24 h, 48 h, and 72 h for co-cultivation in PAS. Prior to each sampling, suspensions containing *A. castellanii* were gently treated with a cell scraper to remove the amoeba adhered to the bottom of the well. As controls PAS, YEB or PYG without any of the microorganisms were incubated under the same conditions as the microbial suspensions.

3.6. Microbiological and cytological methods

3.6.1. Determination of total cell count

1 mL of DAPI solution (25 µg/mL) in 2 % (v/v) formaldehyde was added to 4 mL of diluted or undiluted biofilm suspension, water sample or microbial test suspension. After incubation at room temperature for 20 min in the dark the solution was filtered through a black polycarbonate membrane filter (Millipore, 0.2 µm pore size) using a six-fold vacuum filtration apparatus (Millipore). The filter was stored at 4 °C in the dark until enumeration.

The cells were counted using an epifluorescence microscope (Leitz) at 1000-fold magnification with immersion oil. 20 randomly selected fields of view were examined for each filter with the help of a counting grid (100 µm x 100 µm). Results are given as cells/mL.

3.6.2. Determination of heterotrophic plate count (HPC)

To determine the HPC, decimal dilutions of biofilm suspensions or water samples were prepared in sterile particle-free deionised water and plated in triplicate on R2A agar (Reasoner and Geldreich, 1985). Colonies were enumerated after 7 d of incubation at 20 °C. Results are given as colony-forming units (cfu)/mL. Plates with colony numbers between 30 and 300 were considered for enumeration.

3.6.3. Determination of culturable *P. aeruginosa*

In incorporation experiments (3.3.) and resuscitation experiments in biofilm suspensions (3.4.5), *P. aeruginosa* was quantified according to the standard DIN EN ISO 16266 (2008). Serial dilutions of biofilm suspensions were spread-plated on CN agar in triplicate. Plates were incubated at 36 °C for 2 days. Plates with colony numbers between 30 and 300 were considered for enumeration. Results are expressed as

cfu/cm². In reactor effluent, *P. aeruginosa* was detected by filtering up to 100 mL through 47 mm mixed cellulose ester membrane filters with a pore size of 0.45 µm (Pall). Filters were transferred to CN agar and the plates were incubated at 36 °C for 2 days. Plates with colony numbers between 20 and 200 were considered for enumeration. Results are expressed as cfu/mL.
In experiments on the copper sensitivity of *P. aeruginosa* (3.4.1. – 3.4.4.) or the co-cultivation of *P. aeruginosa, L. pneumophila* and *A. castellanii* (3.5.), *P. aeruginosa* was quantified on nutrient agar by the spread plate method. Determinations were performed in triplicate. Plates were incubated at 36 °C and colonies were enumerated after 24 h and 48 h. Plates with colony numbers between 30 and 300 were considered for enumeration. Results are expressed as cfu/mL.

3.6.4. Determination of culturable *L. pneumophila*

Quantification of *L. pneumophila* was performed according to the standard ISO 11731 (1998). Biofilm suspensions or microbial test suspensions were spread-plated in triplicate on GVPC agar after acid treatment. For acid treatment, 1 mL to 2 mL of biofilm suspension or bacterial suspension was centrifuged for 10 min at 6000 x g and 4 °C. Half volume of the supernatant was discarded and replaced by an equal volume of acid buffer (pH 2.2; see 2.3.1.). The pellet was resuspended and the suspension was incubated at room temperature for 5 min. After incubation, the suspension was diluted if necessary and immediately spread-plated. Triplicate plates were incubated at 36 °C for 10 days. Plates with colony numbers between 30 and 300 were considered for enumeration. Results are expressed as cfu/cm².
Up to 100 mL of the reactor effluent were filtered through black 50 mm mixed cellulose ester membrane filters with a pore size of 0.45 µm (Whatman) and treated with 10 mL of acid buffer (pH 2.2) for 5 min. Filters were rinsed with 10 mL of sterile deionised water and placed onto GVPC agar. Plates were incubated for 10 days at 36 °C and cfu/mL were calculated. Plates with colony numbers between 20 and 200 were considered for enumeration. Results are expressed as cfu/mL.

3.6.5. Determination of *Enterobacter nimipressuralis* (coliform bacteria)

Quantification of *E. nimipressuralis* was performed using the Colilert-18 Quanti-Tray®/2000 system (IDEXX). One vial of the Colilert-18 reagent was dissolved in 100 mL of the diluted or undiluted biofilm suspension, water sample or inoculum and subsequently transferred to a Quanti-Tray®. The Quanti-Tray® was sealed and incubated at 36 °C for 19 ± 1 h. Positive (yellow colored) wells were enumerated and the number was converted to MPN/100 mL according to the manufacturer's instruction.

3.6.6. Characterisation of *P. aeruginosa* isolates

For confirmation of *P. aeruginosa* isolates from the inocula, from biofilms or from the reactor effluent, representative colonies from primary cultures on CN agar were subcultured on nutrient agar (36 °C, 24 h) and identified by typical pigment production, positive cytochrome oxidase reaction (Bactident-Oxidase, Merck) and the biochemical profiles in the API 20 NE system (bioMérieux) according to the manufacturer's instruction (see 3.1.1.). Genotyping of *P. aeruginosa* isolates was performed using pulsed field gel electrophoresis (3.7.2.).

3.6.7. Characterisation of *L. pneumophila* isolates

For confirmation of *L. pneumophila* (serogroup 1), representative colonies from GVPC agar were subcultured on nutrient agar and BCYEα agar at 36 °C for at least 3 days. Those colonies which were able to grow on BCYEα agar, but failed to grow on nutrient agar were regarded as *Legionella*. For the confirmation of *L. pneumophila* serogroup 1, a commercially available latex agglutination test kit (Oxoid) was used according to the manufacturer's instruction. Genotyping of *L. pneumophila* isolates was performed using pulsed field gel electrophoresis (3.7.2.).

3.6.8. Determination of total and viable cells using the LIVE/DEAD® kit

1.5 µl SYTO 9 and 1.5 µl propidium iodide were mixed and added to 1 mL of bacterial test suspension (see 3.4.2. and 3.4.3.). SYTO 9 was applied in the concentration originally supplied by the manufacturer and propidium iodide was diluted 1:200 in DMSO. The mixture was incubated in the dark at room temperature for 20 minutes. After incubation, the stained suspension was diluted in 4 mL sterile particle-free deionised water and filtered through a black polycarbonate filter (Millipore, pore size 0.2 µm).

The cells were counted using an epifluorescence microscope (Leitz) at 1000-fold magnification with immersion oil. 20 randomly selected fields of view were examined for each filter with the help of a counting grid (100 µm x 100 µm). The total cell count was determined by combining both the numbers of green ("viable") and red ("dead") cells and is given in cells/mL. The number of viable cells was given in percentage of green cells in relation to the total cell count.

3.6.9. Quantification of amoebae

The number of *A. castellanii* cells was determined by counting cells using a Neubauer improved counting chamber and phase contrast microscopy. Prior to each sampling, *A. castellanii*-containing microbial suspensions (3.5.) were gently treated

with a cell scraper to remove adhered *A. castellanii*. Differentiation between living and dead cells was performed by means of trypan blue vital staining which stains dead cells blue whereas living cells remain colourless. 100 µL of the microbial suspension were mixed with 100 µL of 0.04% trypan blue solution in PAS (2.3.2.) and incubated in the dark for 4 min. Examination of the stained cultures was performed using light microscopy and a Neubauer improved counting chamber.

3.7. Molecularbiological Methods

3.7.1. Fluorescence in situ hybridisation (FISH)

3.7.1.1. Bacteria

FISH of bacteria was performed, using probe EUB338 (Ammann et al., 1990), Psae16S-182 (Wellinghausen et al., 2005) and probe LEGPNE1 (Grimm et al., 1998) for the detection of eubacteria, *P. aeruginosa* and *L. pneumophila*, respectively. As a negative control a nonsense probe complementary to the sequence of probe EUB338, NONEUB338 (Wallner et al., 1993) was used.

Cells were harvested by centrifugation of up to 16 mL of the samples (biofilm suspensions, inocula, microbial suspensions) (10 min, 6000 x g, 4 °C). The supernatant was discarded and cells were resuspended in half the volume of 4 % paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2. The suspension was incubated for fixation at 4 °C for 1 h. After centrifugation (5 min, 6000 x g, 4 °C), the supernatant was discarded and the pellet was washed in an equal volume of PBS. After centrifugation (5 min, 6000 x g, 4 °C) and rejection of the supernatant, the pellet was resuspended in a mixture of equal volumes of PBS and absolute ethanol. The fixed samples were stored at -20 °C. 10 µL of fixed sample were pipetted onto epoxy-coated 8-well diagnostic slides (Thermo Scientific) and air-dried. After dehydration in 50 %, 80 % and 96 % ethanol (3 min for each step) and air-drying the samples were hybridized by adding 10 µL of hybridisation buffer (2.3.4.) containing 5 ng/µL of the respective oligonucleotide probe to the sample fixed on the 8-well diagnostic slides. Hybridization took place in a humid reaction chamber (Vermicon) at 46 °C for 90 min. In order to remove unbound probe, the 8-well diagnostic slides were then transferred into reaction chambers containing 25 mL pre-heated (46 °C) washing buffer (2.3.4.) and incubated at 46 °C for 15 min. The diagnostic slides were washed in deionised water, air-dried and bacterial cells were counterstained by adding 10 µL of DAPI (1 µg/mL) to each well and incubating for 20 min at room temperature in the dark. The slides were washed in deionised water and stored at 4 °C until enumeration. Cells were counted using an epifluorescence microscope at 1000-fold

magnification. 40 randomly selected fields of view or at least 200 cells were enumerated for each filter with the help of a counting grid (100 µm x 100 µm). The percentage of FISH-positive bacteria in relation to the number of DAPI stained cells was calculated and the concentration of FISH-positive bacteria was calculated from the total cell count determined using the DAPI method (3.6.1.).

For performing FISH of bacteria from microbial co-cultures (3.5.) cells were harvested by centrifugation (10 min, 6000 x g, 4 °C). and washed in 1 mL PBS. After centrifuging (5 min, 6000 x g, 4 °C) and decanting the supernatant, the pellets were resuspended in 1 mL PBS. 20 µl of the sample were pipetted onto epoxy-coated 8-well diagnostic slides and allowed to dry. 10 µL 4 % paraformaldehyde in PBS were pipetted onto the wells of the diagnostic slides and the cells were fixed for 1 h at room temperature in humid reaction chambers. The slides were washed with PBS and dehydration was performed by dipping the slides in 50 %, 80 % and 96 % ethanol for 3 min each. The slides were allowed to dry and hybridisation, counterstaining and microscopic examination followed as described above.

3.7.1.2. Amoebae

FISH of amoeba from drinking water biofilms and from microbial co-cultures (3.5.) was performed, using probe EUK516 (Ammann et al., 1990), probe HART498 (Grimm et al., 2001) and probe GSP (Stothard et al., 1999) for the detection of eukaryotes, *Hartmannella* spp. and *Acanthamoeba* spp., respectively.

Cells were harvested by centrifugation of up to 12 mL (8 min, 500 x g, room temperature). The supernatant was discarded, pellets were washed in an equal volume of PJ saline twice and then resuspended in a tenth of the volume of 0.05 % low melt agarose. 20 µL of the sample were pipetted onto 8-well diagnostic slides and air-dried. Fixation was done with 2 % paraformaldehyde (10 µL per well) for 1 h at room temperature in humid reaction chambers. The diagnostic slides were rinsed twice with PBS and the samples were dehydrated in 80 % ethanol for 10 s. Air-dried samples were hybridized by pipetting 20 µL of the respective hybridisation buffer (2.3.4.) containing 15 ng/µL of specific probe into the wells of the diagnostic slides. Hybridization took place in humid reaction chambers (Vermicon) at 46 °C for 120 min. In order to remove unbound probe, the 8-well diagnostic slides were then transferred into reaction chambers containing 25 mL pre-heated (46 °C) washing buffer (2.3.4.) and incubated at 46 °C for 15 min. The diagnostic slides were washed in deionised water, air-dried and cells were counterstained by adding 10 µL of DAPI (1 µg/mL) to each well and incubating for 20 min at room temperature in the dark. Diagnostic slides were washed in deionised water and stored at 4 °C until enumera-

tion. Cells were counted using an epifluorescence microscope at 1000-fold magnification. 40 randomly selected fields of view or at least 200 cells were enumerated for each filter with the help of a counting grid (100 µm x 100 µm). The percentage of FISH-positive amoeba in relation to the number of DAPI stained cells was calculated and the number of FISH-positive amoeba was calculated from the total cell count determined with the DAPI method (3.6.1.).

3.7.2. Pulsed-field gel electrophoresis (PFGE)

PFGE of *P. aeruginosa* was carried out essentially as described by Head and Yu (2004). 24-h-old *P. aeruginosa* cultures grown on nutrient agar (36 °C) were incubated in 20 mL Lenox broth (LB) in 100-mL Erlenmeyer flasks in a water bath at 36 °C and 180 rpm for 18 h. Approximately 800 µL of the bacterial suspension were transferred to 20 mL fresh LB to an optical density at a wavelength of 600 nm of 0.2. The culture was incubated at 36 °C for 2 h with agitation (180 rpm). Cells were harvested by centrifugation (10 min, 1400 x g, 4 °C). The pellet was washed in 10 mL SE solution twice and then resuspended in 2 mL SE solution.

500 µL of the bacterial suspension were mixed with 500 µL 2 % agarose solution. The mixture was pipetted in portions of 82 µL into plug molds (Bio-Rad). The plugs were allowed to harden at 4 °C for 15-30 min and subsequently incubated in 800 µL of lysis buffer (780 µL SE solution, 20 µL proteinase K solution) for 21 h at 50 °C in order to lyse the cells. The lysis buffer was removed and 800 µL of a protease inhibitor cocktail (PIC; SIGMA, see 2.3.5.) diluted 1:10 in 25 % (v/v) DMSO were added. After incubation for 2 h at 37 °C, the gel plugs were cooled to room temperature and the PIC solution was removed. Gel plugs were equilibrated in TE buffer 4 times for 30 min at room temperature. Gel plugs were stored at 4 °C in TE buffer for further processing.

For DNA hydrolysis, gel plugs were transferred into fresh 1.5 mL reaction tubes and equilibrated in 500 µL 1x NE buffer 2 on ice. Afterwards 300 µL restriction buffer (2.3.5.) were added and the plugs were first incubated for 2.5 h on ice and subsequently for 16 h at 37 °C. The agarose plugs were transferred to a 140 mm x 202 mm 1.2 % agarose gel. The gel was run in 0.5 x TBE buffer (pre-cooled at 4 °C) for 30 h at 12 °C and 5.6 V/cm and an angle of 120 °. The switch time was changed from 5 – 30 s to 5 – 70 s after 19 h. The variable speed pump was operated at 70 % speed. A marker (Lambda Ladder PFG Marker, New England BioLabs) was included in every run. The gel was stained in ethidium bromide solution (1 µg/mL) for 60 min and then destained in deionised water for 20 min. Analysis and documentation of the

agarose gel was performed using a gel documentation system (Universal Hood II, Bio-Rad).

For the confirmation of *L. pneumophila* isolates from inocula, biofilm and water phase the PFGE was performed according to the protocol of Chang et al. (2009). Cell material of 72-h-old *L. pneumophila* cultures grown on BCYEα agar was suspended in 1 mL SE solution to an optical density at 600 nm of 0.5. The suspension was washed in SE solution twice and finally the cell pellet was resuspended in 250 µL SE solution. The bacterial suspension was mixed 1:1 with 2 % agarose solution. The production of agarose plugs and cell lysis were performed in the same way as for *P. aeruginosa* isolates. DNA restriction was performed at 50 °C using the restriction enzyme SfiI. The 140 mm x 127 mm 1.2 % agarose gel was run in 0.5 x TBE buffer (pre-cooled at 4 °C) for 28 h at 13 °C and 5.6 V/cm and an angle of 120 °. The switch time was set to 5 – 60 s. The variable speed pump was operated at 70 % speed. A marker (Lambda Ladder PFG Marker, New England BioLabs) was included in every run. Staining and analysis of the gel was carried out as described above.

3.7.3. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE)

3.7.3.1. Isolation of DNA from drinking water biofilms

Drinking water biofilms grown on coupons of domestic plumbing materials (3.3.1.) were scraped off using a sterile rubber scraper and suspended in 20 mL deionised water. An area of approximately 150 cm² (both sides of 4 coupons) was sampled. For biofilm dispersion, the suspension was vortexed for 2 min. 2 mL of the biofilm suspension were used for the determination of the total cell count (see 3.6.1.). The rest of the sample was centrifuged (20012 x g, 20 min, 4 °C) in order to harvest cells. The pellet was resuspended in 1 mL water for molecular biology (DEPC-treated) and centrifuged again (16060 x g, 30 min, 4 °C). The supernatant was discarded. DNA isolation from the cell pellet was performed using the DNeasy® Plant Mini Kit (QIAGEN) following the protocol for purification of total DNA from plant tissue (Mini Protocol) from step 7. For the final elution of DNA from the spin column, 2 x 50 µL buffer AE were used. The DNA solution was stored at -20 °C.

3.7.3.2. Amplification of 16S rDNA fragments

For the amplification of bacterial 16S rDNA gene fragments a touchdown PCR was performed using primers 27f_GC (Medlin et al., 1988, Murray et al.,1996) and 517r (Murray et al., 1996; see Table 2.6).

A mastermix containing all PCR components except the DNA template was prepared (Table 3.2). 47 µL of the mastermix were mixed with 3 µL of DNA solution in a 0.2 mL reaction tube.

Table 3.3: Components and concentrations of the PCR-reaction used in PCR-DGGE

Constituent	Concentration	Final concentration	Volume (µL)
Taq-Master	5x	1x	10
PCR-Puffer	10x	1x	5
dNTP-Mix	10 mM	200 µM	1
Primer 27f_GC	10 µM	0.5 µM	2.5
Primer 517r	10 µM	0.5 µM	2.5
Taq-Polymerase	5 U/µL	2.5 U	0.5
DNA solution			3
H_2O (molecular biology grade)			25.5
Total volume			50

A negative control without DNA template was included in all PCR reactions.

The PCR program parameters were set as follows:

Initial Denaturation	94 °C	60 s
10 Cycles:		
Denaturation	94 °C	60 s
Annealing*	71 °C	60 s
Elongation	72 °C	90 s

*The temperature in the annealing steps was reduced from 71 °C to 61 °C in 10 cycles (reduction of 1 °C per cycle)

19 Cycles:		
Denaturation	94 °C	60 s
Annealing	61 °C	60 s
Elongation	72 °C	90 s
Final Elongation	72°C	7 min
Cooling	4°C	

The presence and size of the amplicons was analysed using agarose gel electrophoresis (see 3.7.5.). The PCR products consisting of a mixture of 16S rDNA fragments from different bacteria present in the biofilm were used for DGGE without further processing.

3.7.3.3. DGGE

Genetic fingerprinting of drinking water biofilms was performed by denaturing gradient gel electrophoresis using a 7.5 % acrylamide gel with a gradient of 40 % to 60 % denaturation (100% denaturant corresponded to 7M urea and 40% deionised formamide; see 2.3.6.). For one gel (160 mm x 140 mm x 1 mm) 14 mL of a 40 % denaturing solution and a 60 % denaturing solution were prepared (Table 3.3).

Table 3.4: Composition of denaturing acrylamide solutions used for a 40 % - 60 % denaturing gel (for details see chapter 2.3.6.)

Constituent	40 % denaturing solution	60 % denaturing solution
100 % denaturing solution (7.5 % acrylamide, 7M urea and 40% deionised formamide)	5.6 mL	8.4 mL
7.5 % acrylamide solution (0% denaturation)	8.4 mL	5.6 mL
10 % Ammonium persulfate (APS) solution	140 µL	140 µL
TEMED	9 µL	18 µL

After adding TEMED to the solutions, the gel was promptly poured using a gradient delivery system (Bio-Rad). The gel was polymerized to a GelBond PAG film (GE Healthcare) for stabilization. The samples were mixed with 6x loadig dye (Fermentas) and 140 to 280 ng DNA were loaded to the gel. In order to estimate the amount of DNA employed for DGGE, DNA concentrations in the PCR products were measured using the Quant-iT™ PicoGreen® dsDNA Reagent Kit (Invitrogen). A high-range calibration with bacteriophage lambda DNA was performed following the manufacturer's instruction. Samples were diluted 1:10 in TE buffer (Invitrogen). Samples were excited at 485 nm and their relative fluorescence was measured at 520 nm using the fluorimeter SFM 25 (BIO-TEK KONTRON Instrumente). From the standard calibration curve DNA concentrations in µg/mL were calculated.Electrophoresis was carried out in 1 x TAE buffer at 58 °C and 70 V for 17 h using the DCode™ Universal Mutation Detection System (Bio-Rad). The gel was silver stained according to the technique of Blum et al. (1987) as shown in Table 3.4.

Table 3.5: Procedure for silver staining of DNA fragments in DGGE gels (Blum et al., 1987)

Step	Solution	Time of treatment
Fixation	50 % Methanol 12 % Acetic acid 0.5 mL/L 37 % Formaldehyde	≥ 1 h
Washing	50 % Ethanol	3 × 20 min
Pretreatment	0.2 g/L $Na_2S_2O_3 \times 5\ H_2O$	1 min
Rinsing	Deionised water	3 × 20 s
Impregnation	2 g/L $AgNO_3$ 0.75 mL/L 37 % Formaldehyde	20 min
Rinsing	Deionised water	2 × 20 s
Development	60 g/L Na_2CO_3 0.5 mL 37 % Formaldehyde 4 mg/L $Na_2S_2O_3 \times 5\ H_2O$	3-10 min
Washing	Deionised water	2 × 2 min
Stopping	50 % Methanol 12 % Acetic acid	10 min
Washing	50 % Methanol	≥ 20 min

All steps were carried out on a rocking platform at room temperature. Gels were scanned using the Imaging Densitometer GS-700 (Bio-Rad) and subsequently sealed in a polypropylene transparent envelope for storage at 4 °C.

3.7.3.4. Analysis of banding pattern

Analysis of the gels was performed visually using a light table. The number of bands of each sample and the number of similar bands in two samples being compared were counted. Two bands were regarded similar if they migrated the same distance on a gel.

Relatedness of biofilm communities grown on different plumbing materials was determined by calculating the Sørensen Index (C_s; Sørensen, 1948) using the following equation:

$$C_s = 2j / (a+b)$$

a = number of bands in sample A
b = number of bands in sample B
j = number of bands similar in sample A and B

Results are expressed in percent similarity.

3.7.4. Agarose gel electrophoresis

After amplifying 16S rDNA fragments from bacterial pure cultures or drinking water biofilms or 18S rDNA fragments from amoeba pure cultures, the presence and size of the amplicons was analysed by agarose gel electrophoresis. For a 1 % agarose gel, 50 mL of a 1 % Agarose solution in TAE buffer were filled into a gel tray and allowed to harden. A mixture of 5 µL of the PCR products and 1 µl 6x loading buffer (Fermentas) was pipetted into the wells of the gel. If PCR products should be extracted from the agarose gel later, 25 µL of the PCR product were mixed with 2 µl 6x loading buffer and pipetted into the wells of the gel leaving a space of one well between the samples to avoid contamination when the bands were cut out. A DNA size ladder (Fermentas) was included. The separation took place in a HE 33 MiniSubmarine electrophoresis unit in 1x TAE-buffer at 100 V. After electrophoresis, the gel was stained in an ethidium bromide bath (1 µg/mL) for 20 min and then destained in deionised water for 5 min. Analysis and documentation of the agarose gel was performed using a gel documentation system (Universal Hood II, Bio-Rad).

Chapter 4
Results

4.1. Selection of the test strains

All experiments involving the incorporation of bacteria into drinking water biofilms were performed using the test strains *Pseudomonas aeruginosa* AdS, *Legionella pneumophila* AdS and *Enterobacter nimipressuralis* 9827 clone A. All three bacteria were wild-type strains isolated from contamination cases. *P. aeruginosa* AdS was isolated from the residual water within an automatic shut-off valve of a shower in a contaminated domestic plumbing system of a school; *L. pneumophila* AdS (serogroup 1) was isolated from a biofilm in the same automatic shut-off valve. *Enterobacter nimipressuralis* 9827 clone A was isolated from an elevated tank of a drinking water supply system. Identification of *P. aeruginosa* AdS and *E. nimipressuralis* 9827 clone A was performed, using the API 20 NE system (bioMérieux) and GN MicroPlates™ (Biolog) and species identity of *P. aeruginosa* AdS and *L. pneumophila* AdS was confirmed by 16S rDNA sequencing.

P. aeruginosa AdS was identified as *P. aeruginosa* by the API 20 NE system and the GN MicroPlates™ system with a probability of 99.5 % and 100 %, respectively. *E. nimipressuralis* was identified as *E. amnigenus* by the API 20 NE system with a probability of 96 % and as *E. nimipressuralis* by the GN MicroPlates™ system with a probability of 100 %.

The *P. aeruginosa* AdS test strain used in this study showed a 97 % homology to the 16S ribosomal RNA of *Pseudomonas aeruginosa* strain CMG590. A 97 % homology was detected between the *L. pneumophila* test strain used in this study and *Legionella pneumophila* subspecies *pneumophila* strain Philadelphia 1.

4.2. Microbiological characterisation of biofilms grown on domestic plumbing materials

In the first part of the present study the colonisation of materials utilised in domestic plumbing systems by the autochthonous microflora was investigated. For this purpose, biofilms were grown on coupons of two types of elastomeric (EPDM 1, EPDM 2) and plastic (PE-Xb, PE-Xc) materials as well as on copper in a stainless steel tank or in stainless steel flow-through reactors under continuous flow-through with drinking water for 14 days.

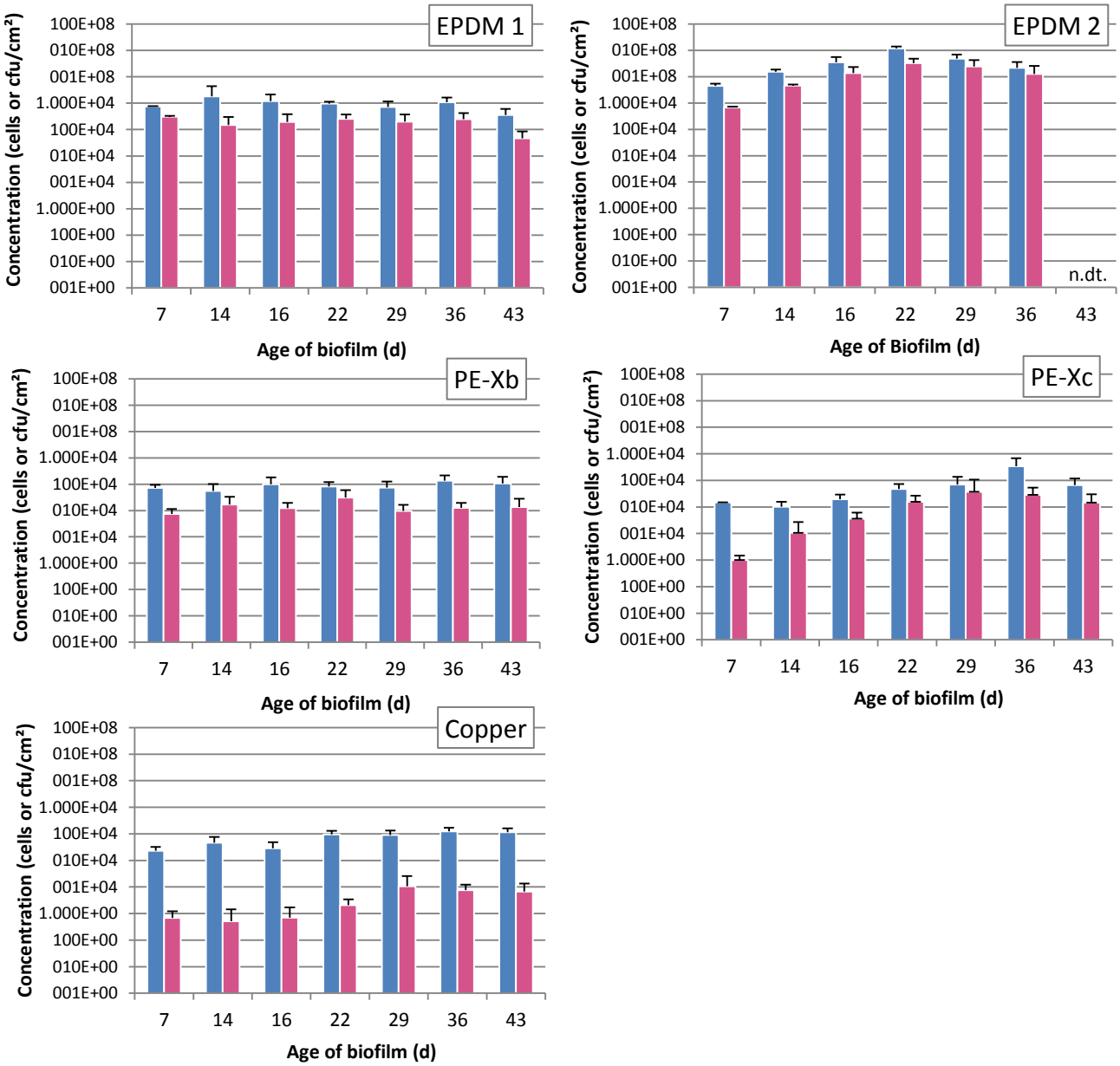

Figure 4.1: Total cell counts and colony counts (HPC) of drinking water biofilms grown on EPDM 1, EPDM 2, PE-Xb, PE-Xc and copper over a period of 43 d under constant flow conditions. Total cell counts and HPC after 7 days were determined in two independent experiments; total cell counts and HPC after 14 days were determined in two independent experiments for EPDM 2, in four independent experiments for PE-Xc and copper and in six independent experiments for EPDM 1 and PE-Xb; total cell counts and HPC after 16, 29 and 43 days were determined in six independent experiments for PE-Xc and copper, in eight independent experiments for EPDM 1 and PE-Xb and in four independent experiments for EPDM 2; total cell counts and HPC after 22 and 36 days were determined in four independent experiments; total cell counts and HPC after 43 days were not determined (n.dt.) for EPDM 2.

EPDM 1 complied with the German standards for plastics in contact with drinking water whereas EPDM 2 did not fulfil these standards. The silane cross-linked PE (PE-Xb) and the electron ray cross-linked PE (PE-Xc) both met the standard requirements (chapter 2.6., Table 2.7). During the period of biofilm growth, the mean temperature of the water inside the tank or reactor was 18.2 °C ± 3.5 °C (range 9.9 °C - 28.8 °C; n = 198). The mean temperature of the influent water was 15.3 °C ± 4.8 °C (range 7.4 °C - 21.9 °C; n = 47) and the average pH of the influent water was 7.9 ± 0.1 (range 7.7 - 8.2; n = 39).

The number of total cells and culturable HPC bacteria of the biofilms on the five plumbing materials was determined over the period of 7 days to 43 days (Fig. 4.1). In biofilms grown on EPDM 1, total cell counts ranged between 3.53 x 10^6 cells/cm² and 1.81 x 10^7 cells/cm² and the concentration of culturable heterotrophic bacteria was 4.64 x 10^5 cfu/cm² to 3.06 x 10^6 cfu/cm². In biofilms grown on EPDM 2, total cell counts of 4.51 x 10^7 cells/cm² to 1.19 x 10^9 cells/cm² were detected, whereas colony counts varied from 6.21 x 10^6 cfu/cm² to 3.27 x 10^8 cfu/cm². On PE-Xb, biofilms with total counts ranging from 5.54 x 10^5 cells/cm² to 1.35 x 10^6 cells/cm² and colony counts of 7.46 x 10^4 cfu/cm² to 3.11 x 10^5 cfu/cm² developed. In biofilms grown on PE-Xc, the concentration of total cells ranged from 1.02 x 10^5 cells/cm² to 3.43 x 10^6 cells/cm² and the concentration of heterotrophic plate count bacteria varied from 1.02 x 10^3 cfu/cm² to 3.70 x 10^5 cfu/cm². On copper biofilms with a total cell count of 2.28 x 10^5 cells/cm² to 1.25 x 10^6 cells/cm² and a colony count of 5.14 x 10^2 cfu/cm² to 1.06 x 10^4 cfu/cm² were detected. The percentage of culturable heterotrophic bacteria on the total cell count ranged from 13.5 % to 40.5 % in biofilms grown on EPDM 1, from 15.3 to 65.0 % in biofilms grown on EPDM 2, from 9.6 % to 34.3 % in biofilms grown on PE-Xb and from 0.7 % to 34.9 % in biofilms grown on PE-Xc. In biofilms grown on copper, the fraction of culturable bacteria on the total count was significantly smaller and varying between 0.2 % and 2.6 %.

The results show that biofilms develop on all of the five plumbing materials within 7 days of constant flow with drinking water at room temperature. Over the period of 43 days biofilms with different total cell counts and colony counts were detected on the five domestic plumbing materials:

Total cell count EPDM 2 > EPDM 1 > PE-Xb > copper > PE-Xc

Colony count EPDM 2 > EPDM 1 > PE-Xb > PE-Xc > copper

(geometric means over the period of 43 days in 4 (EPDM 2), 6 (PE-Xc, copper) or 8 (EPDM 1, PE-Xb) independent experiments).

Over the period of 7 days to 43 days there was no or only a slight (EPDM 2, PE-Xc, copper) increase in total cell counts and colony counts of biofilms grown on the dif-

ferent materials. The fraction of culturable heterotrophic bacteria on the total cell count of biofilms grown on copper was approximately 2 orders of magnitude lower compared to biofilms grown on plastic materials.

In order to assess the number of bacteria that are released by biofilms grown on the different domestic plumbing materials, the reactor effluent from flow-through reactors containing coupons of EPDM 1, EPDM 2, PE-Xb, PE-Xc and copper were collected and the total cell count as well as the colony count were determined over a period of 7 days to 43 days (Fig. 4.2).

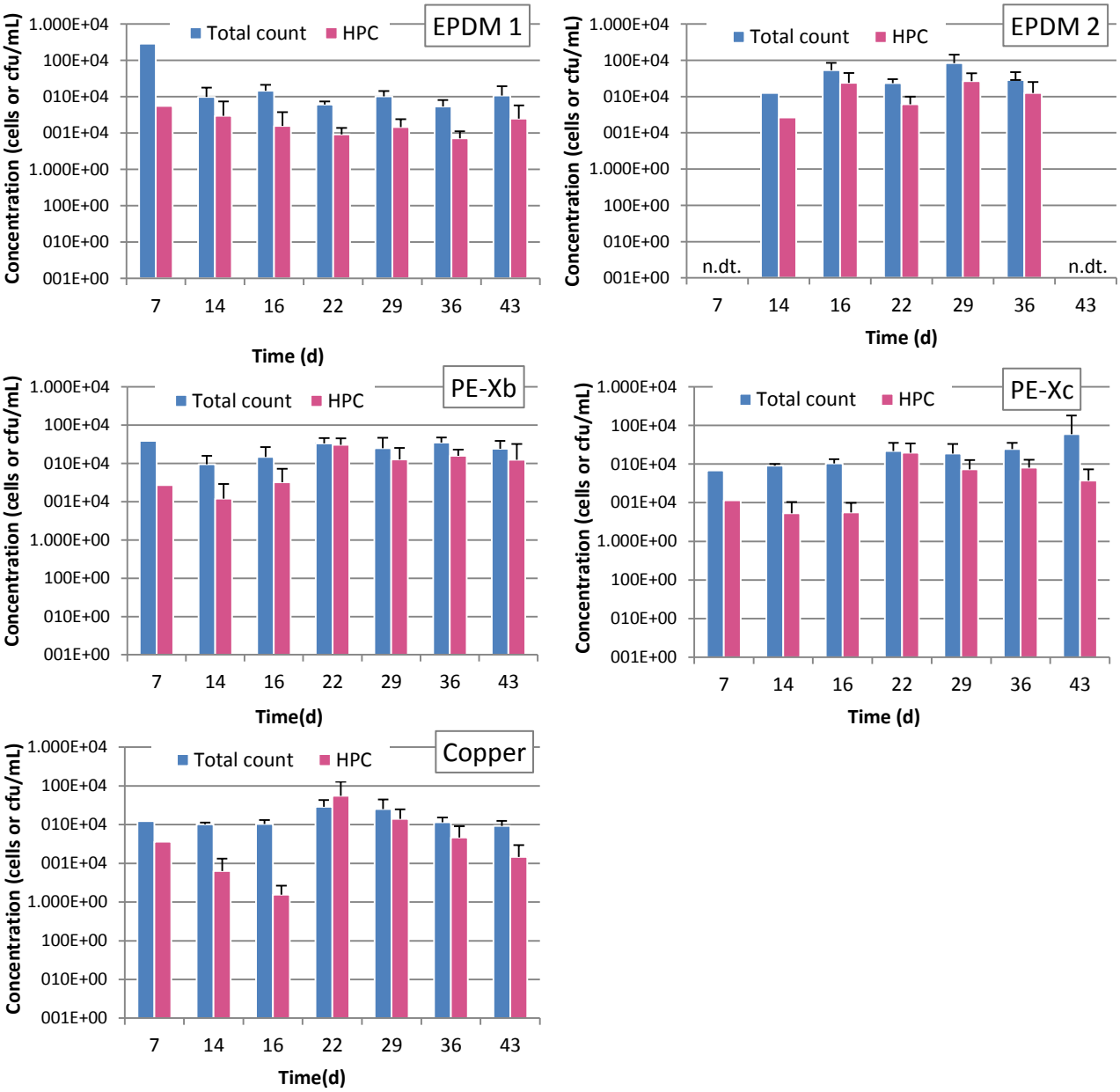

Figure 4.2: Total cell counts and colony counts (HPC) of the reactor effluents of reactors containing coupons of EPDM 1, EPDM 2, PE-Xb, PE-Xc and copper over a period of 43 d under constant flow conditions. Total cell counts and HPC after 7 days were determined in one experiment; total cell counts and HPC after 14 days were determined in one experiment for EPDM 2, in three independent experiments for PE-Xc and copper and in four independent experiments for EPDM 1 and PE-Xb; total cell counts and HPC after 16, 29 and 43 days were determined in six independent experiments for PE-Xc and copper, in eight independent experiments for EPDM 1 and PE-Xb and in four independent experiments for EPDM 2; total cell counts and HPC after 22 and 36 days were determined in four independent experiments; total cell counts and HPC after 7 and 43 days were not determined (n.dt.) for EPDM 2.

On some occasions the total cell count and HPC of the influent drinking water was measured. The average total cell count in the influent drinking water was 5.81 x 10^4 cells/mL (n = 3) and the average colony count was 5.65 x 10^1 cfu/mL (n = 3). Investigations on total cell count and HPC of the effluent water from the reactors containing coupons of the four different plumbing materials (Fig. 4.2) showed that during the passage of drinking water through the reactors containing coupons of domestic plumbing materials, the concentration of culturable heterotrophic bacteria was more enhanced than the total cell number. The total cell count increased by maximally 1.5 log units, whereas the colony count increased by 2 to 4 log units. In contrast to the drinking water biofilms grown on the different materials, there was no significant variation in the total cell count and colony count between the effluents of the five materials. Consequently there was no correlation between total cell counts and colony counts of biofilms and effluent water, respectively.

4.3. The influence of material alterations on biofilm formation

As a second step the influence of possible material alterations of domestic plumbing materials as a consequence of chemical disinfection on biofilm formation was analysed. In order to investigate this aspect, EPDM 1, PE-Xb and PE-Xc were artificially aged by the application of chemical disinfectants simulating a disinfection used as a sanitation measure. Coupons of EPDM 1, PE-Xb and PE-Xc were treated with 2.5 ppm sodium hypochlorite (NaOCl) at 3 bar at 40 °C for 4 weeks. Another set of coupons of EPDM 1 and PE-Xb was treated with 4.0 ppm chlorine dioxide (ClO_2) at 4 bar at 40 °C for 4 weeks. Because of its low quality, EPDM 2 does not comply with the German standards for materials in contact with drinking water and is thus not relevant in German plumbing systems. Therefore, EPDM 2 was not included in the ageing experiments. As copper is not as sensitive against the action of oxidative disinfectants as synthetic plumbing materials, copper coupons were alternatively aged by exposure in a drinking water distribution system for at least 6 months (Wingender et al., 2003). Biofilms were grown on coupons of aged domestic plumbing materials in a stainless steel tank or in stainless steel flow-through reactors under continuous flow-through with drinking water for 14 days. For comparison, biofilms on coupons of the corresponding new domestic plumbing materials were grown in parallel in each experiment. During cultivation and monitoring of biofilms, the mean temperature of the water inside the tank or reactor was 19.0 °C ± 3.0 °C (range 11.4 °C - 25.0 °C; n = 197). The mean temperature of the influent water was 16.5 °C ± 4.9

˚C (range 7.4 ˚C - 21.9 ˚C; n = 27) and the average pH of the influent water was 7.9 ± 0.1 (range 7.7 - 8.2; n = 15).

The number of total cells and culturable HPC bacteria of the biofilms grown on new and artificially aged plumbing materials was determined over the period of 14 days to 43 days. Figures 4.3 to 4.6 show the results of two independent experiments for each of the materials tested separately. No mean values were calculated because only biofilms that were grown in parallel under the same conditions, i.e. during the same period of time, were considered to be comparable.

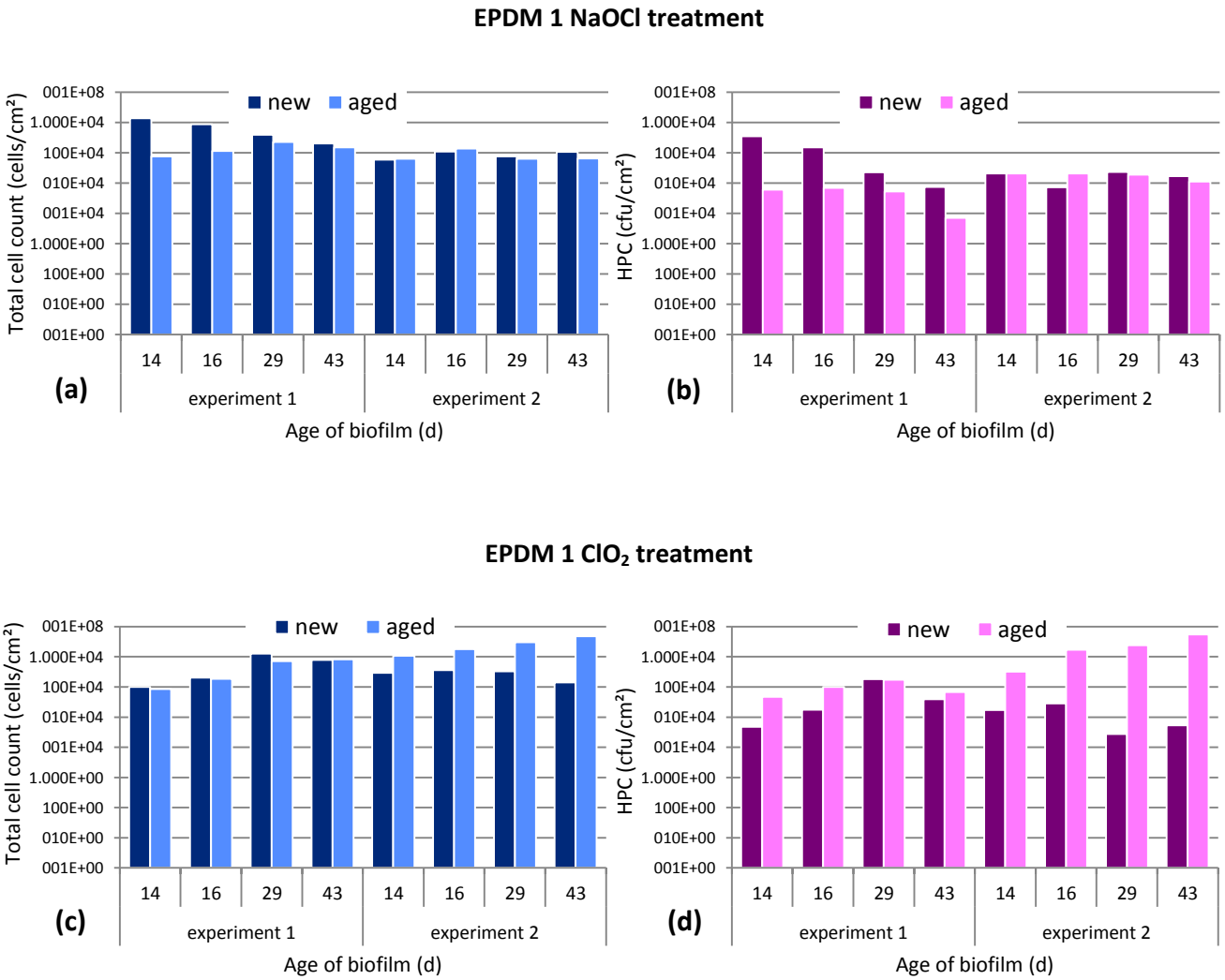

Figure 4.3: Total cell counts (a, c) and colony counts (HPC; b, d) of drinking water biofilms grown on new and aged EPDM 1 over a period of 43 d under constant flow conditions. For ageing EPDM 1 coupons were treated with NaOCl (a, b) or ClO_2 (c, d). Results of two independent experiments are shown.

In biofilms grown on NaOCl-treated EPDM 1 total cell counts ranged from 7.61 x 10^5 cells/cm² to 2.29 x 10^6 cells/cm² in experiment 1 and from 6.31 x 10^5 cells/cm² to

1.37 x 10^6 cells/cm² in experiment 2 (Fig. 4.3 a). The number of culturable heterotrophic bacteria was 7.14 x 10^3 cfu/cm² to 6.94 x 10^4 cfu/cm² in experiment 1 and 1.13 x 10^5 cfu/cm² to 2.09 x 10^5 cfu/cm² in experiment 2 (Fig. 4.3 b). In experiment 1 there was a decline in total cell counts and colony counts of biofilms grown on the new material from day 14 to day 43 whereas in experiment 2 total cell count and HPC of biofilms grown on new EPDM 1 were relatively constant over the whole period of incubation. Biofilms grown on NaOCl-treated EPDM 1 showed relatively constant values for total cell count and HPC over the period of 14 to 43 days in both experiments. In experiment 1 both total counts and HPC were more than one log unit lower in biofilms grown on NaOCl-treated EPDM 1 compared to the new material after 14 d of biofilm growth. The difference in total cell counts decreased over time and after 43 days, total cell counts on new and NaOCl-treated EPDM 1 were almost equal. The colony count in biofilms grown on NaOCl-treated EPDM 1 were still one log unit lower than on new EPDM 1 after 43 days. In experiment 2 no significant difference was observed between total cell counts or colony counts of biofilms grown on new and NaOCl-treated EPDM 1.

On ClO_2-treated EPDM 1 biofilms with total cell counts from 8.50 x 10^5 cells/cm² to 8.14 x 10^6 cells/cm² were detected in experiment 1 and from 1.09 x 10^7 cells/cm² to 4.81 x 10^7 cells/cm² in experiment 2 (Fig. 4.3 c). The number of culturable heterotrophic bacteria varied between 4.76 x 10^5 cfu/cm² and 1.74 x 10^6 cfu/cm² in experiment 1 and between 3.18 x 10^6 cfu/cm² and 5.54 x 10^7 cfu/cm² in experiment 2 (Fig. 4.3 d). In experiment 1, total cell counts of biofilms grown on both new and ClO_2-treated EPDM 1 slightly increased over time. No significant difference occurred in total cell counts of biofilms grown on new or aged material. In experiment 2 total cell counts of biofilms grown on new EPDM 1 were relatively constant over time whereas those of biofilms grown on ClO_2-treated EPDM 1 increased from day 14 to day 43 resulting in a difference of more than one log unit between new and ClO_2-treated EPDM 1. The colony counts of biofilms grown on ClO_2-treated EPDM 1 were about one log unit higher than those of biofilms grown on new EPDM 1 after 14 d in experiment 1. The difference in colony counts of biofilms grown on new and aged EPDM 1 decreased over time and colony counts were almost equal after 43 d. In experiment 2 the HPC of biofilms grown on ClO_2-treated EPDM 1 was significantly higher than in experiment 1 whereas colony counts of biofilms grown on new EPDM 1 were slightly lower in experiment 2 compared to experiment 1. This resulted in an increased difference between new and ClO_2-treated EPDM 1 in experiment 2 compared to experiment 1. After 43 d the HPC of biofilms on ClO_2-treated EPDM 1 was 3 log units higher than that of biofilms on the new material.

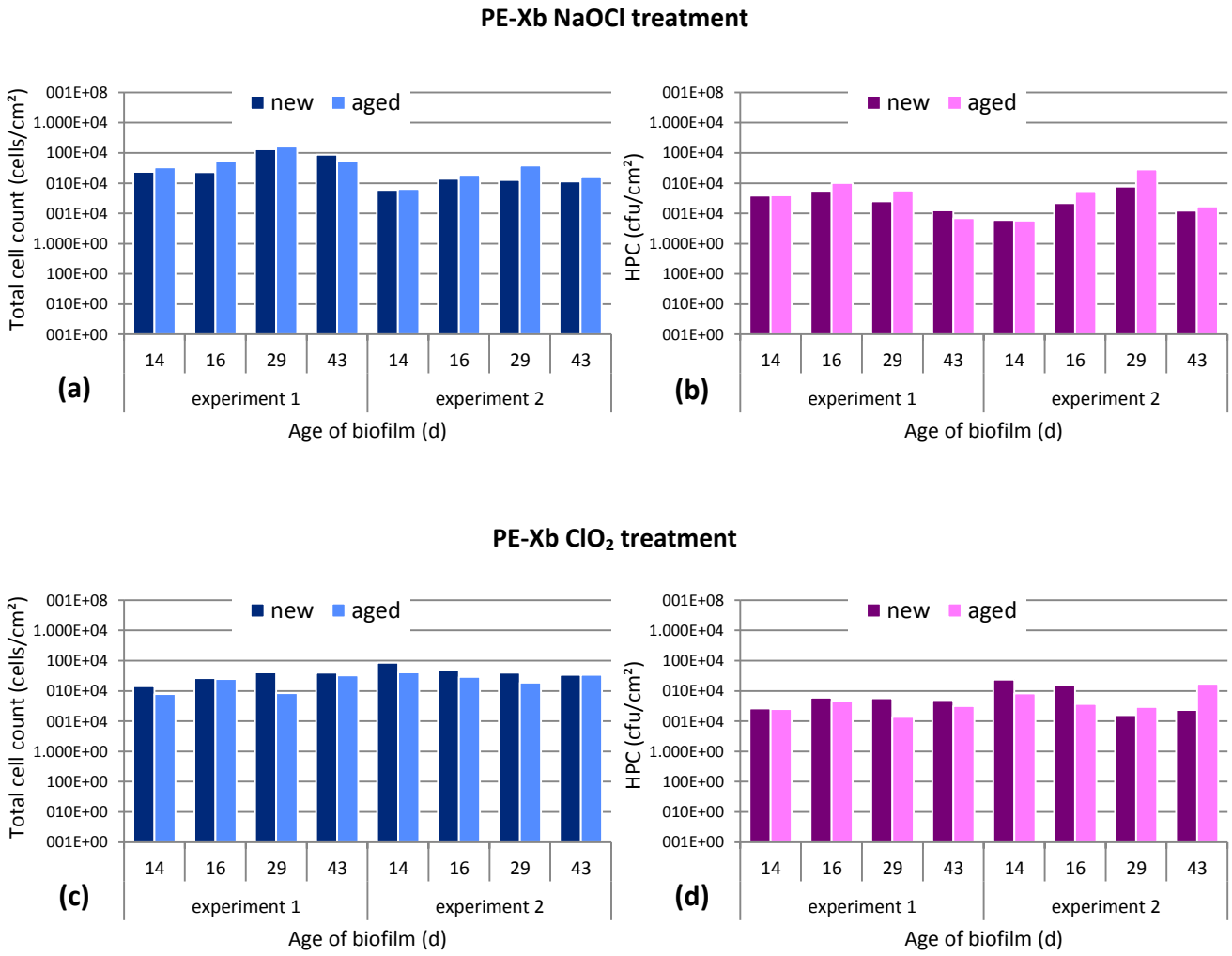

Figure 4.4: Total cell counts (a, c) and colony counts (HPC; b, d) of drinking water biofilms grown on new and aged PE-Xb over a period of 43 d under constant flow conditions. For ageing PE-Xb coupons were treated with NaOCl (a, b) or ClO_2 (c, d). Results of two independent experiments are shown.

In biofilms grown on NaOCl-treated PE-Xb (Fig. 4.4 b) total cell counts ranged from 3.30 x 10^5 cells/cm² to 1.60 x 10^6 cells/cm² in experiment 1 and from 6.34 x 10^4 cells/cm² to 3.86 x 10^5 cells/cm² in experiment 2 (Fig. 4.4 a). The number of culturable heterotrophic bacteria was 6.92 x 10^3 cfu/cm² to 5.69 x 10^4 cfu/cm² in experiment 1 and 5.73 x 10^3 cfu/cm² to 5.44 x 10^4 cfu/cm² in experiment 2. Total cell counts in biofilms grown on new and NaOCl-treated PE-Xb were relatively constant over time in both experiments. In experiment 2 total cell counts were slightly lower compared to experiment 1. No significant difference between total cell counts of biofilms grown on new PE-Xb and those of biofilms grown on NaOCl-treated PE-Xb was detected over the whole period of investigation in any of the two experiments. There was no variation larger than 1 log unit over time in colony counts of biofilms grown on new and NaOCl-treated PE-Xb in both experiments. Only minor differences

were detected between colony counts of biofilms grown on new PE-Xb and colony counts of biofilms grown on NaOCl-treated PE-Xb in both experiments during the period of 14 d to 43 d.

On ClO_2-treated PE-Xb biofilms with total cell counts ranging from 7.94 x 10^4 cells/cm² to 3.26 x 10^5 cells/cm² were detected in experiment 1 and from 1.90 x 10^5 cells/cm² to 4.09 x 10^5 cells/cm² in experiment 2 (Fig. 4.4 c). The number of culturable heterotrophic bacteria varied between 1.39 x 10^4 cfu/cm² and 4.58 x 10^4 cfu/cm² in experiment 1 and between 2.97 x 10^4 cfu/cm² and 1.97 x 10^5 cfu/cm² in experiment 2 (Fig. 4.4 d). Total cell counts in biofilms grown on new and ClO_2-treated PE-Xb remained at the same level from 14 to 43 days in both experiments. No significant difference between total cell counts of biofilms grown on new PE-Xb and those of biofilms grown on ClO_2-treated PE-Xb was detected over the whole period of investigation in any of the experiments.

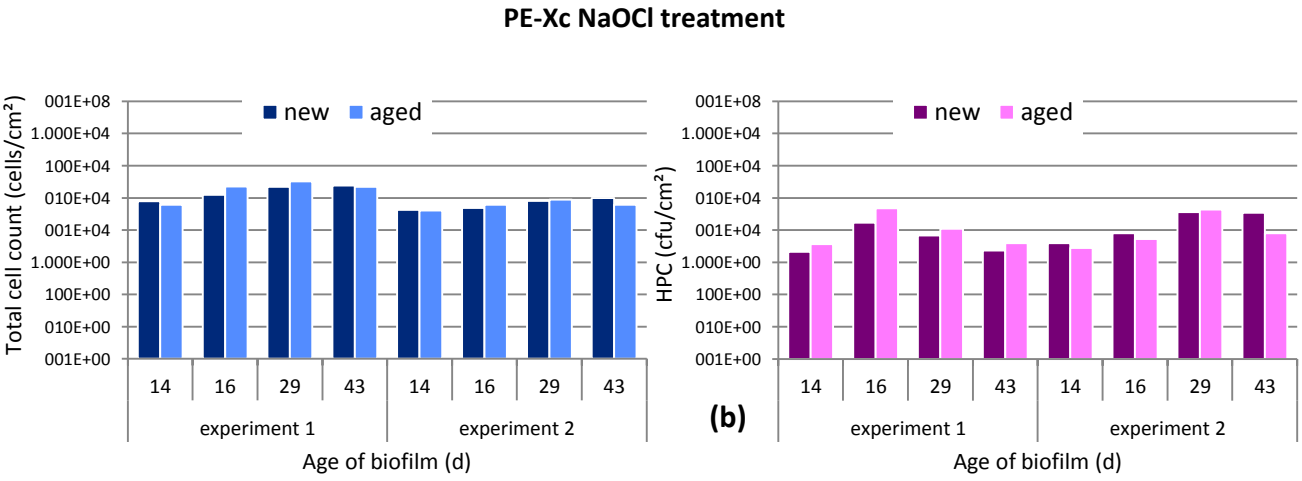

Figure 4.5: Total cell counts (a) and colony counts (HPC; b) of drinking water biofilms grown on new and aged PE-Xc over a period of 43 d under constant flow conditions. For ageing PE-Xc coupons were treated with NaOCl. Results of two independent experiments are shown.

In biofilms grown on NaOCl-treated PE-Xc (Fig. 4.5) total cell counts between 6.18 x 10^4 cells/cm² and 3.29 x 10^5 cells/cm² and between 4.03 x 10^4 cells/cm² and 8.92 x 10^4 cells/cm² were detected in experiment 1 and experiment 2, respectively (Fig. 4.5 a). The number of culturable heterotrophic bacteria ranged from 3.71 x 10^3 cfu/cm² to 4.77 x 10^4 cfu/cm² in experiment 1 and from 2.80 x 10^3 cfu/cm² to 4.34 x 10^4 cfu/cm² in experiment 2 (Fig. 4.5 b). In both experiments total cell counts of biofilms grown on new and NaOCl-treated PE-Xc were relatively constant over time. A significant difference between total cell counts and colony counts of biofilms

grown on new PE-Xc and those of biofilms grown on NaOCl-treated PE-Xc was detected over the whole period of investigation in any of the two experiments.

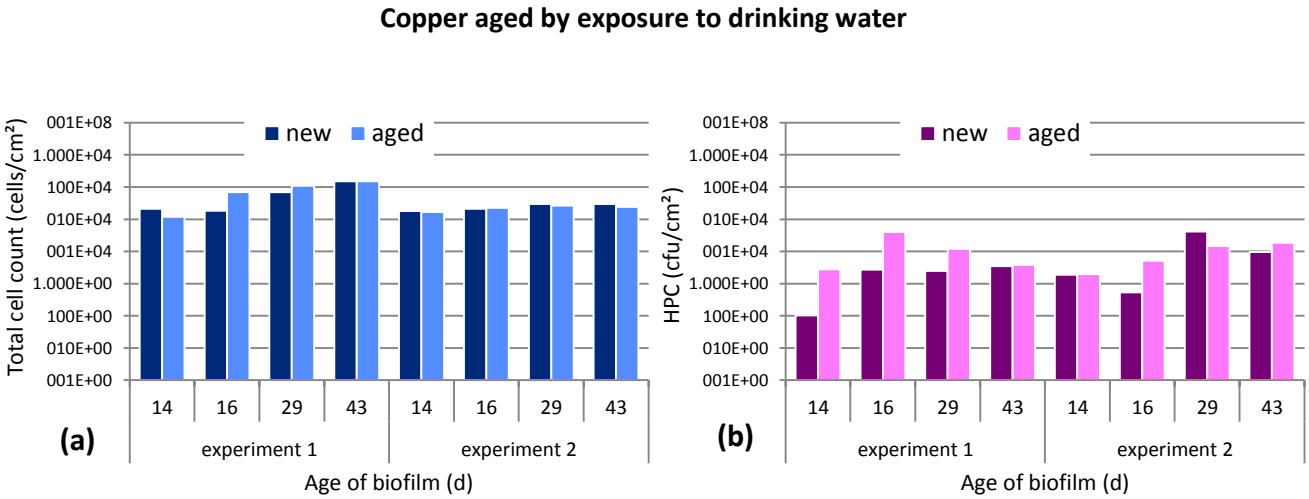

Figure 4.6: Total cell counts (a) and colony counts (HPC; b) of drinking water biofilms grown on new and aged copper over a period of 43 d under constant flow conditions. For ageing copper coupons were exposed to a drinking water distribution system for ≥ 6 months. Results of two independent experiments are shown.

In biofilms grown on copper that was previously aged by exposure to drinking water total cell counts ranged from 1.18 x 10^5 cells/cm² to 1.51 x 10^6 cells/cm² in experiment 1 and from 1.69 x 10^5 cells/cm² to 2.65 x 10^5 cells/cm² in experiment 2 (fig. 4.6 a). The number of HPC bacteria was 2.76 x 10^3 cfu/cm² to 4.09 x 10^4 cfu/cm² in experiment 1 and 1.96 x 10^3 cfu/cm² to 1.86 x 10^4 cfu/cm² in experiment 2 (Fig. 4.6 b).

Total cell counts on new and aged copper slightly increased from day 14 to day 43 of experiment 1. In experiment 2, total cell counts of new and aged copper were slightly lower compared to experiment 1 and did not vary significantly over time. The number of culturable heterotrophic bacteria in biofilms grown on new copper was more than one log unit lower than that of biofilms grown on aged copper. The difference decreased over time and after 43 d of biofilm growth HPC values of biofilms grown on new and aged copper were almost at the same level. In experiment 2 the difference in colony counts between new and aged copper was lower than 1 log unit over the whole period of investigation.

Altogether, the results show that domestic plumbing materials that are treated with disinfectants (sodium hypochlorite, chlorine dioxide) or by exposure to drinking water are colonised by the autochthonous drinking water microflora at concentrations of total cells and culturable heterotrophic bacteria that were comparable to

those of biofilms developed on the corresponding new materials. In case of EPDM the number of culturable HPC bacteria can be significantly higher in biofilms grown on ClO_2-treated EPDM compared to new EPDM.
Determination of the total cell counts and the colony counts of the reactor effluents showed that a release of microorganisms from biofilms grown on aged plumbing materials to the water phase occurred to an extent comparable to that from biofilms grown on new materials (data not shown).

4.4. Biofilm populations on domestic plumbing materials

In order to investigate the influence of the type of material as well as of the ageing of a material on the composition and diversity of drinking water biofilm populations, biofilms were grown for 14 d under constant flow conditions in a stainless steel tank. All new and aged materials were investigated in parallel in one experiment to assure that biofilms on all materials were grown under identical conditions. The DNA of the biofilms was isolated, the 16S rDNA was partially amplified by PCR and the PCR products were separated using DGGE. For every biofilm a specific band pattern was obtained (Fig. 4.7). The number of bands gave information about the species diversity of a biofilm. Relatedness of biofilm communities grown on different plumbing materials was determined by calculating the Sørensen Index (C_s; Sørensen, 1948). All new and aged materials of a single material type were compared with each other.
During the cultivation of biofilms, the mean temperature of the water inside the tank was 13.7 °C ± 1.2 °C (range 11.9 °C - 15.2 °C; n = 6). The average temperature of the influent water was 9.5 °C ± 0.1 °C (range 9.4 °C - 9.5 °C; n = 2) and the mean pH of the influent water was 7.9 ± 0.1 (range 7.8 - 7.9; n = 2). Total cell counts of drinking biofilms grown on the different materials after 14 d are shown in Table 4.1.

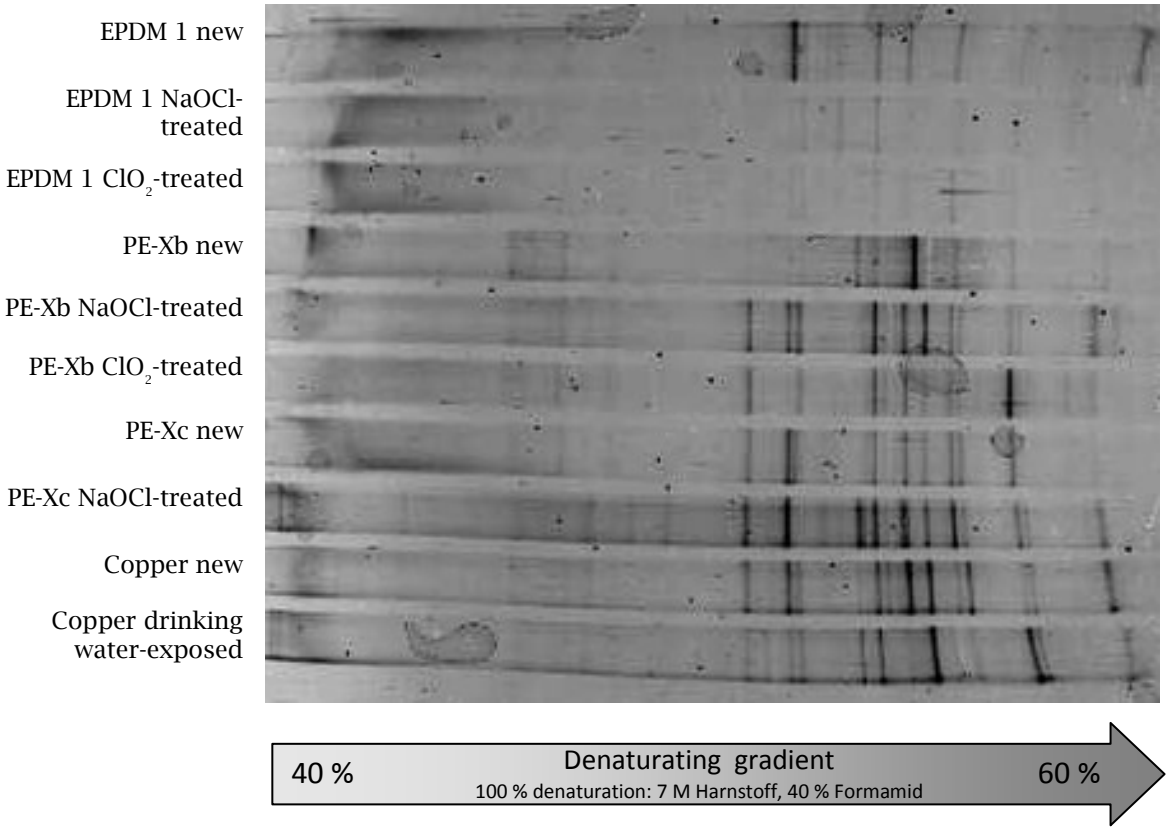

Figure 4.7: DGGE band patterns of PCR-amplified 16S rDNA fragments derived from 14 d-old drinking water biofilms grown on new and aged EPDM 1, PE-Xb, PE-Xc and copper. DGGE gel was silver stained according to Blum et al., 1987.

Table 4.1: Total cell counts and diversity (number of DGGE bands) of 14 d-old drinking water biofilms on new and aged domestic plumbing materials used for DGGE analysis.

Material	Total cell count (cells/cm²)	Diversity (no. of DGGE bands)
EPDM 1 new	1.65×10^6	25
EPDM 1 NaOCl-treated	1.09×10^5	29
EPDM 1 ClO_2-treated	9.71×10^5	31
PE-Xb new	7.68×10^5	24
PE-Xb NaOCl-treated	7.53×10^5	28
PE-Xb ClO_2-treated	7.35×10^5	27
PE-Xc new	2.73×10^5	26
PE-Xc NaOCl-treated	2.52×10^5	11
Copper new	2.96×10^5	14
Copper drinking water exposition	3.49×10^5	18

In biofilms grown on EPDM 1, PE-Xb and copper, a greater diversity (number of DGGE bands) was found in biofilms grown on aged material compared to biofilms grown on new material. Generally, the diversity was higher in biofilms grown on EPDM, PE-Xb and PE-Xc than in biofilms grown on copper with the exception of

biofilms grown on NaOCl-treated PE-Xc (Tab.4.2). There was no correlation between total cell counts and the number of DGGE-bands (R^2 = 0.38).

The highest similarity of band patterns was found between biofilms grown on EPDM 1 and PE-Xb (49.0 %), the least similarity of band patterns existed between biofilms grown on EPDM 1 and copper (35.9 %).

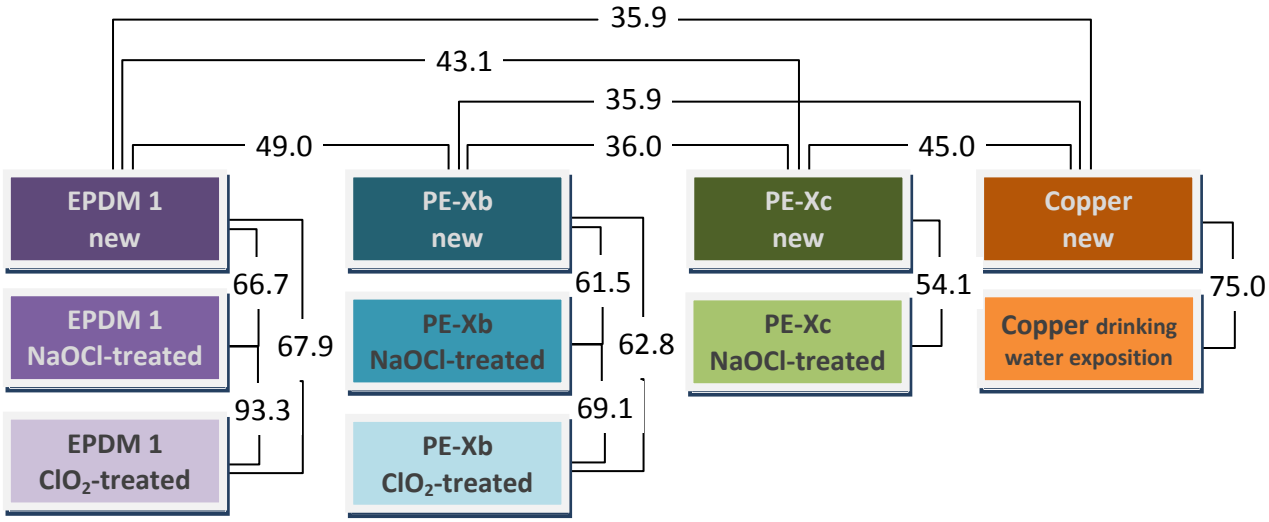

Figure 4. 8: Similarity of band patterns between 14 d-old drinking water biofilms grown on different material types and between new and aged materials. The numbers represent the similarity (Sørensen, 1948; see 3.7.3.4.) expressed in %.

There was no significant difference between the similarity of band patterns of biofilms grown on new and NaOCl-treated EPDM 1 and biofilms grown on new and ClO_2-treated EPDM 1 (66.7 % and 67.9 % similarity, respectively). The similarity of band patterns between biofilms grown on NaOCl-treated EPDM 1 and biofilms grown on ClO_2-treated EPDM 1 was found to be very high (93.3 %). Analogously, the similarity of band patterns between biofilms grown on new and NaOCl-treated PE-Xb was about the same as the similarity of band patterns between biofilms grown on new and ClO_2-treated EPDM 1 (61.5 % and 62.8 % similarity, respectively), whereas a higher similarity of band patterns was observed between biofilms grown on the two aged (NaOCl- or ClO_2-treated) PE-Xb materials (69.1 %). The similarity of band patterns between biofilms grown on new and NaOCl-treated PE-Xc was relatively low (54.1 %). A relatively high similarity of band patterns (75.0 %) was observed between biofilms grown on new and aged copper.

In a comparison of biofilms grown on new and aged plumbing materials of the same type, similarities of band patterns ranged from 54.1 % to 75.0 %. When biofilms

grown on different material types were compared to each other, similarities varied between 35.9 % and 49.0 %. This indicates that the type of material has a greater influence on the composition of the biofilm populations developing on the surface than the possible material alterations resulting from ageing of a material.

In order to investigate to which extent the composition of drinking water biofilms is influenced by the environmental conditions (i. e. physicochemical parameters of the drinking water) biofilms were grown on a set of coupons of EPDM 1 in May 2009 and on another two sets of coupons of EPDM 1 in February 2010, both for 14 days. Biofilm populations were analysed using DGGE and the similarity of band patterns between biofilms grown on EPDM 1 over the same time period as well as the similarity of band patterns of biofilms grown EPDM 1 over the two different time periods were calculated. In May 2009, the mean temperature inside the tank was 19.9 °C ± 0.5 °C (range 19.3 °C - 20.9 °C; n = 12) and in February 2010 a mean temperature of 15.8 °C ± 1.3 °C was measured inside the tank (range 13.6 °C - 16.9 °C; n = 5).

The diversity of the biofilms was at the same order of magnitude regardless of the time period. In the biofilm grown in May 2009, 26 bands were detected and in the two biofilms grown in February 2010, 20 and 21 bands were counted. The two biofilms grown over the same period of time showed a high similarity of band patterns (92.7 %), but when the biofilms grown in February were compared to that grown in May, similarities of only 47.8 % and 42.6 % were detected, even though the biofilms were grown on coupons of the same material (EPDM 1). These results show that environmental conditions prevailing during biofilm growth may have a great impact on the composition of the biofilm populations. This impact seems to be higher than that of material ageing.

4.5. Incorporation of hygienically relevant bacteria into drinking water biofilms grown on new and aged domestic plumbing materials

4.5.1. Adaptation of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* to drinking water conditions

To investigate the fate of *P. aeruginosan* AdS, *L. pneumophila* AdS (serogroup 1) and *E. nimipressuralis* 9827 clone A in drinking water biofilms, 14 day-old biofilms grown on new and aged EPDM 1, PE-X b, PE-X c and copper coupons were spiked with a mixture of *E. nimipressuralis*, *P. aeruginosa* and *L. pneumophila*. In order to adapt the bacteria to drinking water conditions, a suspension of each bacterium with a concentration of 3 x 10^6 cells/mL in sterile drinking water was incubated ("starved") for 24 h at 20 °C (*P. aeruginosa* and *E. nimipressuralis*) or 30 °C (*L. pneumophila*). After starving, the three bacterial suspensions were combined resulting in

an inoculum with a concentration of approximately 3 x 10^6 cells/mL and of 1 x 10^6 cells/mL of each *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis*. The concentration of the bacteria at the time of inoculation of the biofilms was determined using DAPI staining, cultivation and FISH for the detection of *P. aeruginosa* and *L. pneumophila* and the Colilert-18 Quanti-Tray®/2000 system for quantification of *E. nimipressuralis* (Fig. 4.9).

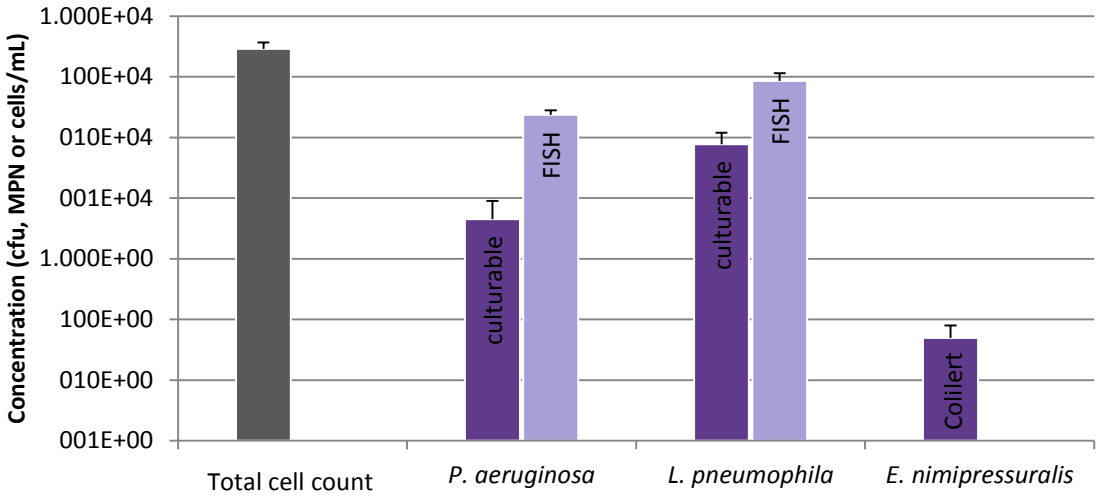

Figure 4.9: Total cell count and concentrations of *P. aeruginosa* AdS, *L. pneumophila* AdS and *E. nimipressuralis* 9827 clone Ain bacterial suspensions used for inoculating biofilms. *P. aeruginosa* was quantified using cultivation on CN agar and using FISH (probe Psae 16S-182). *L. pneumophila* was quantified using cultivation on GVPC agar and using FISH (probe Legpne1). The concentration of *E. nimipressuralis* was determined using the Colilert-18 system. Total cell counts and concentrations of *P. aeruginosa* were determined in three independent experiments. Concentrations of *L. pneumophila* and *E. nimipressuralis* were determined in two independent experiments.

The average total cell count of the inoculum was 2.89 x 10^6 cells/mL. The number of culturable *P. aeruginosa* was more than 2 log units lower than the concentration adjusted before starving, whereas the application of the FISH method showed a concentration of *P. aeruginosa* that was only slightly lower than the originally adjusted value. The concentration of culturable *L. pneumophila* in the inoculum was only slightly lower than the number of FISH-positive *L. pneumophila*, which was almost as high as the originally adjusted value before starvation. The concentration of *E. nimipressuralis* detected using the Colilert-18 system was 4.98 x 10^1 MPN/mL. As no oligonucleotide probe specific for this bacterial species was available, FISH could not be performed to detect possibly unculturable organisms.

These results show that incubation in drinking water for 24 h decreased the culturability of the target organisms. After the starving period, the reduction in culturability was most pronounced in *E. nimipressuralis* followed by *P. aeruginosa* and *L. pneumophila*.

After inoculation, the presence of the target bacteria in the drinking water biofilms was monitored with standard cultural methods as well as with culture-independent FISH (for *P. aeruginosa* and *L. pneumophila*) over a period of 4 weeks under continuous flow-through with drinking water in two independent experiments performed successively. Prior to inoculation, *P. aeruginosa*, *L. pneumophila* serogroup 1 and coliform bacteria were not detected culturally in 14 day-old biofilms and in 100-mL volumes of influent drinking water.

During the 4-week-period of monitoring the target organisms in the biofilms, the mean temperature of the water inside the flow-through reactors was 19.0 °C ± 3.1 °C (range 11.4 °C - 25.0 °C; n = 176). The average temperature of the influent water was 16.2 °C ± 5.3 °C (range 7.4 °C - 21.9 °C; n = 23) and the mean pH of the influent water was 7.9 ± 0.1 (range 7.7 - 8.2; n = 15).

4.5.2. Incorporation of *P. aeruginosa* into drinking water biofilms

In order to investigate the incorporation of *P. aeruginosa*, 14 d-old drinking water biofilms were inoculated with *P. aeruginosan* AdS, *L. pneumophila* AdS (serogroup 1) and *E. nimipressuralis* 9827 clone A. The fate of *P. aeruginosa* in the biofilms under flow-through conditions was monitored for 4 weeks using cultivation on CN agar (3.6.3.) and using the culture-independent FISH (3.7.1.1.). *P. aeruginosa* was able to incorporate into biofilms grown on new and NaOCl-treated EPDM 1 (Fig. 4.10).

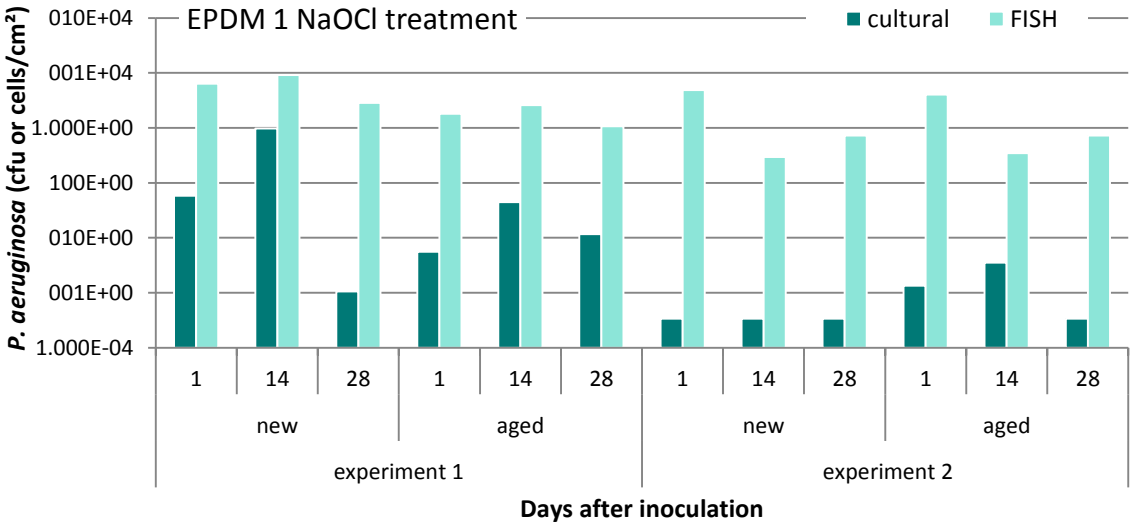

Figure 4.10: Persistence of *P. aeruginosa* AdS in drinking-water biofilms on new and aged (NaOCl-treated) EPDM 1 under constant flow conditions. *P. aeruginosa* in the biofilms was quantified using cultivation on CN agar and FISH (probe Psae16S-182). Results of two independent experiments are shown.

It was detected by culture and with the culture-independent FISH method over the whole period of investigation of 28 days in both experiments in varying concentrations. The concentrations of FISH-positive *P. aeruginosa* were 1 to 4 log units higher than those of culturable *P. aeruginosa*.

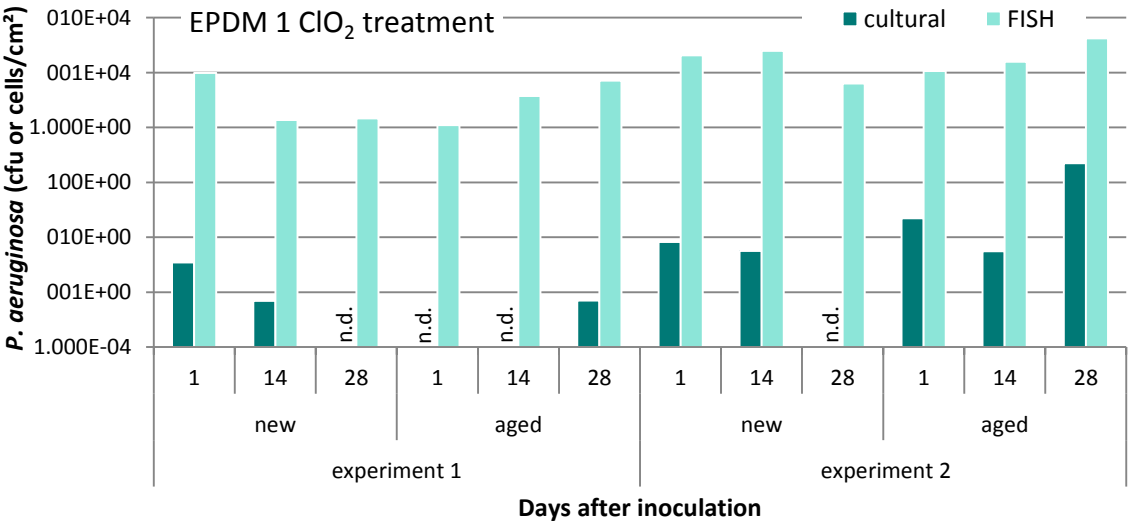

Figure 4.11: Persistence of *P. aeruginosa* AdS in drinking-water biofilms on new and aged (ClO_2-treated) EPDM 1 under constant flow conditions. *P. aeruginosa* in the biofilms was quantified using cultivation on CN agar and FISH (probe Psae16S-182). Results of two independent experiments are shown. n.d. - not detected (detection limit of cultivation 0.34 cfu/cm²).

In both experiments comparing new and ClO_2-treated EPDM 1 (Fig. 4.11), *P. aeruginosa* was detectable by culture for only 14 days in biofilms grown on new EPDM 1. In biofilms grown on ClO_2-treated EPDM 1, culturable *P. aeruginosa* were not detected until 28 d after inoculation in experiment 1, whereas in experiment 2 culturable *P. aeruginosa* in concentrations of up to 2.23 x 10^2 cfu/cm² were detected in biofilms grown on ClO_2-treated EPDM 1. Two to 4 log units higher concentrations of *P. aeruginosa* were detected using FISH compared to cultivation on CN agar.

Biofilms grown on new and NaOCl-treated PE-Xb were colonised by *P. aeruginosa* in varying concentrations and periods of persistence (Fig. 4.12). In biofilms grown on new PE-Xb, *P. aeruginosa* was reproducibly detected for only 14 d both by culture and FISH. In biofilms grown on NaOCl-treated PE-Xb, culturable *P. aeruginosa* were detected up to day 14 after inoculation in experiment 1 and up to day 28 after inoculation in experiment 2. FISH-positive *P. aeruginosa* were found in biofilms grown on NaOCl-treated PE-Xb for only one day in experiment 1, but for 28 d in experiment 2. Concentrations of *P. aeruginosa* detected with FISH were in most cases higher (0.5 to 3 orders of magnitude) than those detected by cultivation.

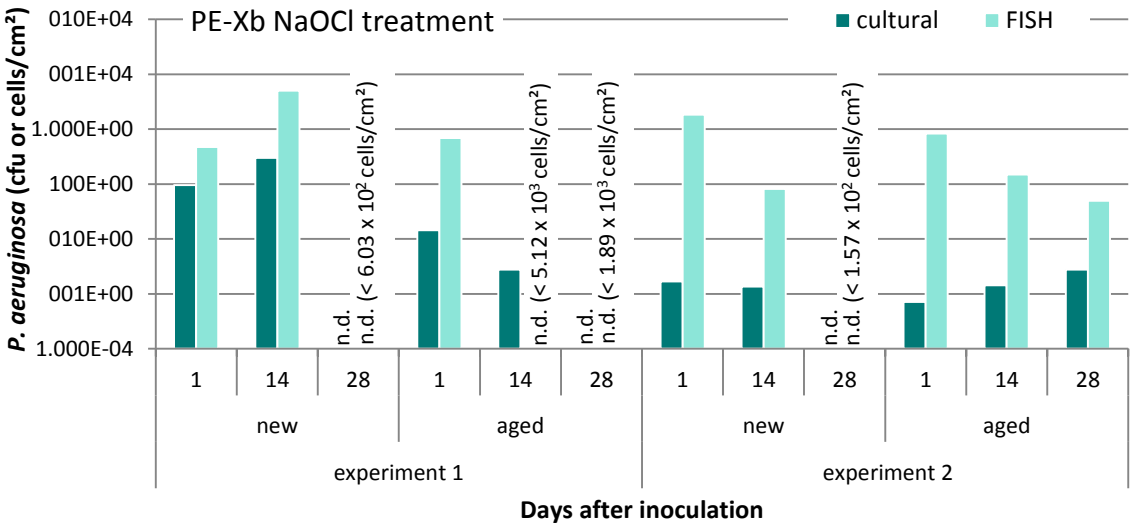

Figure 4.12: Persistence of *P. aeruginosa* AdS in drinking-water biofilms on new and aged (NaOCl-treated) PE-Xb under constant flow conditions. *P. aeruginosa* in the biofilms was quantified using cultivation on CN agar and FISH (probe Psae16S-182). Results of two independent experiments are shown. n.d. - not detected (detection limit of cultivation 0.34 cfu/cm²; the detection limit of the FISH method was calculated individually for each sample and is indicated in the diagram).

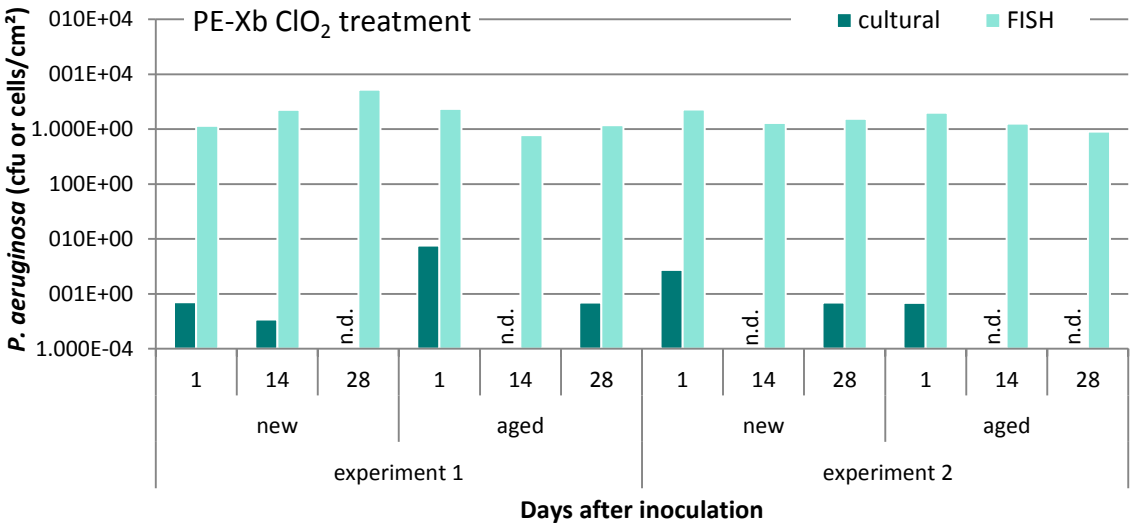

Figure 4.13: Persistence of *P. aeruginosa* AdS in drinking-water biofilms on new and aged (ClO_2-treated) PE-Xb under constant flow conditions. *P. aeruginosa* in the biofilms was quantified using cultivation on CN agar and FISH (probe Psae16S-182). Results of two independent experiments are shown. n.d. - not detected (detection limit of cultivation 0.34 cfu/cm²).

In the experiments comparing the persistence of *P. aeruginosa* in biofilms grown on new and ClO_2-treated PE-Xb (Fig. 4.13), *P. aeruginosa* was detected by FISH over the whole period of investigation in biofilms grown on new and ClO_2-treated PE-Xb in

both experiments. The concentrations of FISH-positive *P. aeruginosa* were comparable in biofilms on both materials in the two experiments and they were 2 to 4 orders of magnitude higher compared to the number of culturable *P. aeruginosa*. Using cultivation, *P. aeruginosa* was detected in biofilms grown on new PE-Xb for 14 d in experiment 1; in experiment 2 biofilms sampled after 1 and 28 d, but not after 14 d were positive for culturable *P. aeruginosa*. In biofilms grown on ClO_2-treated PE-Xb, *P. aeruginosa* could be recovered by culture after 1 and 28 d after inoculation, but not after 14 d after inoculation in experiment 1. In experiment 2, *P. aeruginosa* persisted in a culturable state for only one day in biofilms grown on ClO_2-treated PE-Xb.

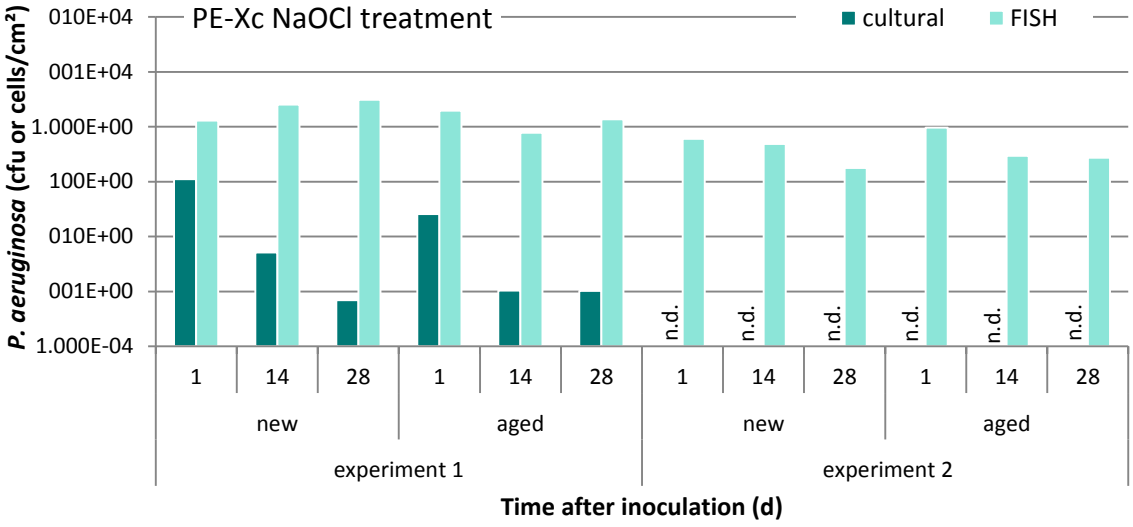

Figure 4.14: Persistence of *P. aeruginosa* AdS in drinking-water biofilms on new and aged (NaOCl-treated) PE-Xc under constant flow conditions. *P. aeruginosa* in the biofilms was quantified using cultivation on CN agar and FISH (probe Psae16S-182). Results of two independent experiments are shown. n.d. - not detected (detection limit of cultivation 0.34 cfu/cm²).

In the first experiment on the integration of *P. aeruginosa* into biofilms grown on new and NaOCl-treated PE-Xc (Fig. 4.14), culturable *P. aeruginosa* were detected in biofilms on both materials over the whole period of investigation with a tendency to decrease over time. The number of FISH-positive *P. aeruginosa* was relatively constant (about 10^3 cells/cm²) during the period of 1 d to 28 d after inoculation in biofilms on new and aged material. In experiment 2, the concentration of *P. aeruginosa* in biofilms grown on new and NaOCl-treated PE-Xc detected with FISH was slightly lower compared to experiment 1. No culturable *P. aeruginosa* were detected in biofilms on new and NaOCl-treated PE-Xc in experiment 2.

In biofilms grown on new and aged copper, *P. aeruginosa* could not be detected, neither by cultivation nor by FISH.

Table 4.3 summarises the maximum time of persistence of *P. aeruginosa* in drinking water biofilms grown on the different new and aged plumbing materials and the maximum concentrations of *P. aeruginosa* detected by culture and using FISH.
Altogether, the results show that *P. aeruginosa* is able to incorporate into and persist in biofilms grown on new and aged EPDM 1, PE-Xb and PE-Xc. Concentrations of culturable *P. aeruginosa* were relatively low (< 1 KBE/cm² in 52 % of the samples and < 10 KBE/cm² in 82 % of the samples) and varied depending on material type and material ageing as well as between single experiments with new and aged plumbing materials. A direct influence of material ageing by treatment with NaOCl or ClO_2 on the incorporation of *P. aeruginosa* into drinking water biofilms grown on the domestic plumbing materials was not observed.

Table 4.2: Maximum time of persistence and maximum concentration at maximum time of persistence of *P. aeruginosa* AdS in drinking water biofilms grown on new and aged EPDM 1, PE-Xb, PE-Xc and copper. *P. aeruginosa* in the biofilms was quantified using cultivation on CN agar and FISH (probe Psae16S-182). Maximum results of four (new EPDM 1 and PE-Xb) or two (new PE-Xc and copper; NaOCl-treated EPDM 1, PE-Xb and PE-Xc; ClO_2-treated EPDM 1 and PE-Xb) independent experiments are shown.

Material	Maximum time of persistence (d)		Maximum concentration at maximum time of persistence	
	Detected culturally	Detected with FISH	Cultural (cfu/cm²)	FISH (cells/cm²)
EPDM 1 new	28	28	1.06	6.34×10^3
EPDM 1 NaOCl-treated	28	14	1.16×10^1	2.60×10^3
EPDM ClO_2-treated	28	28	2.23×10^2	4.21×10^4
PE-X b new	28	28	0.69	5.21×10^3
PE-X b NaOCl-treated	28	28	2.78	4.99×10^1
PE-X b ClO_2-treated	28	28	0.69	1.17×10^3
PE-X c new	28	28	0.69	3.09×10^3
PE-X c NaOCl-treated	28	28	1.02	1.38×10^3
Copper new	0	0	< 0.34	$< 10^3$
Copper drinking water exposition	0	0	< 0.34	$< 10^3$

Application of the culture-independent FISH method showed up to 4 log units higher concentrations of *P. aeruginosa* than the cultivation method. In many cases, only 0.01 % of the FISH-positive *P. aeruginosa* were detected by culture; in 58 % of the samples the fraction of culturable *P. aeruginosa* on FISH-positive *P. aeruginosa* was < 0.1 % and in 82 % of the samples it was < 1 %. On some occasions, *P. aeruginosa* could not be detected using the culture method, but was only detected by FISH as observed on new and ClO_2-treated EPDM 1 (Fig. 4.11), on new and ClO_2-treated PE-Xb (Fig. 4.11) and on new and NaOCl-treated PE-Xc (Fig. 4.14).

4.5.3. Incorporation of *L. pneumophila* into drinking water biofilms

The incorporation of *L. pneumophila* was investigated by inoculating a 14 d-old drinking water biofilm with *P. aeruginosa* AdS, *L. pneumophila* AdS (serogroup 1) and *E. nimipressuralis* 9827 clone A. The fate of *P. aeruginosa* in the biofilms under flow-through conditions was monitored for 4 weeks using cultivation on GVPC agar (3.6.4.) and using the culture-independent FISH (3.7.1.1.). *L. pneumophila* was able to incorporate into biofilms grown on new and NaOCl-treated EPDM 1. It was detected by culture and with the culture-independent FISH method over the whole period of investigation of 28 days in both experiments (Fig. 4.15).

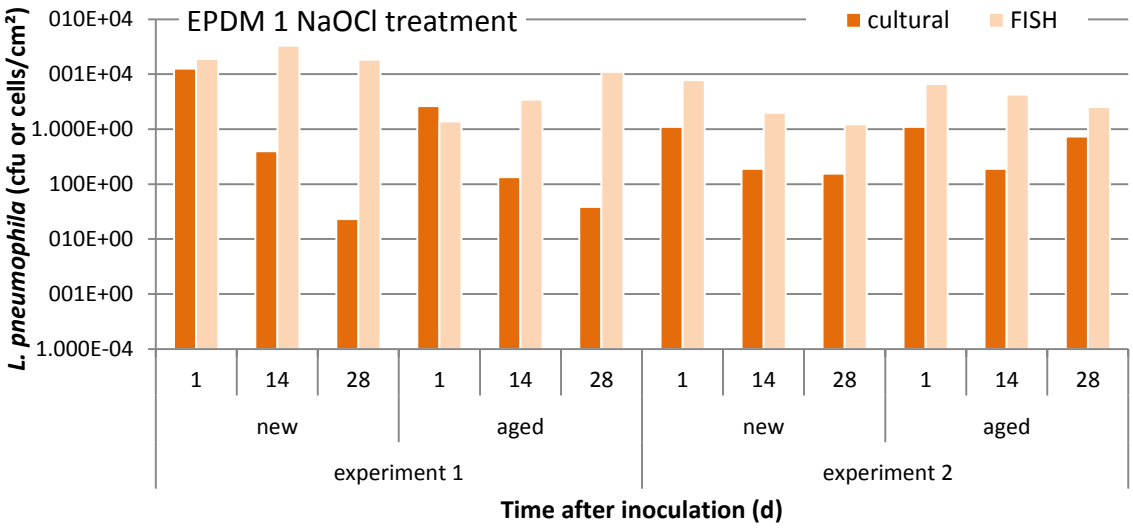

Figure 4.15: Persistence of *L. pneumophila* AdS in drinking-water biofilms on new and aged (NaOCl-treated) EPDM 1 under constant flow conditions. *L. pneumophila* in the biofilms was quantified using cultivation on GVPC agar and FISH (probe Legpne1). Results of two independent experiments are shown.

The concentrations of culturable *L. pneumophila* decreased from day 1 after inoculation to day 28 after inoculation. In most cases the concentrations of FISH-positive *L. pneumophila* were higher than those of culturable *L. pneumophila*.

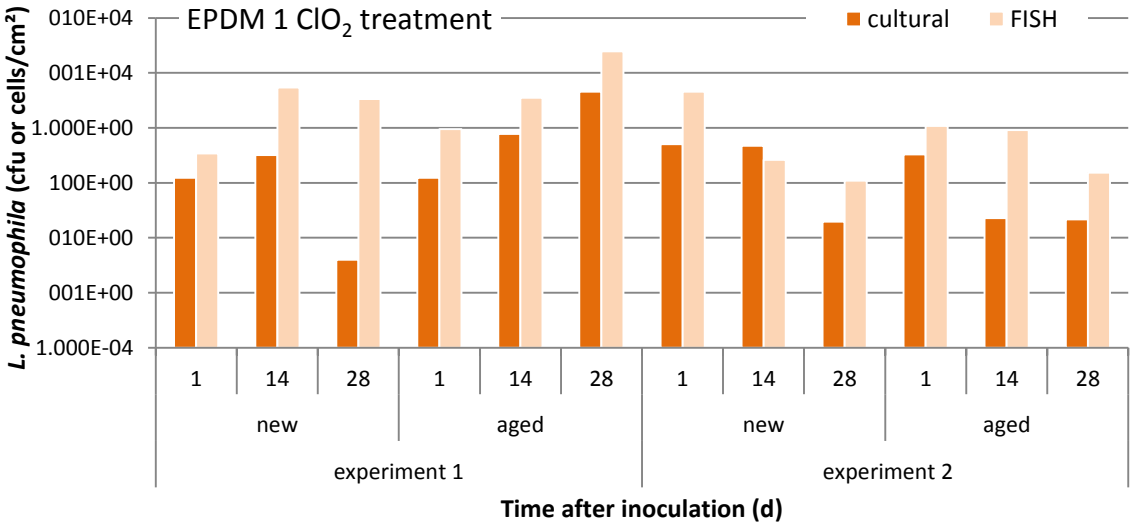

Figure 4.16: Persistence of *L. pneumophila* AdS in drinking-water biofilms on new and aged (ClO_2-treated) EPDM 1 under constant flow conditions. *L. pneumophila* in the biofilms was quantified using cultivation on GVPC agar and FISH (probe Legpne1). Results of two independent experiments are shown.

In both experiments comparing new and ClO_2-treated EPDM 1 (Fig. 4.16), *L. pneumophila* was detectable by culture and FISH for up to 28 days in biofilms grown on both materials. The concentrations of FISH-positive *L. pneumophila* were up to 3 log units higher than the concentrations of culturable *L. pneumophila*.

L. pneumophila was able to colonise biofilms grown on new and NaOCl-treated PE-Xb (Fig. 4.17). In biofilms grown on new PE-Xb, *L. pneumophila* was detected for only 14 d using the FISH method whereas by culture it was detected for 28 d in experiment 1. In experiment 2, both culturable and FISH-positive *L. pneumophila* were detected over the whole period of investigation in biofilms grown on new PE-Xb. In biofilms grown on NaOCl-treated PE-Xb, culturable *L. pneumophila* were detected up to day 28 after inoculation in both experiments; FISH-positive *L. pneumophila* were detected for 1 d in experiment 1 and for up to 28 d in experiment 2.

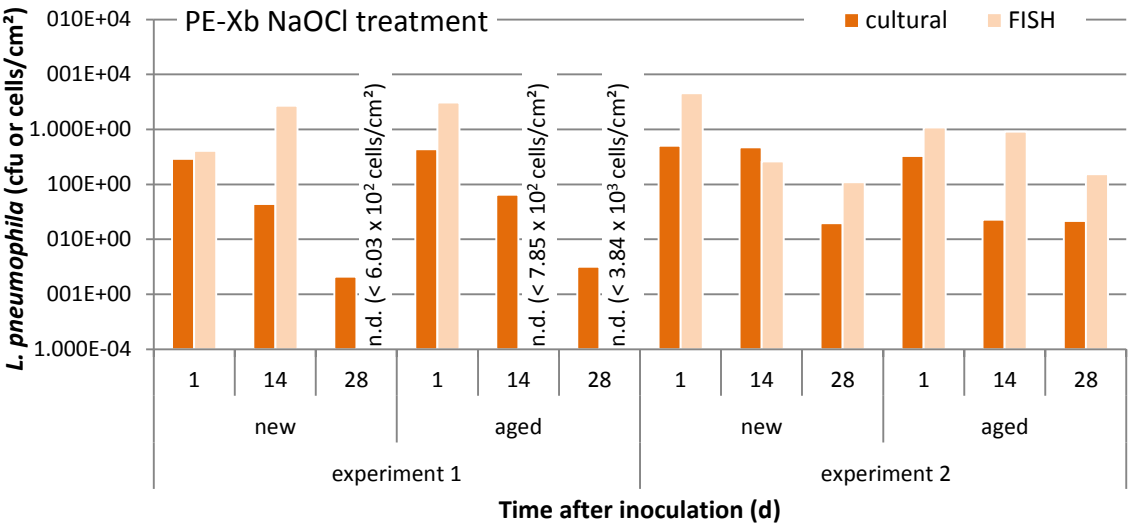

Figure 4.17: Persistence of *L. pneumophila* AdS in drinking-water biofilms on new and aged (NaOCl-treated) PE-Xb under constant flow conditions. *L. pneumophila* in the biofilms was quantified using cultivation on GVPC agar and FISH (probe Legpne1). Results of two independent experiments are shown. n.d. - not detected (the detection limit of the FISH method was calculated individually for each sample and is indicated in the diagram).

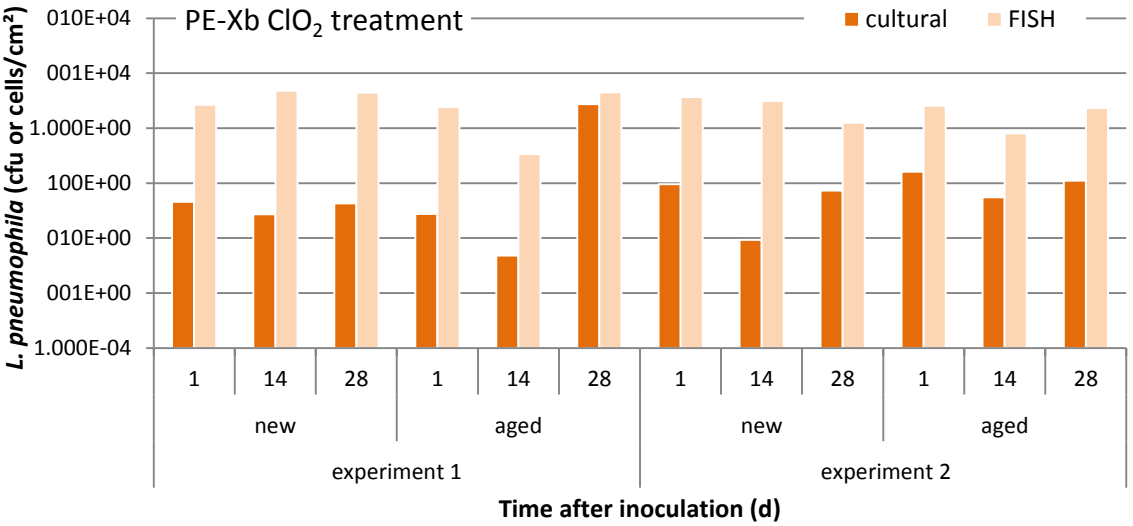

Figure 4.18: Persistence of *L. pneumophila* AdS in drinking-water biofilms on new and aged (ClO_2-treated) PE-Xb under constant flow conditions. *L. pneumophila* in the biofilms was quantified using cultivation on GVPC agar and FISH (probe Legpne1). Results of two independent experiments are shown.

L. pneumophila was able to incorporate into biofilms grown on new and ClO_2-treated PE-Xb (Fig. 4.18). It was detected by culture and with the culture-independent FISH method over the whole period of investigation of 28 days in both experiments in

biofilms grown on new and aged material. The concentrations of FISH-positive *L. pneumophila* were 0.2 to 2 log units higher than those of culturable *L. pneumophila*.

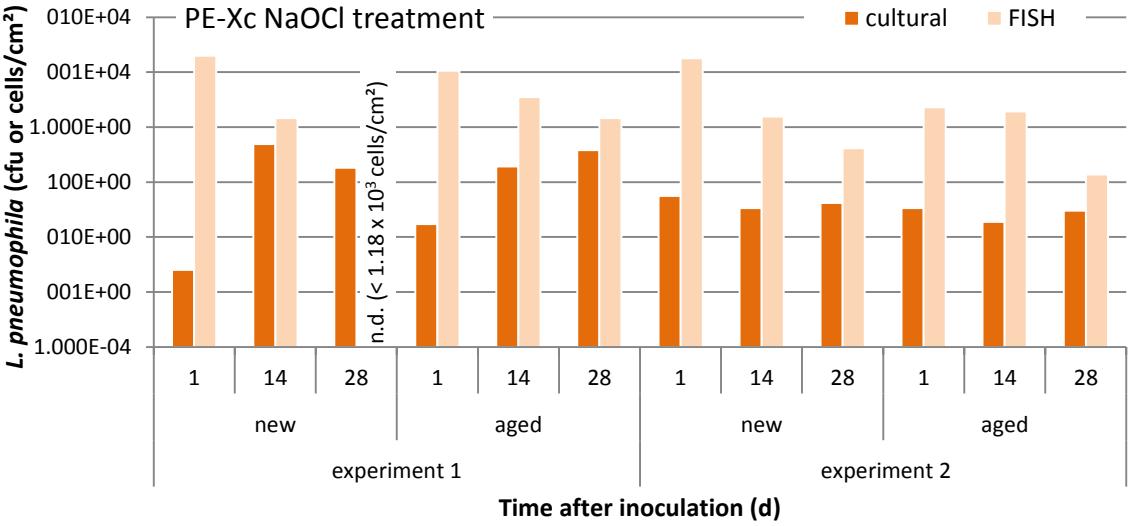

Figure 4.19: Persistence of *L. pneumophila* AdS in drinking-water biofilms on new and aged (NaOCl-treated) PE-Xc under constant flow conditions. *L. pneumophila* in the biofilms was quantified using cultivation on GVPC agar and FISH (probe Legpne1). Results of two independent experiments are shown. n.d. - not detected (the detection limit of the FISH method was calculated individually for each sample and is indicated in the diagram).

In both experiments comparing the persistence of *L. pneumophila* in biofilms grown on new and NaOCl-treated PE-Xc (Fig. 4.19), *L. pneumophila* was recovered from biofilms on both materials by cultivation over the whole period of investigation. In experiment 1, the concentrations of culturable *L. pneumophila* increased over time whereas in experiment 2 they were relatively constant. In biofilms grown on new PE-Xc, *L. pneumophila* was detected using the FISH method for 14 d in experiment 1 and for 28 d in experiment 2. In biofilms grown on NaOCl-treated PE-Xc FISH-positive *L. pneumophila* were detected over the whole period of 1 to 28 d in both experiments. The concentrations of FISH-positive *L. pneumophila* were in most cases higher (0.6 to 4 log units) than those of culturable *L. pneumophila*.

L. pneumophila was capable of colonising biofilms grown on new and aged copper. In biofilms grown on new copper culturable *L. pneumophila* were detected for up to 28 d in experiment 1 and for only 1 d in experiment 2; FISH-positive *L. pneumophila* were detected for 14 d in experiment 1 and for 28 d in experiment 2. In biofilms grown on aged copper quantification of culturable and FISH-positive *L. pneumophila* was possible for up to 28 d after inoculation; over the whole period of investigation *L. pneumophila* was detected using FISH but not using culture-based methods in experiment 2.

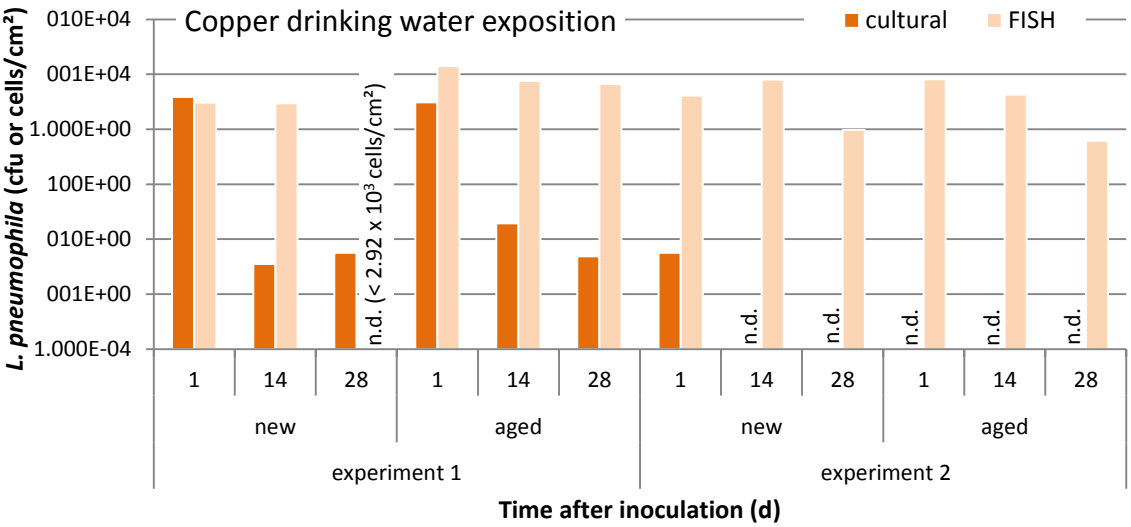

Figure 4.20: Persistence of *L. pneumophila* AdS in drinking-water biofilms on new and aged (exposed to drinking water for ≥ 6 months) copper under constant flow conditions. *L. pneumophila* in the biofilms was quantified using cultivation on GVPC agar and FISH (probe Legpne1). Results of two independent experiments are shown. n.d. - not detected (detection limit of cultivation 0.34 cfu/cm²; the detection limit of the FISH method was calculated individually for each sample and is indicated in the diagram).

Table 4.4 summarises the maximum time of persistence of *L. pneumophila* in drinking water biofilms grown on the different new and aged plumbing materials and the maximum concentrations of *L. pneumophila* detected by culture and using FISH.

Taken together the results of the incorporation experiments, *L. pneumophila* was detected in biofilms on any of the four materials and persisted there for up to 28 days. The concentrations of both culturable and FISH-positive *L. pneumophila* varied between new and aged material, between different sampling events and between the two independent experiments. In most of the experiments with the exception of ClO_2-treated EPDM and PE-Xb and NaOCl-treated PE-Xc, the concentration of culturable *L. pneumophila* decreased over time; this effect was most pronounced in biofilms grown on new and aged copper. A direct influence of material ageing by treatment with NaOCl or ClO_2 on the incorporation of *L. pneumophila* into drinking water biofilms grown on domestic plumbing materials was not observed.

Table 4.3: Maximum time of persistence and maximum concentration at maximum time of persistence of *L. pneumophila* AdS in drinking water biofilms grown on new and aged EPDM 1, PE-Xb, PE-Xc and copper. *L. pneumophila* in the biofilms was quantified using cultivation on GVPC agar and FISH (probe Legpne1). Maximum results of four (new EPDM 1 and PE-Xb) or two (new PE-Xc and copper; NaOCl-treated EPDM 1, PE-Xb and PE-Xc; ClO_2-treated EPDM 1 and PE-Xb) independent experiments are shown.

Material	Maximum time of persistence (d)		Maximum concentration at maximum time of persistence	
	Detected culturally	Detected with FISH	Cultural (cfu/cm²)	FISH (cells/cm²)
EPDM 1 new	28	28	1.55×10^2	1.84×10^4
EPDM 1 NaOCl-treated	28	28	7.38×10^2	1.10×10^4
EPDM ClO_2-treated	28	28	4.55×10^3	1.46×10^5
PE-X b new	28	28	7.27×10^1	4.44×10^3
PE-X b NaOCl-treated	28	28	$2.14\ 10^1$	1.54×10^2
PE-X b ClO_2-treated	28	28	2.73×10^3	4.51×10^3
PE-X c new	28	28	1.81×10^2	4.11×10^2
PE-X c NaOCl-treated	28	28	3.80×10^2	1.46×10^3
Copper new	28	28	5.62	9.70×10^2
Copper drinking water exposition	28	28	4.89	6.61×10^3

The concentrations of culturable *L. pneumophila* were generally higher compared to culturable *P. aeruginosa*. In only 7 % of the samples the concentrations of culturable *L. pneumophila* were < 1 cfu/cm², whereas they were > 100 cfu/cm² in 44 % of the samples. The difference between the number of *L. pneumophila* detected by culture and by FISH was not as high as in the case of *P. aeruginosa*. However, in some of the samples concentrations of FISH-positive *L. pneumophila* were 3 log units higher than the concentrations of culturable *L. pneumophila* and in 85 % of the samples, the percentage of culturable *L. pneumophila* on FISH-positive *L. pneumophila* was < 25 % (< 1 in 42 % of the samples).

4.5.4. Incorporation of *E. nimipressuralis* into drinking water biofilms

In order to investigate the incorporation of *E.nimipressuralis*, 14 d-old drinking water biofilms were inoculated with *P. aeruginosa* AdS, *L. pneumophila* AdS (serogroup 1) and *E. nimipressuralis* 9827 clone A. *E. nimipressuralis* in the biofilms was moni-

tored for 4 weeks using the Colilert-18 Quanti-Tray®/2000 system (IDEXX; 3.6.5.) and using the culture-independent FISH (3.7.1.1.). The coliform bacterium *E. nimipressuralis* was not detected culturally in any of the biofilms grown on new and aged plumbing materials. Since no oligonucleotide probe specific for this bacterial species was available, FISH could not be performed to detect possibly unculturable organisms in the biofilms.

4.5.5. Characterisation of *Pseudomonas* and *Legionella* isolates

P. aeruginosa and *L. pneumophila* were isolate from drinking water biofilms and effluent water. In order to confirm that the strains that were originally inoculated had persisted in the biofilms the isolates were characterised. For *P. aeruginosa* isolates pigment production, cytochrome oxidase reaction and the biochemical profiles in the API 20 NE system were investigated. All *P. aeruginosa* isolates from the inoculum, from biofilms and from reactor effluents exhibited the typical green pigmentation on CN agar and a positive cytochrome oxidase reaction. The biochemical profiles of all biofilm and water isolates of *P. aeruginosa* were identical to that of the *P. aeruginosa* isolates from the inoculum.

L. pneumophila isolates were confirmed by growth on BCYEα agar and by their inability to form colonies on nutrient agar at 36 °C within 3 d and their serogroup was determined using a commercially available latex agglutination test kit. All *L. pneumophila* isolates from the inoculum, from biofilms and from reactor effluents failed to grow on nutrient agar and were shown to belong to serogroup 1.

In addition to microbiological and biochemical characterisation and serotyping, genotyping of *P. aeruginosa* and *L. pneumophila* isolates was performed by pulsed-field gel electrophoresis (PFGE). The electrophoretic patterns (the “genetic fingerprints”) generated by this technique are highly specific and allow the identification of distinct strains.

In all cases, PFGE analysis of *P. aeruginosa* and *L. pneumophila* re-isolated from biofilms and reactor effluent over the 4-week period after inoculation always showed the same macrorestriction patterns compared to those of the bacteria used for inoculation of the biofilms (figs. 4.21 and 4.22), confirming that the original strains had persisted in the biofilms and no secondary contamination with other *P. aeruginosa* or *L. pneumophila* clones from the influent water had occurred. Figures 4.21 and 4.22 exemplarily show the macrorestriction patterns of *P. aeruginosa* and *L. pneumophila* isolates from biofilms grown on new and NaOCl-treated EPDM 1 and PE-Xb and from the corresponding reactor effluents.

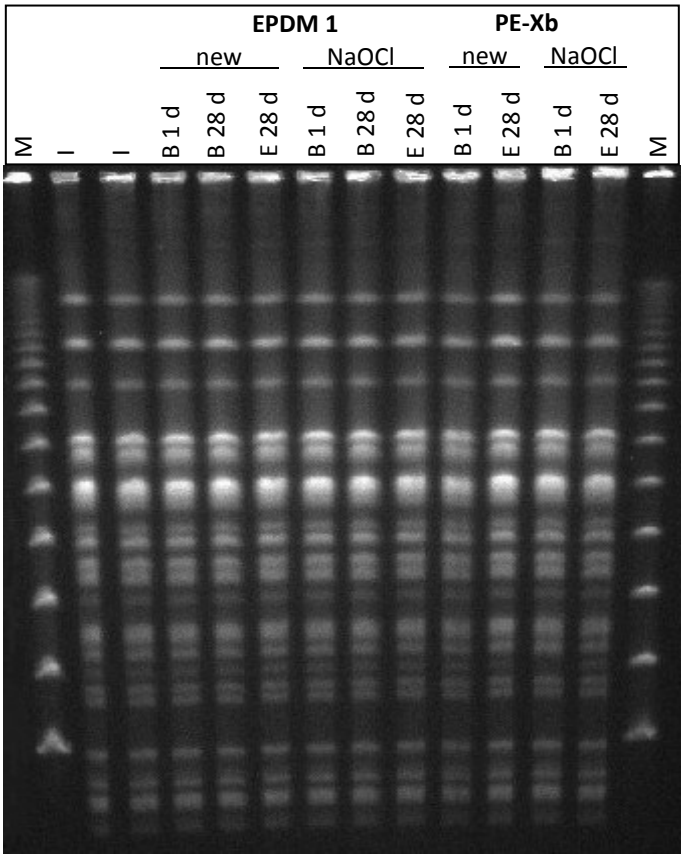

Figure 4.21: PFGE analysis of *P. aeruginosa* isolates from biofilms grown on new and NaOCl-treated EPDM 1 and on new and NaOCl-treated PE-Xb and from the corresponding reactor effluents. M – DNA size marker, I - inoculum, B - biofilm, E - effluent water.

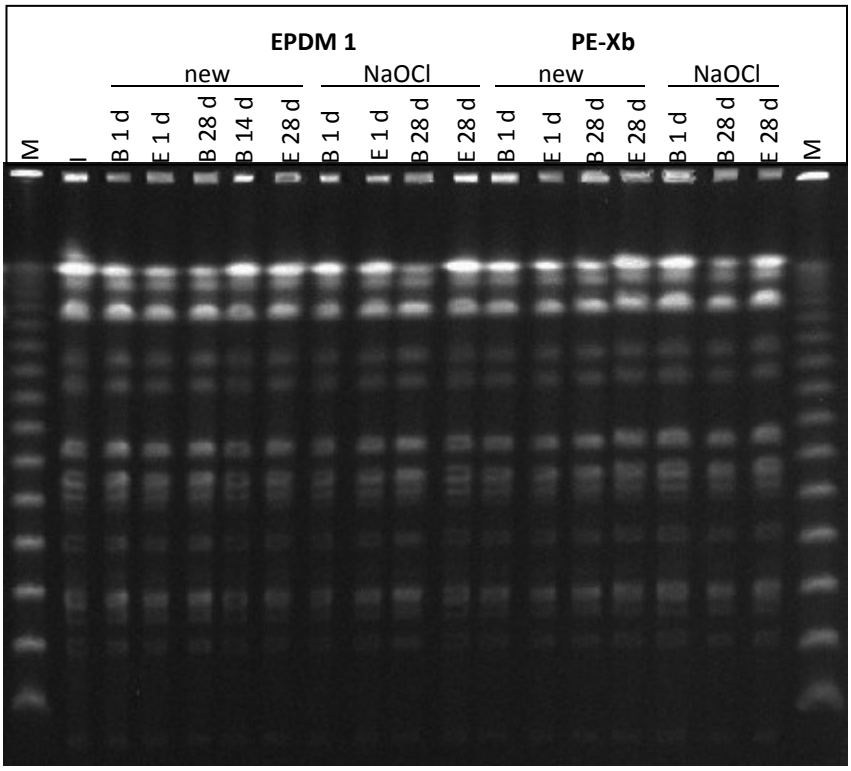

Figure 4.22: PFGE analysis of *L. pneumophila* isolates from biofilms grown on new and NaOCl-treated EPDM 1 and on new and NaOCl-treated PE-Xb and from the corresponding reactor effluents. M - DNA size marker, I - inoculum, B - biofilm, E - effluent water.

4.6. Amoebae in drinking water biofilms grown on domestic plumbing materials

In drinking water biofilms free-living amoebae play an important role in the control of biofilms as well as in the persistence and multiplication of certain bacteria. By grazing, amoebae can significantly reduce biomass, but certain bacteria such as *L. pneumophila* survive ingestion by amoebae and use them as host cells and for intracellular multiplication. Therefore, the presence of amoebae in drinking water biofilms grown on domestic plumbing materials was investigated. Biofilms were grown on new EPDM 1, PE-Xb, PE-Xc and copper under constant flow conditions and inoculated with *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* after 14 d. The presence of protozoa in general and amoebae was determined using the FISH method with oligonucleotide probes specific for *Eukarya*, *Hartmannella* spp. and *Acanthamoeba* spp.. During the period of biofilm growth and monitoring protozoa and amoebae in the biofilm, the mean temperature of the water inside the flow-through reactors was 19.2 °C ± 1.1 °C (range 18.3 °C - 21.0 °C; n = 6). The average temperature of the influent water was 17.6 °C ± 1.9 °C (range 16.3 °C - 20.7 °C; n = 5) and the mean pH of the influent water was 8.0 ± 0.1 (range 7.9 - 8.0; n = 3).

The average total cell counts on EPDM 1, PE-Xb, PE-Xc and copper were 3.82 x 10^6 cells/cm², 4.85 x 10^5 cells/cm², 1.41 x 10^5 cells/cm² and 1.55 x 10^5 cells/cm², respectively, before inoculation with *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis*. No significant change in the concentrations of total cells was observed after inoculation. *Eukarya* as well as *Hartmannella* and *Acanthamoeba* spp. were detected in biofilms grown on all materials both before and after inoculation (Fig. 4.23).

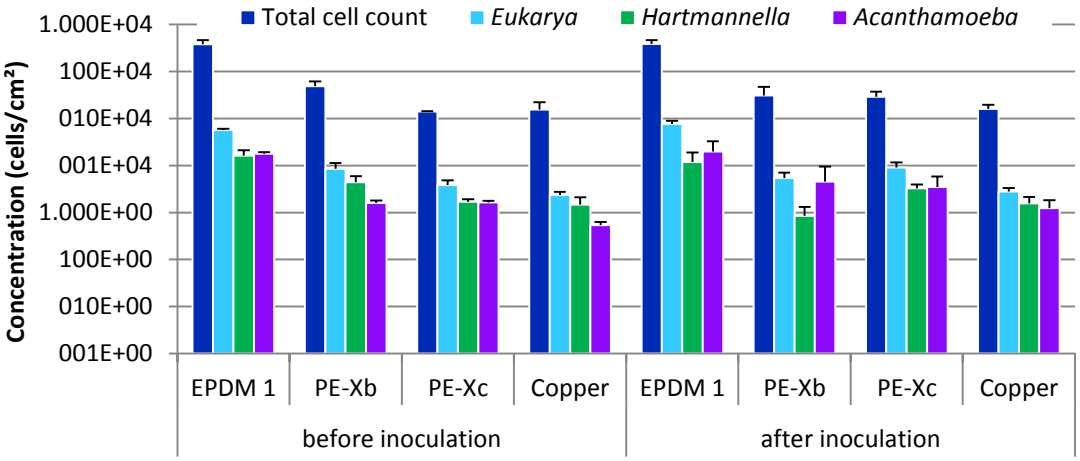

Figure 4.23: Total cell counts and concentrations of *Eukarya*, *Hartmannella* spp. and *Acanthamoeba* spp. in drinking water biofilms grown on EPDM 1, PE-Xb, PE-Xc and copper before and 1 d after inoculation with *P. aeruginosa* AdS, *L. pneumophila* AdS and *E. nimipressuralis* 9827 clone A. The total cell count was determined by DAPI-staining. The concentrations of *Eukarya*, *Hartmannella* spp. and *Acanthamoeba* spp. were determined using FISH (probes EUK516, HART498 and GSP, respectively). The results are averages of two independent experiments.

All organisms detected using the probe specific for *Eukarya* were identified as protozoa under the epifluorescence microscope; no multicellular organisms were observed. Before and after inoculation the highest concentrations of *Eukarya* and *Acanthamoeba* spp. were detected in biofilms grown on EPDM 1 and the lowest in biofilms grown on copper. The concentration of *Hartmannella* spp. was highest on EPDM before and after inoculation; the lowest concentration of *Hartmannella* spp. before inoculation was measured in biofilms on copper and after inoculation it was lowest on PE-Xc. A strong correlation between total cell count and the concentration of *Eukarya* (R^2 = 0.95), *Hartmannella* spp. (R^2 = 0.89) and *Acanthamoeba* spp. (R^2 = 0.84) was observed. The concentrations of FISH-positive *Acanthamoeba* spp. were slightly higher in biofilms on all materials after inoculation than before. There was no significant difference in the concentrations of *Eukarya* and *Hartmannella* spp. in biofilms before or after inoculation. 10.3 % to 80.3 % of FISH-positive amoebae belonged to the genus *Hartmannella*. The number of FISH-positive *Acanthamoeba* spp. accounted for 16.7 % to 67.4 % of the total FISH-positive amoebae detected by the *Eukarya* probe.

The results show that protozoa, predominantly amoebae are present in drinking water biofilms grown on all tested plumbing materials. These amoebae may either serve as a host or feed on bacteria in the biofilms. The presence of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* does not significantly influence the presence or concentrations of *Eukarya*, *Hartmannella* spp. and *Acanthamoeba* spp. in drinking water biofilms over the short time of 2 d examined in the present study.

4.7. Interaction of *P. aeruginosa*, *L. pneumophila* and *A. castellanii*

It is well known that amoebae and potentially pathogenic bacteria such as *Legionella* spp. can share drinking water biofilms as a habitat. In the present study, it has been shown that amoebae such as *Acanthamoeba* spp. are present in drinking water biofilms (4.6.) and that the potentially pathogenic bacteria *P. aeruginosa* and *L. pneumophila* are able to integrate into established drinking water biofilms (4.5.2. and 4.5.3.). In order to analyse the possible interactions between the bacteria *P. aeruginosa* and *L. pneumophila* and the amoeba *Acanthamoeba castellanii,* the three organisms were co-incubated in liquid culture under nutrient-rich conditions and under nutrient limitation. Table 4.4 gives an overview of the interactions between the *P. aeruginosa*, *L. pneumophila* and *A. castellanii* under the different nutrient conditions. The detailed results are described and shown in section 4.7.2. and 4.7.2. and in Figures 4.24 to 4.29.

Table 4.4: Growth and survival of *P. aeruginosa*, *L. pneumophila* and *A. castellanii* incubated in in pairs and in combination of all three organisms under nutrient-rich and nutrient-limited conditions over a period of 7 d (nutrient-rich) or 3 d (nutrient-limited). *P. aeruginosa* and *L. pneumophila* were quantified by cultivation on CN and GVPC agar, respectively, and using FISH. *A. castellanii* was quantified using FISH.

Conditions during incubation	**Pa + Lp**		**Pa + Ac**		**Lp + Ac**		**Pa + Lp + Ac**		
nutrient-rich	↑↑↑	↓↓	↑↑↑	↓↓↓	↑↑	↓↓↓	↑↑↑	↓↓↓	↓↓↓
nutrient-limited	–	↓	↑↑	–	↑	–	↑	↑	–

– constant cell numbers
↑ increase by <2 log units ↑↑ increase by >2 to <4 log units ↑↑↑ increase by >4 log units
↓ decrease by <2 log units ↓↓ decrease by >2 to <4 log units ↓↓↓ decrease to below detection limit

4.7.1. Interaction of *P. aeruginosa*, *L. pneumophila* and *A. castellanii* under nutrient-rich conditions

Interactions between *P. aeruginosa*, *L. pneumophila* and *A. castellanii* were investigated in co-cultivation experiments under growth-promoting conditions in proteose peptone yeast extract glucose (PYG) broth. Preliminary experiments on the cultivation of the three organisms showed that this medium was suitable for growth or at least survival of *P. aeruginosa*, *L. pneumophila* and *A. castellanii* over a period of 7d. *P. aeruginosa*, *L. pneumophila*, and *A. castellanii* were incubated in PYG in pure and mixed cultures in six-well microtitre plates under static conditions at 30 °C. Initial concentrations of the bacteria were 1 x 10^6 cells/mL and the concentration of *A. castellanii* was adjusted to 1 x 10^5 cells/mL. Sampling was performed at defined time points (0 h, 24 h and 7 d). Growth of *P. aeruginosa* and *L. pneumophila* was examined by determination of colony forming units (cfu) on nutrient agar and GVPC selective agar, respectively. *A. castellanii* growth was evaluated by vital staining with trypan blue and microscopic counting using a Neubauer improved chamber. In addition to that, the three organisms were quantified by FISH using probe Psae16S-182 for quantifying *P. aeruginosa*, probe Legpne1 for quantifying *L. pneumophila* and probe GSP for the quantification of *A. castellanii.*

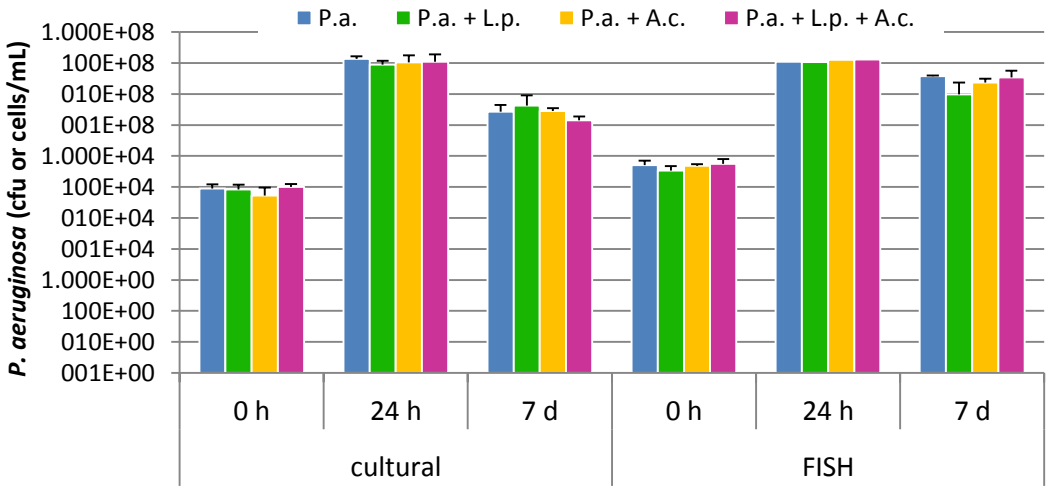

Figure 4.24: Growth of *P. aeruginosa* AdS either as pure culture (P.a.) or co-cultivated with *L. pneumophila* AdS (P.a. + L.p.) or *A. castellanii* ATCC 30234 (P.a. + A.c.) or both (P.a. + L.p. + A.c.) in PYG medium. Cultures were incubated under static conditions at 30 °C. *P. aeruginosa* was quantified by cultivation on nutrient agar and FISH using probe Psae 16S-182. Results are averages of two independent experiments with the exception of the 24 h values obtained by the FISH method, which only represent one single experiment.

P. aeruginosa was able to grow to high numbers (up to 10^{10} cfu/mL or FISH-positive cells/mL after 24 h of incubation) in PYG medium (Fig. 4.24). Concentrations of FISH-positive *P. aeruginosa* in co-cultures with *L. pneumophila*, *A. castellanii* or both did not differ from those of *P. aeruginosa* pure cultures indicating that under nutritionally favourable conditions the growth of *P. aeruginosa* is neither significantly inhibited nor enhanced by the presence of *L. pneumophila* or *A. castellanii* or both.

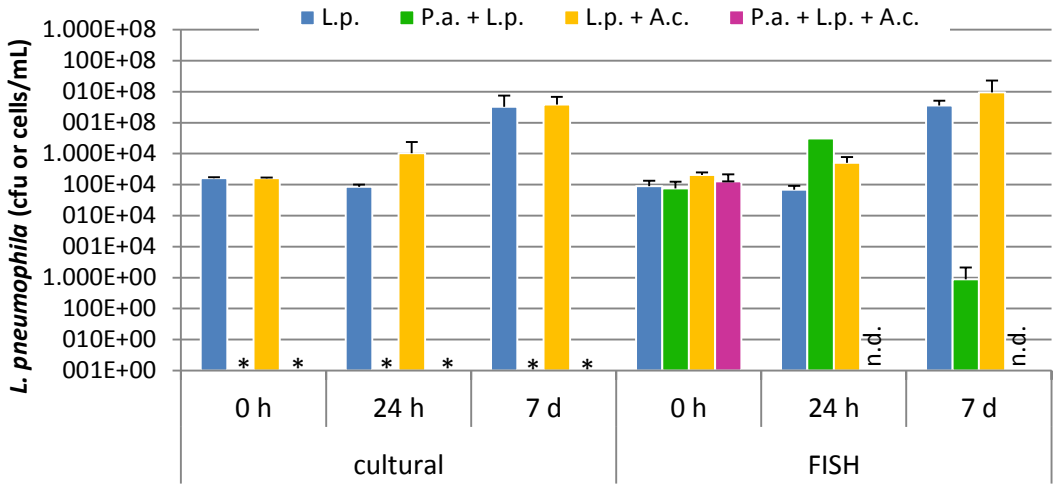

Figure 4.25: Growth of *L. pneumophila* AdS either as pure culture (L.p.) or co-cultivated with *P. aeruginosa* AdS (L.p. + P.a.) or *A. castellanii* ATTC 30234 (L.p. + A.c.) or both (P.a. + L.p. + A.c.) in PYG medium. Cultures were incubated under static conditions at 30 °C. *L. pneumophila* was quantified by cultivation on GVPC agar with acid treatment and FISH using probe Legpne1. Results are averages of two independent experiments with the exception of the 24 h value of P.a. + L.p. obtained by the FISH method, which only represents one single experiment. n.d. - not detected. Detection limit FISH 10^3 cells/mL. * cultivation of *L. pneumophila* was not possible in suspensions containing *P. aeruginosa* because of overgrowth of *P. aeruginosa* on GVPC agar.

Survival and growth of *L. pneumophila* in pure culture and in co-culture with *P. aeruginosa* or *A. castellanii* or both under nutrient rich conditions is shown in Fig. 4.25. *L. pneumophila* could not be quantified in cultures containing *P. aeruginosa*, because GVPC plates were overgrown by *P. aeruginosa* within a few days despite acid treatment of samples in order to inhibit growth of bacteria other than *Legionella*e. The concentrations of *L. pneumophila* after 24 h and 7 d of incubation in PYG were lower compared to those of *P. aeruginosa* (Fig. 4.24). Growth of *L. pneumophila* in PYG medium was observed after incubation of > 24 h. After 7 d of incubation in pure culture the concentration of *L. pneumophila* reached 3 x 10^8 cfu/mL or FISH-positive cells/mL. In co-cultures with *A. castellanii*, approximately 3 x 10^8 cfu/mL and 10^9 FISH-positive cells/mL were detected. In co-culture with *P. aeruginosa*, the concentration of FISH-positive *L. pneumophila* had decreased by almost 5 log units until day 7 of incubation. No FISH-positive *L. pneumophila* were detected in suspensions containing *P. aeruginosa*, *L. pneumophila* and *A. castellanii* after 24 h and after 7 d of incubation. The results show that under nutrient-rich conditions growth of *L. pneumophila* is neither enhanced nor inhibited by the presence of *A. castellanii*, but decreased in the presence of *P. aeruginosa*.

Growth and survival of *A. castellanii* detected by the FISH method are shown in Fig. 4.26. Using trypan blue staining and phase contrast microscopy because of the low number of amoebal cells, only a general evaluation concerning the increase or decrease of the number of amoeba cells and cysts could be made.

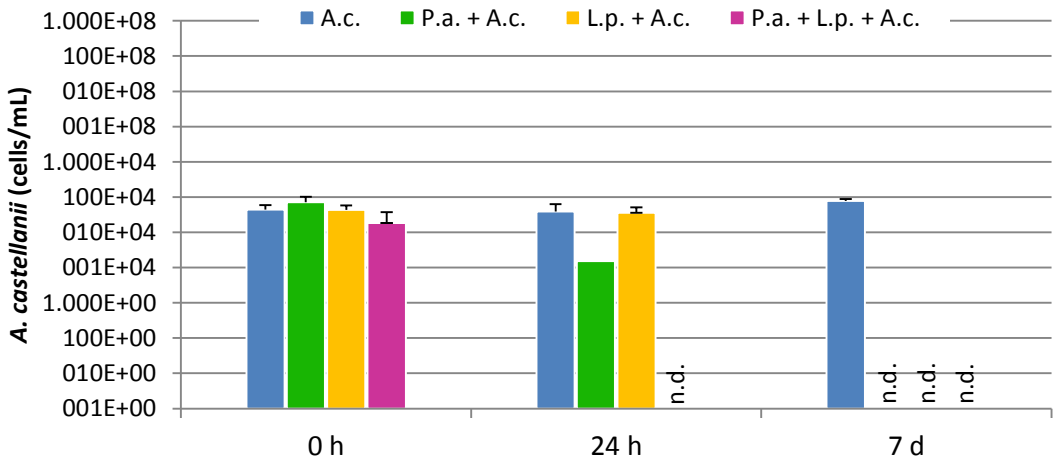

Figure 4.26: Growth of *A. castellanii* ATCC 30234 either as pure culture (A.c.) or co-cultivated with *P. aeruginosa* AdS (P.a. + A.c.) or *L. pneumophila* AdS (L.p. + A.c.) or both (P.a. + L.p. + A.c.) in PYG medium. Cultures were incubated under static conditions at 30 °C. *A. castellanii* was quantified by FISH using probe GSP. Results are averages of two independent experiments with the exception of the 24 h value of P.a. + A.c., which only represents one single experiment. n.d. - not detected. Detection limit FISH 10^3 cells/mL.

Microscopic observations (phase contrast microscopy) showed a slight increase in *A. castellanii* cells and in the number of cysts for the pure culture over the whole period of the experiment. After 7 d of incubation the number of vegetative cells was still higher than the number of cysts and Trypan blue staining (3.6.9.) showed far more viable than dead cells. The concentration of FISH-positive *A. castellanii* in pure culture remained relatively constant with a slight increase after 7 d of incubation in PYG (Fig. 4.26). In co-culture with *P. aeruginosa* the number of FISH-positive *A. castellanii* decreased to 10^4 cells/mL after 24 h and to below the detection limit after 7 d of incubation (Fig. 4.26). No amoebal cells were detected already after 24 h of incubation using trypan blue staining and phase microscopy. In the culture containing *A. castellanii* and *L. pneumohpila* encystment of trophozoites and bacteria-containing cysts were observed under the phase microscope after 24 h. The majority of amoebal cells were determined to be alive according to trypan blue staining. After 7 d of incubation, no living *A. castellanii* were observed anymore. The concentration of FISH-positive *A. castellanii* in the co-culture with *L. pneumophila* remained constant during the first 24 h of incubation, but fell to below detection limit after 7 d of incubation (Fig. 4.26). In the culture containing *A. castellanii*, *P. aeruginosa,* and *L. pneumophila* no amoebal cells were detected after 24 h of incubation, neither by phase contrast microscopy nor by FISH.

Altogether, the results of the co-cultivation of *P. aeruginosa*, *L. pneumophila* and *A. castellanii* under nutrient-rich conditions show that *P. aeruginosa* is the dominant organism impairing growth and survival of both *L. pneumophila* and *A. castellanii*.

When co-cultured only with *A. castellanii, L. pneumophila* was shown to enter the amoebae within 24 h of incubation and to completely kill *A. castellanii* within 7 d. However, intracellular growth of *L. pneumophila* did not result in a strong increase in the number of culturable or FISH-positive *L. pneumophila* compared to *L. pneumophila* grown in pure culture under nutritionally favourable conditions (Fig. 4.25). In co-cultures of all three organisms the presence of *A. castellanii* did not protect *L. pneumophila* from the bactericidal action of *P. aeruginosa*, but led to an even stronger decrease in *L. pneumophila* concentrations compared to those in the co-culture of *P. aeruginosa* and *L. pneumophila* without amoebae (Fig. 4.25). Accordingly, a stronger suppression of amoebae was observed in the presence of both *P. aeruginosa* and *L. pneumophila* compared to co-cultures of *A. castellanii* with *P. aeruginosa* alone (Fig. 4.26).

4.7.2. Interaction of *P. aeruginosa*, *L. pneumophila* and *A. castellanii* under non-growing conditions

In order to investigate the interaction of *P. aeruginosa*, *L. pneumophila*, and *A. castellanii* under nutrient-limited conditions where no proliferation of any of the three organisms was expected, Page's Amoeba Saline (PAS; 2.3.2.) was chosen as an alternative medium for co-incubation experiments.

P. aeruginosa, *L. pneumophila*, and *A. castellanii* were incubated, sampled and detected as described in section 4.7.1. Sampling was performed at 0, 24, 48 and 72 h after incubation.

P. aeruginosa was able to survive in PAS in the presence or absence of *L. pneumophila* without any significant increase or decrease in the concentrations of culturable and FISH-positive cells over a period of 72 h (Fig. 4.27). In co-culture with *A. castellanii*, the number of culturable and FISH-positive *P. aeruginosa* increased to concentrations (5.6 x 10^6 cfu/mL and 8.7 x 10^8 cells/mL) almost as high as the concentration of *P. aeruginosa* that was reached under nutrient-rich conditions (Fig. 4.24) and more than 2 log units higher than the concentration of *P. aeruginosa* as a pure culture in PAS. In the suspension containing all three organisms, the concentration of culturable and FISH-positive *P. aeruginosa* constantly increased during the whole period of incubation and reached 4.6 x 10^7 cfu/mL and 1.2 x 10^8 cells/mL, respectively.

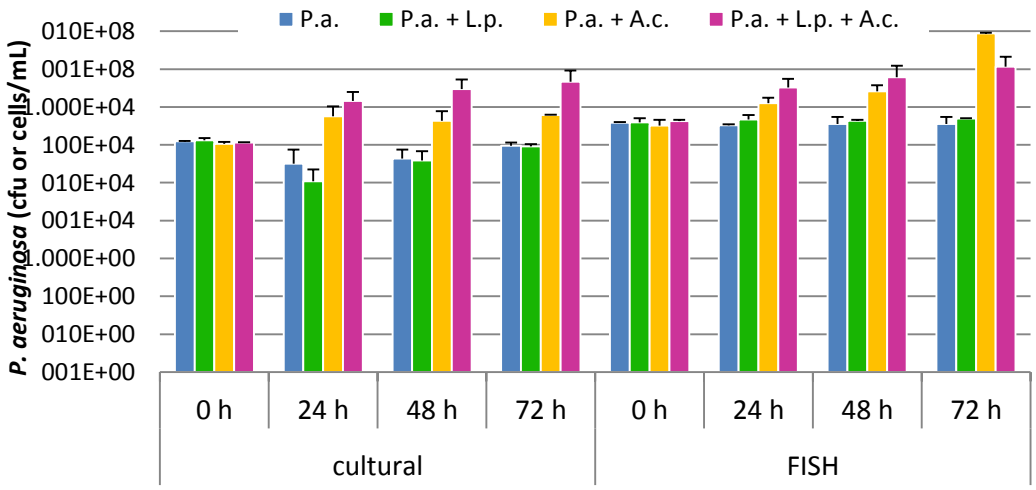

Figure 4.27: Quantification of *P. aeruginosa* AdS either as pure culture (P.a.) or co-cultivated with *L. pneumophila* AdS (P.a. + L.p.) or *A. castellanii* ATCC 30234 (P.a. + A.c.) or both (P.a. + L.p. + A.c.) in PAS. Cultures were incubated under static conditions at 30 °C. *P. aeruginosa* was quantified by cultivation on nutrient agar and FISH using probe Psae 16S-182. Results are averages of two independent experiments.

These results indicate that the presence of *A. castellanii* enables *P. aeruginosa* to multiply without any source of organic carbon and to reach concentrations comparable to those obtained in the nutrient-rich medium PYG.

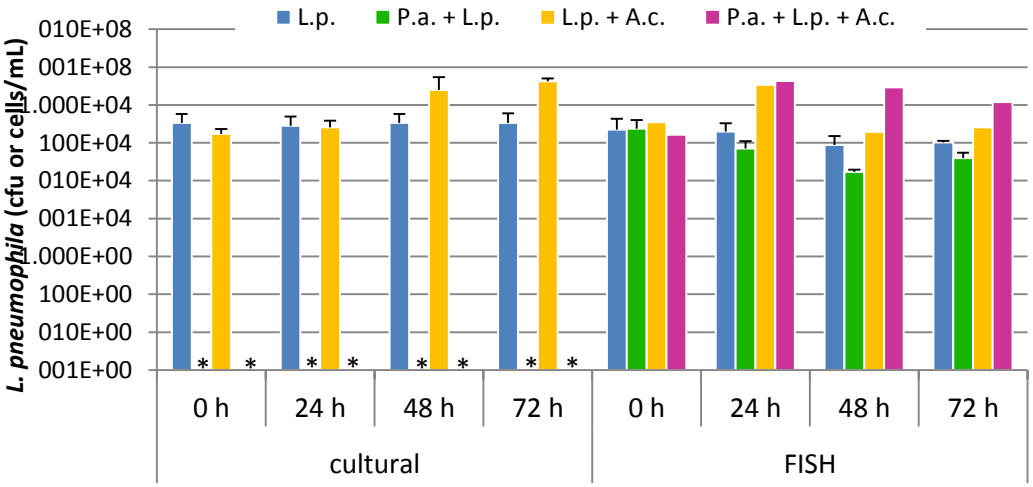

Figure 4.28: Quantification of *L. pneumophila* AdS either as pure culture (L.p.) or co-cultivated with *P. aeruginosa* AdS (L.p. + P.a.) or *A. castellanii* ATCC 30234 (L.p. + A.c.) or both (P.a. + L.p. + A.c.) in PAS. Cultures were incubated under static conditions at 30 °C. *L. pneumophila* was quantified by cultivation on GVPC agar with acid treatment and FISH using probe Legpne1. Results are averages of two independent experiments with the exception of the values for L.p. + A.c. and P.a. + L.p. + A.c. obtained by the FISH method, which only represents one single experiment. * cultivation of *L. pneumophila* was not possible in suspensions containing *P. aeruginosa* because of overgrowth of *P. aeruginosa* on GVPC agar.

Survival of *L. pneumophila* in pure culture and in co-culture with *P. aeruginosa* or *A. castellanii* or both under nutrient-limited conditions is shown in Fig. 4.28. As mentioned above, *L. pneumophila* could not be quantified in cultures containing *P. aeruginosa*, because of overgrowth of *P. aeruginosa* on GVPC plates. No growth occurred in the *L. pneumophila* pure culture. In the presence of *A. castellanii*, *L. pneumophila* started to multiply after 48 h and a concentration of 4.2 x 10^7 cfu/mL and was reached after 72 h of incubation. An increase in FISH-positive *L. pneumophila* was observed in co-cultures of *L. pneumophila* and *A. castellanii* and in cultures containing all three organisms whereas in co-cultures of *L. pneumophila* and *P. aeruginosa* a decrease in the concentration of FISH-positive *L. pneumophila* was detected. The results show that *L. pneumophila* was able to survive under nutrient-limited conditions at 30 °C over a period of 72 h. In the presence of *A. castellanii* or both *A. castellanii* and *P. aeruginosa*, *L. pneumophila* was able to multiply. The presence of *P. aeruginosa* alone slightly impaired the survival of *L. pneumophila*.

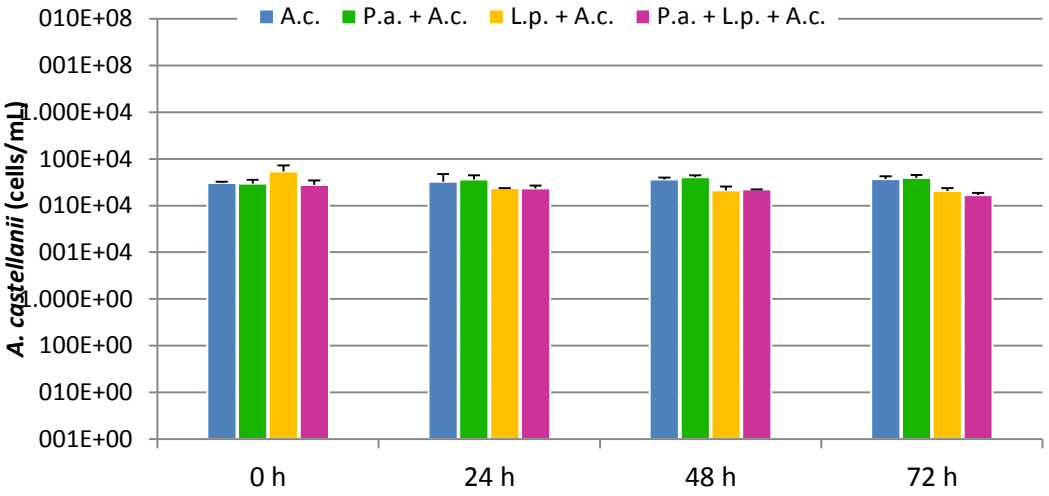

Figure 4.29: Quantification of *A. castellanii* ATCC 30234 either as pure culture (A.c.) or co-cultivated with *P. aeruginosa* AdS (P.a. + A.c.) or *L. pneumophila* AdS (L.p. + A.c.) or both (P.a. + L.p. + A.c.) in PAS. Cultures were incubated under static conditions at 30 °C. *A. castellanii* was quantified by FISH using probe GSP. Results are averages of two independent.

No growth of amoebae was observed in the *A. castellanii* in any of the three microbial suspensions (Fig. 29). In *A. castellanii* pure cultures encystation started after 24 h of incubation, after 72 h of incubation the number of cysts exceeded the number of vegetative cells in the culture. In the culture containing *L. pneumophila* and *A. castellanii,* encystment of trophozoites occurred after 24 h and amoebal cells containing bacteria as well as dead amoebae were observed. After 48 h of incubation almost all amoebal cells present in the culture were cysts. In the culture containing *P. aeruginosa* and *A. castellanii* amoebae cells remained in the vegetative and vital form to a great extent over the whole period of incubation. Examination of the culture containing *P. aeruginosa, L. pneumophila*, and *A. castellanii* showed a decrease of amoebal cells over the period of 72 h. After 24 h of incubation, trophozoites started to encyst and cysts containing bacteria were observed. After 72 h the numbers of intact amoebae equalled the number of dead amoebae according to trypan blue vital staining.

The experiment showed that *A. castellanii* is capable to survive in PAS over a period of 72 h at 30 °C. Although vital staining showed part of the amoebal population present in pure and mixed cultures to be damaged, they still possessed enough ribosomal RNA to be detectable using FISH, indicating that they were metabolically active.

On the whole, the results of the co-cultivation of *P. aeruginosa*, *L. pneumophila* and *A. castellanii* show that *P. aeruginosa* did not significantly impair the survival of *L. pneumophila* or *A. castellanii* under nutrient-limited conditions. Multiplication of *P.*

aeruginosa without addition of nutrients was only possible in the presence of *A. castellanii* or of both *A. castellanii* and *L. pneumophila*. Similarly, the presence of *A. castellanii* or *A. castellanii* and *P. aeruginosa* lead to an increase in *L. pneumophila* concentrations in PAS.

4.8. Copper sensitivity of *P. aeruginosa*

The preparation of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* for the inoculation of drinking water biofilms included a starving period of 24 h in sterile drinking water in order to adapt the bacteria to drinking water conditions. A significant drop in culturability was observed for *P. aeruginosa* after incubation in drinking water (see Fig. 4.9). In addition to that, the concentrations of culturable *P. aeruginosa* in drinking water biofilms were considerably lower than the number of FISH-positive *P. aeruginosa* (see figs. 4.10 - 4.14). For the preparation of target organisms for inoculation as well as for cultivation of drinking water biofilms drinking water from a copper plumbing system containing average copper concentrations of 27 µM (0.017 mg/L; Tab.4.5) was used. Thus, the hypothesis that copper ions may impair bacterial viability was established. The influence of copper ions on the survival and physiological state of bacteria was analysed exemplarily for *P. aeruginosa*.

4.8.1. Effect of copper on the culturability of *P. aeruginosa*

Investigations of the impact of copper on bacterial survival have frequently been performed in growth media or mineral salt solutions (Harrison et al., 2005; Teitzel and Parsek, 2003). In the present study the influence of copper on *P. aeruginosa* was to be investigated under concentrations that are relevant to drinking water environments. For this purpose, bacterial suspensions of *P. aeruginosa* AdS with a density of approximately 10^6 cells/mL were exposed to different drinking water samples with variation in their copper content for 24 h. Deionized water served as a reference medium; it was used (i) without an additive, representing water not containing any substances which might possibly interfere with the biological effect of copper and (ii) supplemented with copper ($CuSO_4$ x 5 H_2O) added in defined concentrations. The copper concentrations of the various types of water were determined on the occasion of several samplings (Tab.4.5).

Table 4.5: Copper concentration of test waters determined according to DIN EN ISO 11885 (2007). Mean values of three samplings.

Origin of water samples	Abbreviation	Copper content	
		mg/L	µM
Deionized water	D	< 0.002	< 0.03
Drinking water, Stadtwerke Duisburg, Biofilm Centre, laboratory tap	DW DU	0.19	2.99
Drinking water, waterworks Styrum-Ost (RWW), IWW Water Centre, laboratory tap	DW MH	0.017	0.27
Finished water, waterworks Styrum-Ost, RWW	FW	< 0.002	< 0.03

The results of the copper analysis showed that drinking water collected in Duisburg contained an about eleven-fold higher level of copper (0.19 mg/l) than drinking water from Mülheim (0.017 mg/l), while copper concentrations in finished water from the waterworks were below the detection limit (< 0.002 mg/L).

Survival of *P. aeruginosa* in the three different real water samples and in deionised water was investigated. Total cell counts were determined before and after exposure of the bacteria in the test waters (Tab. 4.5) for 24 h at 20 °C and colony counts were determined after 24 h of exposure.

In all bacterial test suspensions, total cell counts were approximately 10^6 cells/mL before and after 24 h exposure to the different water samples.

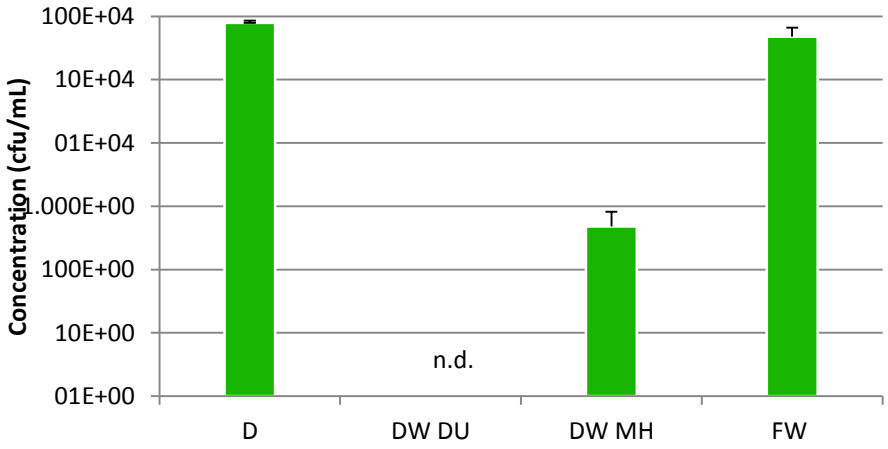

Figure 4.30: Survival of *P. aeruginosa* AdS exposed to deionised water (D), drinking water collected from Duisburg (DW DU), drinking water from Mülheim (DW MH) and finished water (FW) at 20 °C for 24 h. Initial cell numbers were 1 x 10^6 cells/mL. The results are averages of two or three independent experiments. n.d. - not detected (detection limit 0.33 cfu/mL).

Recovery of *P. aeruginosa* after exposure to deionised water was equal to the initial cell numbers of 1 x 10^6 cells/mL. After exposure to drinking water from Duisburg which contained the highest level of copper (0.19 mg/L = 2.99 µM) no culturable *P. aeruginosa* could be observed. When bacteria were exposed to drinking water from Mülheim which contained copper in lower concentrations colony counts decreased by about three log units. Recovery of *P. aeruginosa* was not significantly impaired by finished water with copper concentrations below the detection limit (< 0.03 µM).

The results show that the culturability of *P. aeruginosa* decreased with increasing copper concentrations in natural drinking water. This indicated that the presence of copper might inhibit growth of *P. aeruginosa*. To investigate whether copper was actually the inhibiting factor, an experiment was performed using water, which contained only copper and no other substances which might impair culturability. For this purpose, *P. aeruginosa* with a density of approximately 10^6 cells/mL were exposed for 24 h to deionised water, supplemented with copper of increasing concentrations (Fig. 4.31).

In all test waters, total cell numbers were approximately 1 x 10^6 cells/mL before and after exposure to copper containing water for 24 h. Up to a copper concentration of 1 µM colony numbers were approximately equal to the total cell numbers (1 x 10^6 cfu/mL). The culturability of *P. aeruginosa* started to decrease by about three log units at a copper concentration of 1 µM. When exposed for 24 h to deionised water containing 10 µM copper and higher concentrations, bacteria were not culturable anymore (0 cfu/1.5 mL).

Altogether, the results support the hypothesis that copper inhibits the culturability of *P. aeruginosa*.

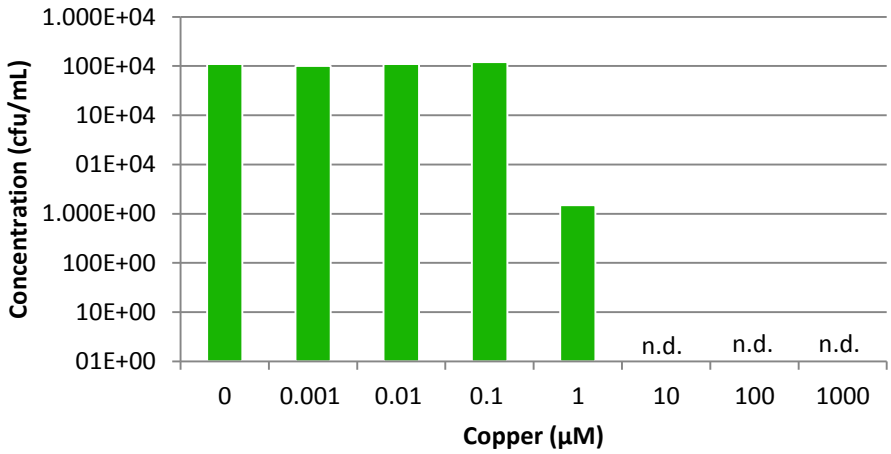

Figure 4.31: Survival of *P. aeruginosa* AdS exposed to deionised water containing increasing copper concentrations (0 – 1000 µM copper) at 20 °C for 24 h. Initial cell numbers were 1 x 10^6 cells/mL. n.d. – not detected (detection limit 0.33 cfu/mL).

4.8.2. Protective effect of the copper chelator sodium diethyldithiocarbamate

The concentration of free copper ions can be diminished by suitable metal chelators. Sodium diethyldithiocarbamate trihydrate (DDTC) was reported to be an efficient copper-complexing compound (Harrison et al., 2005). In order to investigate the protective effect of DDTC on the culturability of *P. aeruginosa* in the presence of copper, bacterial suspensions of *P. aeruginosa* AdS with a density of approximately 10^6 cells/mL were exposed for 24 h to (i) deionised water (ii) drinking water collected from Duisburg, (iii) drinking water from Mülheim, and (iv) finished water and each of the test waters supplemented with DDTC (final concentration 100 µM). Total cell counts were determined before and after exposure to different water samples. Enumeration of colony counts (Fig. 4.32) was performed after 2 d of incubation on nutrient agar.

Total cell numbers in all bacterial test suspensions were approximately 1 x 10^6 cells/mL before and after exposure for 24 h to different water samples. In all test suspensions, the culturability could be increased by adding DDTC (by 4 log units in deionised water containing 1 µM copper, by > 5 log units in drinking water from Duisburg, by 3 log units in drinking water collected in Mülheim, by 0.8 log units in finished water). There was no significant difference in the colony count of *P. aeruginosa* exposed to deionised water containing DDTC compared to deionised water without DDTC showing that the copper chelator itself did not impair or enhance the culturability of *P. aeruginosa*.

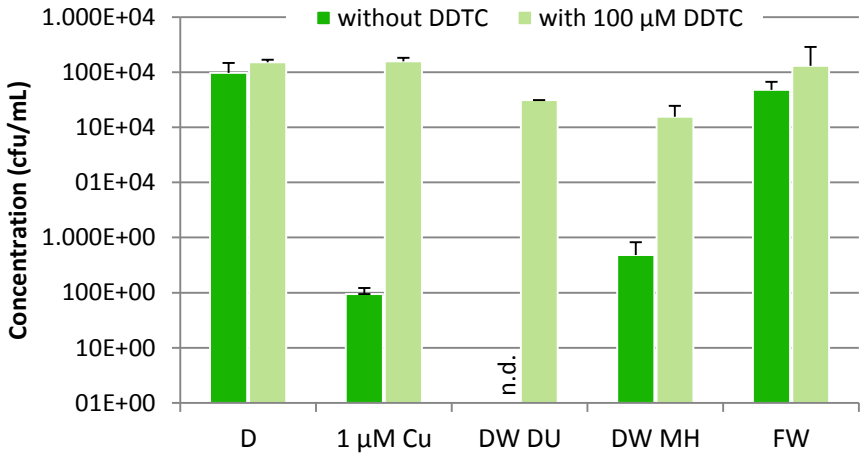

Figure 4.32: Protective effect of DDTC for the survival of *P. aeruginosa* AdS exposed to deionised water (D), deionised water supplemented with 1 µM copper (1 µM Cu), drinking water collected from Duisburg (DW DU), drinking water from Mülheim (DW MH) and finished water (FW) at 20 °C for 24 h. Initial cell numbers: 1 x 10^6 cells/mL. The results are averages (cfu/mL) of two or three independent experiments. n.d. - not detected (detection limit 0.33 cfu/mL).

The experiment demonstrated that the recovery of *P. aeruginosa* in copper-containing water can be improved by the copper chelator DDTC. This is another indication for the inhibiting effect of copper on the culturability of *P. aeruginosa*.

4.8.3. Effect of copper on the viability of *P. aeruginosa* in deionised water and in natural copper-containing drinking water

Viability determination using the LIVE/DEAD kit was performed to study the effect of increasing copper concentrations on *P. aeruginosa* in deionised water. For this purpose *P. aeruginosa* with a density of approximately 10^6 cells/mL were exposed for 24 h at 20 °C to deionised water with supplemented copper ($CuSO_4$) of increasing concentrations (0 – 100 µM). Total cell counts and colony counts were determined. The LIVE/DEAD® Bacterial Viability Kit (*Bac*Light™) was applied to estimate the number of viable *P. aeruginosa* (Fig. 4.33). The kit is composed of a mixture of two nucleic acid-binding stains: the green-fluorescent SYTO 9 dye and red-fluorescent propidium iodide. SYTO 9 penetrates all bacterial membranes and stains all cells green, while propidium iodide only penetrates cells with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Using epifluorescence microscopy, viable cells, i.e. cells with an intact cytoplasmic membrane, can be differentially quantified from cells with a damaged membrane defined as dead cells (Haugland, 2005).

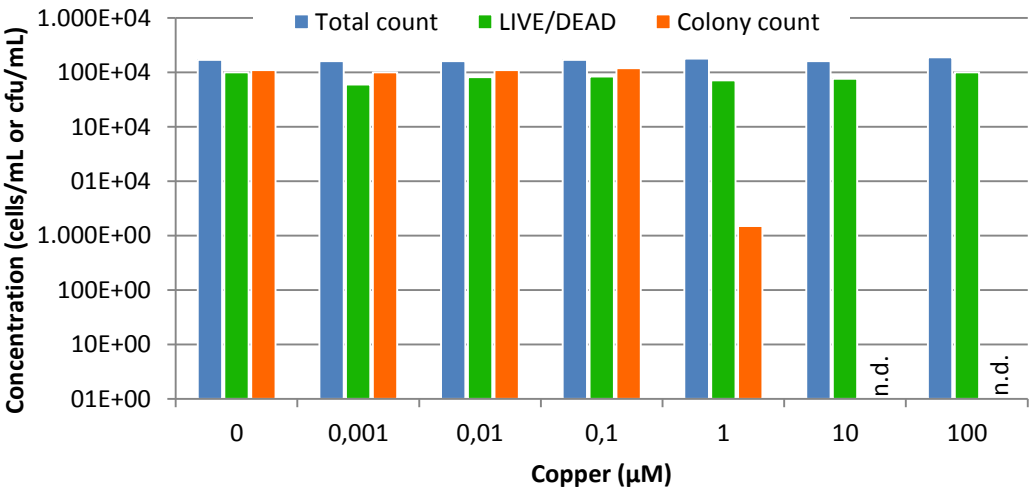

Figure 4.33: Total counts, colony counts and viable counts of *P. aeruginosa* AdS in the presence of increasing copper concentrations. Total cell numbers were determined with the DAPI method, colony counts were enumerated on nutrient agar (36 °C, 2 d) and viable counts were performed using the LIVE/DEAD kit. n.d. - not detected (detection limit of colony count 0.33 cfu/mL).

Total cell counts were approximately 1 x 10^6 cells/mL, regardless of the copper concentration, whereas the culturability decreased by three log units at a copper con-

centration of 1 µM and by more than six log units at concentrations ≥ 10 µM (Fig. 4.33). The results of viable cell enumeration using the LIVE/DEAD kit did not reveal any dependence on the copper concentration tested (0 – 100 µM). The numbers of green cells were generally ≥ 5 x 10^5 cells/mL and did not decline at higher copper concentrations. The results show that *P. aeruginosa* was not culturable when exposed to ≥ 1 µM copper whereas the proportion of cells with intact cell membranes remained constant with increasing copper concentration and exceeded the colony numbers at ≥ 1 µM copper.

The impact of copper on the viability of *P. aeruginosa* and the neutralizing effect of the copper chelator DDTC was also studied in natural water samples (Fig. 4.34).

P. aeruginosa with a density of approximately 10^6 cells/mL was exposed to different drinking water samples with variation in their copper content for 24 h. Total cell counts, colony counts and viable counts were determined as described above.

Total cell numbers in all bacterial test suspensions were approximately 1 x 10^6 cells/mL after exposure to different water samples for 24 h. In all test suspensions, the culturability could be improved by adding DDTC (final concentration 100 µM).

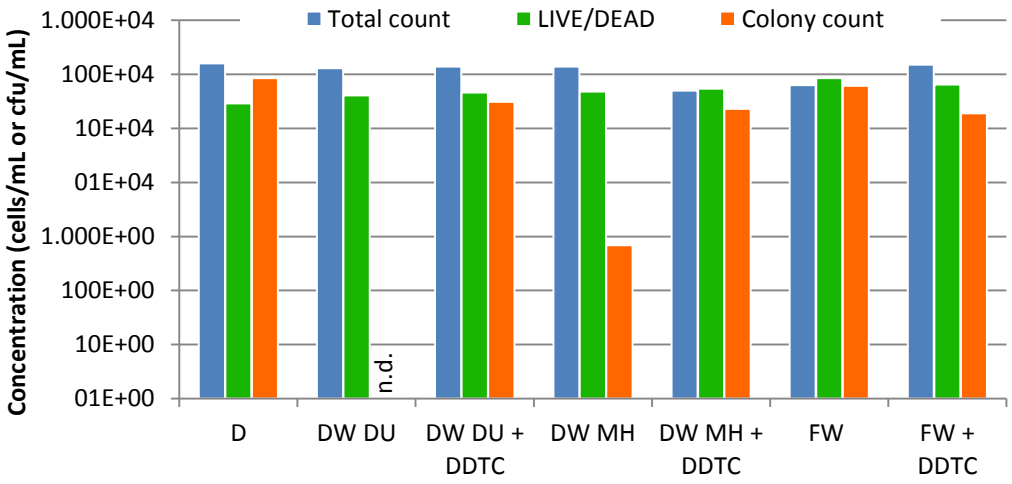

Figure 4.34: Determination of the viability of *P. aeruginosa* AdS exposed to (i) deionised water (D), (ii) drinking water from Duisburg (DW DU) (iii) drinking water from Duisburg with added DDTC (100 µM), (iv) drinking water collected in Mülheim (DW MH), (v) drinking water from Mülheim with added DDTC, (vi) finished water (FW) and (vii) finished water with added DDTC. Total cell numbers were determined with the DAPI method, colony counts were enumerated on nutrient agar (36 °C, 2 d) and viable counts were performed using the LIVE/DEAD kit. n.d. - not detected (detection limit of colony count 0.33 cfu/mL).

Viability measurement using the LIVE/DEAD kit revealed a relatively unchanged viability of *P. aeruginosa*, regardless in which water the cells were incubated; the proportions of green cells were determined to be in the range of 5 x 10^5 to 1 x 10^6 cells/mL. The addition of DDTC to the different waters did not result in an increase

in viable cells. The results showed again that the number of viable cells determined using the LIVE/DEAD significantly exceeded the colony numbers in drinking water from Duisburg and Mülheim indicating that the proportion of *P. aeruginosa* cells that is not able to form colonies on nutrient agar still possess an intact cytoplasmic membrane.

4.8.4. Resuscitation of copper-stressed *P. aeruginosa* in pure culture

The purpose of this experiment was to investigate the extent of resuscitation of copper-stressed *P. aeruginosa* in deionised water containing the copper-neutralizing agent DDTC.

P. aeruginosa were exposed to deionised water containing 1 µM copper for 24 h at 20 °C (cell density 2 x 10^6 cells/mL). For resuscitation, copper-stressed bacteria were incubated in deionised water containing 100 µM DDTC for up to 7 d at 20 °C. After 2 h, 1 d, 4 d and 7 d of incubation, total cell numbers were determined with DAPI and colony numbers were enumerated on nutrient agar (Fig. 4.36). The initial cell density was 1 x 10^6 cells/mL.

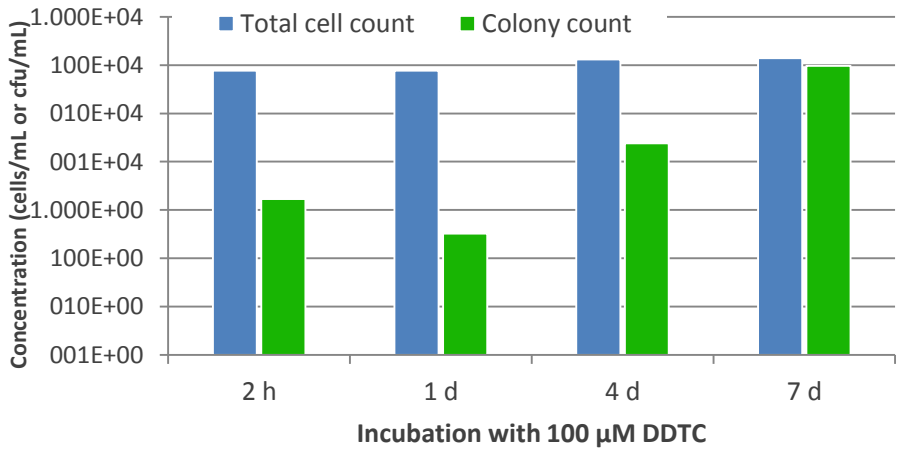

Figure 4.35: Resuscitation of copper-stressed *P. aeruginosa* in 100 µM DDTC solution. Total cell numbers were determined with the DAPI method, colony counts were enumerated on nutrient agar (36 °C, 2 d). Initial cell numbers were 1 x 10^6 cells/mL.

Total cell numbers were approximately 1 x 10^6 cells/mL after 2 h of incubation in the presence of DDTC, colony counts were approximately 10^3 cfu/mL. Total cell numbers remained unchanged at any time (1 x 10^6 cells/mL), never exceeding the initial cell numbers. After 2 h and 24 h of incubation, the colony numbers in DDTC suspension were recorded to be 1 x 10^3 cfu/mL, after 4 d about 1 x 10^4 cfu/mL and finally, after 7 d of incubation, the colony numbers were 1 x 10^6 cfu/mL, which is equal to the initial cell density of 1 x 10^6 cell/mL and the total cell numbers enu-

merated at 7 d. This result showed that copper-stressed *P. aeruginosa* are able to regain culturability in the presence of the copper chelator DDTC within 7 days.

4.8.5. Resuscitation of copper-stressed *P. aeruginosa* in drinking-water biofilms

Experiments on the survival of *P. aeruginosa* in copper-containing drinking water and in deionised water supplemented with copper showed that *P. aeruginosa* enters a non-culturable state in the presence of copper ions (see figs. 4.30 and 4.31). Upon the addition of the copper chelator NDDC, *P. aeruginosa* became culturable again within 7 d (see Fig. 4.36). In order to investigate whether this process is also possible in biofilms, drinking water biofilms were grown under constant flow in drinking water on coupons of EPDM 1. After 14 d the biofilms were inoculated with *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis*. Seven days after inoculation biofilms were suspended in deionised water (control) or in deionised water containing the copper chelator NDDC (final concentration 100 µM) and incubated at 20 ˚C for 7 d. The concentration of *P. aeruginosa* in biofilm suspension before and after incubation with NDDC was determined using cultivation on CN agar and using the culture-independent FISH method (Fig. 4.37).

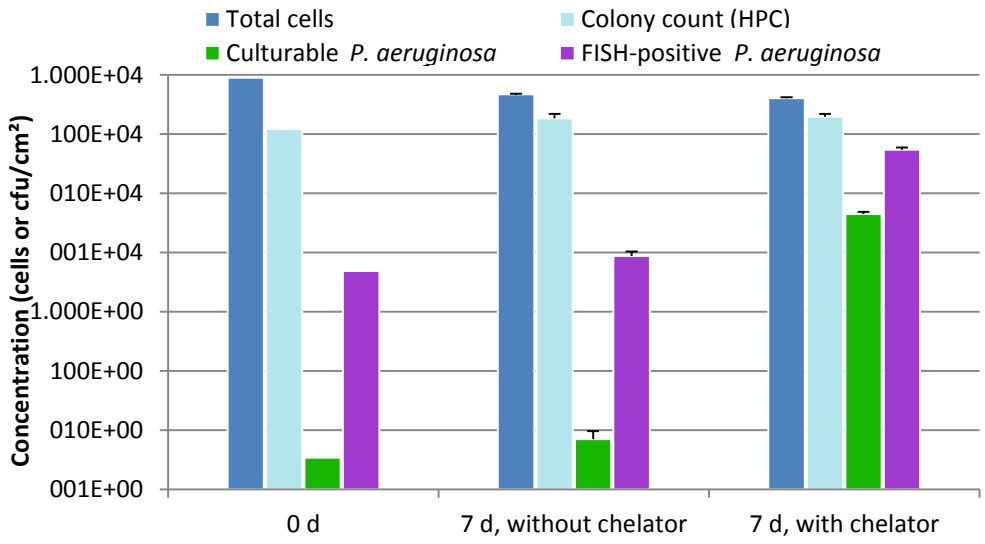

Figure 4.36: Total cell counts and colony counts as well as concentrations of *P. aeruginosa* AdS in drinking water biofilms grown on EPDM 1 before and after incubation with or without the copper chelator NDDC. Concentrations of *P. aeruginosa* were determined by cultivation on CN agar and using FISH (probe Psae16S-182). The results are averages of three independent experiments.

During the period of biofilm growth and inoculation of the biofilm, the mean temperature of the water inside the tank or reactor was 14.8 ˚C ± 0.8 ˚C (range 13.7 ˚C - 15.7 ˚C; n = 6). The mean temperature of the influent water was 9.5 ˚C ± 0.1 ˚C (range

9.4 °C - 9.5 °C; n = 2) and the average pH of the influent water was 7.9 ± 0.1 (range 7.8 - 7.9; n = 2). The copper concentration in the biofilms varied between 11.08 and 46.00 µg/cm² corresponding to a concentration of 0.017 µmol/cm² and 0.072 µmol/cm², respectively.

Before incubation with or without NDDC, the concentration of culturable *P. aeruginosa* was 3.41 cfu/cm² whereas 4.84 x 10^3 cells/mL were detected using FISH. Total cell count and colony count of the biofilm were 8.96 x 10^6 cells/cm² and 1.23 x 10^6 cfu/cm², respectively. After 7 d of incubation in deionised water without NDDC, the concentrations of both culturable and FISH-positive *P. aeruginosa* slightly increased (4.07 cfu/cm² and 8.78 x 10^3 cells/cm², respectively). After 7 d of incubation with 100 µM NDDC, the concentration of culturable *P. aeruginosa* increased by 4 log units to 4.45 x 10^4 cfu/cm² and the number of *P. aeruginosa* detected using FISH increased by 2 log units to 5.45 x 10^5 cells/cm². The percentage of culturable *P. aeruginosa* on FISH-positive *P. aeruginosa* increased from 0.08 % to 8.2 %. No significant change in total cell count and colony count was observed after incubation with or without the copper chelator. The results show that non-culturable *P. aeruginosa* present in a drinking water biofilm can become culturable again upon release of the copper stress.

Chapter 5
Discussion

5.1. Development of drinking water biofilms on domestic plumbing materials

Most research on biofilms in technical water systems has focused on materials of public drinking water distribution systems, not including domestic plumbing systems in public and private buildings (Eboigbodin et al., 2009). The choice of materials in domestic plumbing systems is less regulated than in water mains, which leads to a wide variety of materials used. Some of these materials might strongly enhance biofilm formation (Colbourne 1985; Schönen 1986). According to the WHO and the European Community directives, drinking water should meet the quality requirements at the point of consumption (WHO, 2008; The Council of the European Union directive, 1998). A high water quality has to be maintained throughout the distribution, including passage through domestic plumbing. Therefore it is important to investigate and understand biofilms in both distribution mains and domestic plumbing.

In the present study, the colonisation of plumbing materials by the autochthonous drinking water microflora was investigated. For this purpose, biofilms were grown on coupons of elastomeric (EPDM 1, EPDM 2) and plastic (PE-Xb, PE-Xc) materials as well as on copper under continuous flow-through with drinking water for 14 days. The plastic materials and EPDM 1 met specific physical and chemical requirements (Anonymous, 1985; UBA, 2008), but also the microbiological specifications relating to their biofilm formation potentials (DVGW, 2007), that are the basis for the recommendation of their use in drinking water systems in Germany. EPDM 2 did not

fulfil any of these requirements. Biofilm development was followed over a period of 43 days under flow-through of cold tap water. Biofilm formation based on the determination of total cell counts and colony counts of HPC bacteria was more pronounced on EPDM materials compared to PE-Xb, PE-Xc and copper. Similar findings were reported by Benölken et al. (2010) comparing the biofilm formation potential of EPDM, PE-Xb, PE-Xc and copper in a pilot scale domestic plumbing system under cold (12 °C) and warm (37 °C) water conditions (Benölken et al., 2010). Enhanced microbial colonisation of EPDM and other elastomeric materials has also been observed in previous field and laboratory studies (Kilb et al. 2003; Rogers et al., 1994b). Using a chemostat model of a plumbing system inoculated with sludge from a calorifier, Rogers et al. (1994b) found higher colony counts of heterotrophic bacteria (1.08×10^7 cfu/cm^2) in drinking water biofilms grown on ethylene-propylene material compared to PE (2.75×10^6 cfu/cm^2) and other plastic and steel materials over an exposure period of 28 days. In many studies enhanced biofilm formation on synthetic polymers has been attributed to the leaching of biodegradable organic compounds (plasticisers, antioxidants, lubricants, heat stabilizers) supplying additional nutrients to the biofilm organisms (Keevil, 2002; Kilb et al., 2003; Rogers et al., 1994b; van der Kooij, 2005). For instance, Lehtola et al. (2004a) found an increase in both total and microbially available phosphorous in the effluent water of PE pipes during the first 20 days of drinking water flow-through, which was traced back to an organophosphorous stabiliser that leached into the water. Favourable nutrient conditions may be an explanation for the relatively high fraction of culturable bacteria in biofilms grown on EPDM, PE-Xb and PE-Xc compared to copper in the present study.

Biofilm formation on EPDM 2 which did not comply with the German recommendations for plastics in contact with drinking water was considerably (up to 2 orders of magnitude) higher than on EPDM 1 that fulfilled the recommendations, indicating that the application of state-of-the-art materials can decrease the risk of massive biofilm formation. The biomass concentration detected on EPDM 2 in the present study correlates well with the findings of Bressler et al. (2009) who reported total cell counts and HPC values of 1.2×10^9 cells/cm^2 and 1.0×10^8 cfu/cm^2, respectively, for 14 day-old drinking water biofilms on a type of EPDM which complied with the physical and chemical requirements, but not with the microbiological requirements of German recommendations for rubber materials in contact with drinking water. A lower degree of leaching of biodegradable compounds as nutrients for microbial growth from EPDM (Rogers et al., 1994b) may be an explanation for the lower values of total cell counts and HPC in the biofilms grown on EPDM 1 in the present study

compared to EPDM 2 and to those of the other studies mentioned. These observations indicate that significant variations in biofilm formation have to be expected depending on the chemical composition of EPDM materials.

Total cell counts of biofilms grown on copper were comparable to those of biofilms on PE-Xb and PE-Xc over the period of 7 to 43 days. The HPC of biofilms grown on PE-Xc and copper was lower than that of biofilms grown on PE-Xb after 7 days. The HPC of biofilms grown on PE-Xc increased relatively rapidly and was at the same level as the HPC on PE-Xb after 16 days, whereas the development of culturable heterotrophic bacteria in biofilms grown on copper was delayed. These results suggest that drinking water biofilms of cell densities comparable to those on plastic materials can develop on copper surfaces, but the fraction of culturable bacteria in copper-associated biofilms can be significantly reduced.

In only a few other studies, copper and PE-X or PE, sometimes in combination with other materials, were directly compared with respect to biofilm formation. Biofilms detected on copper exposed in a drinking water distribution system for 12 to 15 days showed lower total cell counts and colony counts (HPC) than biofilms developed on hardened PE (HD-PE) coupons (Schwartz et al., 1998). Similar to the results of the present study, the HPC values of biofilms grown on HD-PE were about one order of magnitude less than the corresponding total cell counts, whereas the fraction of HPC bacteria in copper biofilms was significantly lower. Based on total cell count, Lehtola et al. (2004a) observed a slower formation of drinking water biofilms in copper pipes compared to PE pipes, but after 200 days there was no difference in the cell numbers between the pipe materials; however, in contrast to the results of our work, HPC of biofilms on copper and PE did not differ significantly over the whole experimental period. In another study, Lehtola et al. (2007) found similar or even higher numbers of culturable heterotrophic bacteria in biofilms grown in copper pipes compared to biofilms grown in PE pipes in a pilot scale distribution system. Wingender and Flemming (2004) found the same or even slightly higher levels of total cells in biofilms grown on copper compared to PE exposed for 18 months in a drinking water distribution system. Van der Kooij et al. (2005) reported that levels of HPC and ATP of biofilms on PE-X were higher than those of biofilms grown on copper in a model warm water system over a 2-year period. In static (batch) experiments, biofilm formation based on ATP analysis was lowest on copper compared to PE-X or PE and other plastic and steel materials after an incubation at 30 °C for 112 days (Tsvetanova and Hoekstra, 2009) or at 25 °C for 90 days (Yu et al., 2010). Taken together, when total cell counts were used as a parameter to monitor biofilm formation, biofilms of cell densities comparable to those on plastic materials can develop

after longer periods of weeks or months. However, biofilm formation on copper analysed by colony counts or ATP content has often been observed to be limited or delayed compared to other materials. A possible explanation for the low fraction of culturable or metabolically active bacteria in relation to the total number of cells may be the inhibitory effect of dissolved copper ions which can affect metabolic functions, cause cell injury or induce a VBNC state of the biofilm organisms (Avery et al., 1996; Teitzel et al., 2006). In contrast to synthetic polymers such as EPDM and PE, copper does not leach any biodegradable organic compounds serving as additional nutrients for enhanced biofilm growth.

Investigations on the reactor effluent water showed that during the passage of drinking water through the reactors containing coupons of domestic plumbing materials, the proportion of culturable heterotrophic bacteria related to the total cell count increased from 0.1 % in the influent drinking water to 31.3 % culturable bacteria in the reactor effluent. In contrast to the drinking water biofilms grown on the different materials, there was no great variation in the total cell count and colony count between the effluents of the five materials. These results indicate that during the passage of water through the reactor the culturability of the autochthonous microflora of the drinking water is enhanced, probably by the release of substances by the materials or by the biofilm itself.

5.2. The influence of material ageing on biofilm formation

In the present study, the influence of possible material alterations of domestic plumbing materials as a consequence of chemical disinfection on biofilm formation was analysed. Domestic plumbing materials were artificially aged by the application of chemical disinfectants (NaOCl or ClO_2) simulating intensive disinfection measures in the framework of the sanitation of a domestic plumbing system. As these disinfectants are strong oxidants, they can affect the structural, physical and chemical integrity of construction materials, especially of synthetic polymers such as EPDM and PE (Denberg et al., 2007). As copper was not expected to be sensitive against the action of oxidative disinfectants, copper coupons were alternatively aged by exposure in a drinking water distribution system for at least 6 months (Wingender et al., 2003). Biofilms were grown on coupons of aged domestic plumbing materials under continuous flow-through with drinking water for 43 days.

PE-Xb and PE-Xc materials that were treated with NaOCl or ClO_2 did not show any difference in biofilm formation (total cell count, HPC) compared to the corresponding new materials. In case of EPDM 1 the effect of material ageing was much more

pronounced in the ClO_2-treated material than in the NaOCl-treated material. Especially the number of culturable heterotrophic bacteria was strongly enhanced in biofilms grown on ClO_2-treated EPDM 1 resulting in a very high fraction of HPC bacteria on the total cell count. As the physical and chemical characteristics of aged and new materials were not investigated in the present study, it is not clear whether this can be attributed to a higher surface roughness facilitating the attachment of bacteria or to an increased release of biodegradable substances favouring enhanced proliferation of microorganisms, or both.

In the literature there are only few data dealing with the comparison of biofilm formation on new and aged plumbing materials. Schaule et al. (2010) demonstrated that total cell counts of biofilms grown on ClO_2-treated EPDM and PE-Xb (5 ppm ClO_2 at 40 °C and 4 bar for 4 weeks) in a batch system in drinking water at 8 °C and 37 °C were less than 1 order of magnitude lower compared to total cell counts on the corresponding new materials (Schaule et al., 2010).

Previous studies on the ageing of plumbing materials have focused on changes in the physical, chemical and structural characteristics of plastic materials including PVC, PB and PE (Vibien et al., 2001; Skjevrak et al., 2003; Bigg et al., 2004; Hassinen et al., 2004; Castagnetti et al., 2008). These studies demonstrated that disinfectants attack the additives of synthetic polymers (Skjevrak et al., 2003) and lead to the formation of a porous degradation layer with micro-cracks that expand with further action of the disinfectant (Vibien et al., 2001; Chung et al., 2003; Bigg et al., 2004; Hassinen et al., 2004). For some of the materials tested in the present study (EPDM, PE-Xb, PE-Xc) ageing by exposure to NaOCl or ClO_2 has been shown increase surface roughness and hydrophility and to change the chemical composition of the material surface (Schaule et al., 2010). There is evidence that surface parameters such as hydrophobic or hydrophilic nature as well as roughness of a surface do influence the attachment of microorganisms (Pedersen et al., 1990; Melo and Bott, 1997; Percival et al., 1998; Shin et al., 2007). However, in the present study, no influence of material ageing on biofilm formation on NaOCl-treated EPDM and NaOCl- or ClO_2-treated PE-Xb and PE-Xc was detected.

Biofilm formation was compared on new copper and copper coupons that had been aged by exposure to drinking water for at least 6 months. No significant difference was observed between total cell counts of biofilms grown on new and aged copper, whereas the number of culturable heterotrophic bacteria on aged copper was often higher than on new copper, especially during the first 16 days of biofilm growth. In contact with drinking water copper surfaces start to oxidize and a protective layer develops (von Franqué et al., 1975). This phenomenon decreases the levels of copper

ions being released into the water (Rogers et al., 1994a). Bacteria attaching to new copper surfaces have to deal with higher concentrations of dissolved copper ions which can affect metabolic functions, cause cell injury or induce a VBNC state (Avery et al., 1996; Teitzel et al., 2006). After 16 days of contact with drinking water, oxidation of new copper surfaces may have proceeded and the concentration of dissolved copper ions decreased. This might explain the lower culturability of biofilms grown on new copper coupons during the first 16 days compared to the subsequent period.

5.3. Population diversity of drinking water biofilms on domestic plumbing materials

16S rDNA gene fingerprinting performed using terminal restriction fragment length polymorphism (T-RFLP), single strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) has been proven to be a useful tool for the analysis and comparison of biofilm or drinking water communities (Emtiazi et al., 2004; Hoefel et al., 2005; Eichler et al., 2006; Bressler et al., 2009; Roeder et al., 2010).

In the present study, the influence of the type of material as well as of the ageing of a material on the diversity of drinking water biofilm populations was investigated. Biofilms were grown on new and aged coupons of EPDM 1, PE-Xb, PE-Xc and copper for 14 d under constant flow conditions in a stainless steel tank and analysed by PCR-DGGE. Different banding patterns were obtained for each of the new and aged domestic plumbing materials revealing a material dependent development of drinking water biofilm communities. A higher diversity of biofilm populations was observed in biofilms grown on synthetic materials (EPDM, PE-Xb, PE-Xc) compared to biofilms grown on copper.

As copper is known to inhibit attachment and growth of bacteria (Rogers et al., 1994a; van der Kooij et al., 2005; Mathys et al., 2008; Elguindi et al., 2009) copper as a substrate for growth in domestic plumbing might select for copper-resistant bacteria resulting in a less diversified drinking water biofilm community. Yu et al. (2010) reported similar results from an investigation of biofilm communities grown on materials utilised in drinking water distribution. The diversity of drinking water biofilms was higher on plastic materials such as PE, PVC and PB compared to biofilms on copper (Yu et al., 2010). In situ hybridisation with fluorescence labelled, group-specific rRNA targeted oligonucleotide probes revealed that the quantitative composition of the α-, β-, γ- and δ- subclasses of proteobacteria differed between

biofilms grown on HD-PE, PVC, steel and copper (Schwartz et al., 1998). A material-dependent development of specific biofilm communities assessed by FISH was also reported by Kalmbach et al. (1997): the diversity of drinking water biofilms attached to glass surfaces was higher than that of biofilms attached to PE, but the difference diminished when surfaces were exposed for more than 40 days (Kalmbach et al., 1997).

A comparison of genetic fingerprints of biofilms grown on new materials and materials aged by the action of oxidative disinfectants has not been published yet. The present study revealed that the similarity between the biofilm populations on new and aged (NaOCl- or ClO_2-treated) material of the same type was always higher than the similarity between biofilm populations of different material types. These results indicate that the type of material has a greater influence on the composition of biofilm populations developing on the surface of domestic plumbing materials than the possible material alterations resulting from ageing of a material.

Drinking water biofilms grown on the same material in different reactor runs showed a relatively low similarity (45.2 % on average). A higher similarity (71 % on average) of drinking water biofilms grown on EPDM in flow-through reactors connected to a domestic pumbing system was shown by Bressler et al. (2009). Compared to the differences between biofilm communities developed in different reactor runs - and thus under different environmental conditions - the differences in biofilm diversity between biofilms grown on new and aged material were relatively low.

Apparently, the species composition of a biofilm is not exclusively influenced by the type and age of the substrate. Additional factors such as temperature, pH, redox potential, nutrient content and redox potential of the bulk water (Hempel et al., 2009; Bai et al., 2010), disinfection practice (Mathieu et al., 2009; Bai et al., 2010; Roeder et al., 2010) and the age of a biofilm (Deines et al., 2010) play an important role in the development of a specific biofilm community.

In case of EPDM 1, PE-Xb and copper, biofilms grown on aged material showed a slightly higher species diversity compared to new materials. This shows that the surface characteristics of aged materials represent a substratum suitable for the attachment and growth of a broader spectrum of bacteria.

Biofilms grown on NaOCl-treated and ClO_2-treated EPDM 1 and biofilms grown on NaOCl-treated and ClO_2-treated PE-Xb showed a relatively high similarity. This suggests that changes in material characteristics due to disinfection and the resulting changes in biofilm community composition are independent of the type of disinfectant used.

Some of the observed banding pattern differences may be associated with inherent biases of the PCR-DGGE method including preferential DNA extraction and inefficient or selective DNA amplification or with limitations in the DGGE technique, such as poor resolution and insufficient staining for visualization of low quantity population bands (Hoefel et al., 2005).

5.4. Integration and fate of hygienically relevant bacteria in drinking water biofilms on new and aged domestic plumbing materials

5.4.1. Survival of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* in drinking water

To investigate the fate of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* in drinking water biofilms, 14 day-old biofilms grown on new and aged EPDM 1, PE-Xb, PE-Xc and copper coupons were spiked with a mixture of *E. nimipressuralis*, *P. aeruginosa* and *L. pneumophila*. In order to adapt the bacteria to drinking water conditions, a suspension of each bacterium with a concentration of 3 x 10^6 cells/mL in sterile drinking water was incubated ("starved") for 24 h at 20 °C (*P. aeruginosa* and *E. nimipressuralis*) or 30 °C (*L. pneumophila*).

Quantification of the bacteria after 24 h of incubation in sterile drinking water showed that incubation in drinking water for 24 h influenced the culturability of the target organisms. Quantification of *P. aeruginosa* and *L. pneumophila* was performed using culture-based methods as well as the culture independent FISH and *E. nimipressuralis* was quantified using the Colilert-18 system. The decrease in detectability was most pronounced in *E. nimipressuralis*. The number of culturable *E. nimipressuralis* after incubation in drinking water was more than 4 orders of magnitude lower than before incubation. As no other method was used for detection of *E. nimipressuralis*, it is not clear, whether the major part of the *E. nimipressuralis* population in the suspension was lethally damaged by incubation in drinking water or whether it was still viable, but inactivated and thus not detected by culture. Certain *Enterobacter* spp. such as *E. cloacae* and *E. agglomerans* are able to enter a VBNC state upon exposure to unfavourable conditions (Oliver et al., 2005). Thus, it is possible that the *E. nimipressuralis* strain used in the present study is also capable of undergoing such a transition.

The number of culturable *P. aeruginosa* decreased by more than two orders of magnitude after incubation in sterile drinking water, whereas the number of FISH-positive *P. aeruginosa* was less than 1 order of magnitude lower than the original cell number before incubation. As *P. aeruginosa* belongs to the bacteria that are able

to undergo transition into a VBNC state (Oliver et al., 2005), this result indicates that in contact with the drinking water used in the present study part of the *P. aeruginosa* population in the suspension may have entered a VBNC state. *P. aeruginosa* cells in this state did not form colonies on CN agar but still showed signs of metabolic activity such as the presence of ribosomal RNA which was detected by the FISH method.

Observations from practical situations and experimental systems prove that both *P. aeruginosa* and coliform bacteria are able to survive and replicate under low-nutrient and low-temperature conditions in a culturable state (Boyle et al., 1991; Wricke et al., 2007; Wingender et al., 2009; LeChevallier et al., 1987; Camper et al., 1991; Kämpfer et al., 2008). Thus, the low concentration of nutrients present in drinking water and the low temperature (20 °C) during incubation are unlikely to be responsible for the low culturability of planktonic *P. aeruginosa* and *E. nimipressuralis*. Other factors such as residual chlorine (Bej et al., 1991; Dussere et al., 2008; Wang et al., 2010) or toxic concentrations of copper originating from the copper plumbing system (Grey and Steck, 2001; Teitzel and Parsek, 2003; Huang et al., 2008; Dwidjosiswojo et al., 2010) may have damaged or killed part of the bacteria or induced a VBNC state. This aspect has been investigated in more detail (see 4.8) and will be discussed further in section 5.7.

In contrast to *E. nimipressuralis* and *P. aeruginosa*, the number of culturable *L. pneumophila* after incubation in sterile drinking water for 24 h at 30 °C was only one order of magnitude lower than the original cell number before incubation; the number of FISH-positive *L. pneumophila* was about as high as the concentration of *L. pneumophila* set in the suspension before incubation. This demonstrates that *L. pneumophila* is not as much affected by incubation in sterile drinking water as *P. aeruginosa* and *E. nimipressuralis*. Similar results were obtained for *L. pneumophila* strain Philadelphia 2 exposed to sterile tap water at 37 °C (Hussong et al., 1987) and for *L. pneumophila* strain Philadelphia JR32 incubated in sterile drinking water at room temperature (Steinert et al., 1997). When exposed to drinking water for > 10 d, colony counts of *L. pneumophila* started to decrease, but culturable *L. pneumophila* were still detected in sterile drinking water after 40 d 37 °C (Hussong et al., 1987) and for 120 d at room temperature (Steinert et al., 1997).

Altogether the results show that at the time of inoculation of the biofilms, the bacterial suspension (inoculum) probably consisted of diverse populations of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* that exhibit different physiological states. It was not investigated in the present study, whether the ability to integrate into and persist in drinking water biofilms varied between bacteria in different

physiological states. Boyle et al. (1991) showed that *P. aeruginosa* incubated in distilled water retained culturability for up to 5 months and that starved *P. aeruginosa* were able to form a biofilm on stainless steel surfaces (Boyle et al., 1991). In addition to that, there is evidence that starved cells exhibit a "sticky" phenotype enabling them to adhere to solid surfaces or to established biofilms (Toutain et al., 2004). In laboratory experiments it has been shown that the integration and persistence of *Enterobacter* spp. and *Klebsiella* spp. added to established mixed species drinking water biofilms depends on the growth rate of the coliforms in the inoculum; coliforms pre-cultured under oligotrophic conditions with a slow growth rate persisted longer and in higher concentrations (Camper et al., 1996; Szabo et al., 2006). Thus, the way of preparation of bacteria for the inoculation of biofilms in laboratory experiments and consequently the physiological state of bacteria at the time of inoculation can play an important role in the integration and survival of these bacteria in biofilms.

5.4.2. Integration and fate of *P. aeruginosa* in drinking water biofilms

14 day old drinking water biofilms grown on new and aged EPDM 1, PE-Xb, PE-Xc and copper were inoculated with a mixture of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis*. The persistence of *P. aeruginosa* in these biofilms was monitored over a period of 4 weeks with the standard cultural method as well as with the culture-independent FISH.

P. aeruginosa was able to incorporate into and persist in biofilms grown on new and aged EPDM 1, PE-Xb and PE-Xc. Concentrations of culturable *P. aeruginosa* were relatively low (< 1 KBE/cm² in 52 % of the samples and < 10 KBE/cm² in 82 % of the samples). In the literature, relatively little information exists regarding the occurrence of *P. aeruginosa* in biofilms on materials which are relevant to plumbing systems and which have been considered in the present study. In municipal drinking water systems, *P. aeruginosa* was only sporadically found in biofilms, for instance on galvanized iron surfaces (Lee and Kim, 2003), on steel coupons (Emtiazi et al., 2004), and in one (concentration of *P. aeruginosa* 0.4 cfu/cm^2) of 13 biofilms grown on EPDM-coated valves in German drinking water distribution systems (Kilb et al., 2003). In a pilot scale domestic plumbing system *P. aeruginosa* was shown to incorporate into pre-existing drinking water biofilms on EPDM, PE-Xb and PE-Xc pipes and persist there for up to 8 weeks whereas the concentrations of culturable *P. aeruginosa* rapidly decreased (Benölken et al., 2010). In testing microbial performance of materials, ethylene propylene polymer was among the elastomers which were found to support growth of *P. aeruginosa* (Colbourne, 1985). In a chemostat model of a

plumbing system, *P. aeruginosa* was identified in naturally occurring mixed-population biofilms cultivated at 20 °C, 40 °C or 50 °C on polybutylene (Rogers et al., 1994a). In the same experimental system, biofilms grown at 30 °C were shown to include *P. aeruginosa* already after 24 hours on polypropylene and PE at concentrations of 1.9 and 260 cfu/cm^2, respectively (Rogers et al., 1994b); after 21 days, the bacteria were also present in biofilms on mild steel (30 cfu/cm^2). Seeding experiments in a flow-through reactor demonstrated the integration and persistence of *P. aeruginosa* at densities of approximately 1 x 10^2 to 3 x 10^2 cfu/cm^2 in established drinking water biofilms on EPDM coupons for 28 days (Bressler et al., 2009). These observations and the results from the present study indicate that *P. aeruginosa* may occur in a culturable state in drinking water biofilms, mostly at relatively low concentrations compared to the general biofilm microflora, on new and aged materials which are employed in plumbing systems.

A direct influence of material ageing by treatment with NaOCl or ClO_2 on the incorporation of *P. aeruginosa* into drinking water biofilms grown on domestic plumbing materials was not observed. There are no studies investigating the influence of material alterations on the incorporation of *P. aeruginosa* into drinking water biofilms grown on these materials. From the results of the present work it can be concluded that material ageing by disinfectant treatment does not significantly influence the integration and survival of *P. aeruginosa* in drinking water biofilms.

Quantification of *P. aeruginosa* using FISH gave orders of magnitude higher concentrations of the organisms on EPDM 1, PE-Xb and PE-Xc compared to results obtained by the cultivation method. In many cases, only 0.01 % of the FISH-positive *P. aeruginosa* were detected by culture; in 58 % of the samples the fraction of culturable *P. aeruginosa* on FISH-positive *P. aeruginosa* was < 0.1 % and in 82 % of the samples it was < 1 %; on some occasions, *P. aeruginosa* could not be detected using the culture method, but was only detected by FISH (new and ClO_2-treated EPDM 1, new and ClO_2-treated PE-Xb and new and NaOCl-treated PE-Xc. These results suggest that *P. aeruginosa* may occur in a VBNC state in drinking water biofilms in the present study.

In biofilms grown on new and aged copper, *P. aeruginosa* could be detected neither by cultivation nor by FISH. Recently, it has been demonstrated that pure cultures of *P. aeruginosa* were rapidly killed upon adhesion to different copper cast alloys (Elguindi et al., 2009). In a bacterial suspension, 0.1 - 0.8 mg/L of copper ions ($CuCl_2$) achieved more than 99.999 % reduction of culturable *P. aeruginosa* within 1.5 h (Huang et al., 2008). Thus, a possible explanation for the absence of *P. aeruginosa* in the copper-associated biofilms of our study may be the release of copper ions which

were toxic to *P. aeruginosa*, and prevented the colonisation of the copper surface or rapidly killed adhered *P. aeruginosa* cells. In contrast to these observations, *P. aeruginosa* has been identified among the microorganisms isolated from biofilms grown on corroded copper pipes of a hospital (Wagner et al., 1992) and a nuclear power plant (Wallace et al., 1994) and was shown to incorporate into drinking water biofilms on copper pipes of a pilot scale domestic plumbing system at 12 °C and persist there for 24 h in a culturable state (Benölken et al., 2010). Using FISH, *P. aeruginosa* was detected on copper pipes for 36 weeks (Benölken et al., 2010). These observations indicate that *P. aeruginosa* nevertheless has the potential to survive in mixed-population biofilms on copper under conditions, which have to be defined in future experiments.

5.4.3. Integration and fate of *L. pneumophila* in drinking water biofilms

L. pneumophila was detected in biofilms on any of the four materials and persisted there for up to 28 days in different concentrations. A number of studies have considered the fate of *L. pneumophila* either as a component of naturally occurring mixed-population biofilms or following artificial spiking of drinking water biofilms. But the experimental conditions often differed from those of our study. The inclusion and persistence of naturally occurring *L. pneumophila* at cold-water temperatures and multiplication of these bacteria at warm-water temperatures in biofilms of autochthonous water bacteria has been observed on various plumbing materials including ethylene-propylene, PE-X, unplasticised PVC and copper (Keevil, 2002; Rogers et al., 1994a, 1994b; van der Kooij et al., 2002, 2005; Gião et al., 2009a, b). Seeding experiments reported in the literature indicated that *L. pneumophila* could become integrated in pre-existing biofilms and survive or even multiply in these biofilms, depending on the environmental conditions (Armon et al. 1997; Långmark et al., 2005b; Lehtola et al., 2007). Elastomeric materials were shown to promote growth of *L. pneumophila* in biofilms (Colbourne and Ashworth, 1986; Schönen, 1986; Benölken et al., 2010) compared to stainless steel, copper and glass surfaces. In the present study, high concentrations of *L. pneumophila* were found in biofilms grown on EPDM 1, but the concentrations were at the same level as those in biofilms grown on plastic materials and copper. Schönen et al. (1986) also reported strong proliferation of *L. pneumophila* in biofilms on PVC, polytetrafluorethylene (PTFE) and PE.

Increased concentrations of *L. pneumophila* are preferentially found in warm water systems where the temperature is favourable for growth of these bacteria (25 °C - 45 °C). The results of the seeding experiments of the present study that were conducted

at water temperatures below 25 °C demonstrate that integration and persistence of *L. pneumophila* in biofilms can also occur under cold water conditions. This is in accord with observations of other studies. Armon et al. (1997) found that culturable *L. pneumophila* survived in biofilms of heterotrophic drinking water bacteria on glass and PVC at 24 °C for more than 40 days. *L. pneumophila* inoculated into 1 month-old drinking water biofilms grown on PVC coupons in a Propella reactor was shown to persist in the biofilms under high-shear turbulent flow at an average temperature of 15 °C for at least 4 weeks and was also found in the water during this period (Lehtola et al., 2007). Under cold water conditions (12 °C), *L. pneumophila* was only sporadically detected in drinking water biofilms grown on EPDM, PE-X and copper, no multiplication of *L. pneumophila* was observed (Benölken et al., 2010). At lower water temperatures of 5.0 to 8.5 °C, addition of *L. pneumophila* to 8 week-old drinking water biofilms on glass surfaces in a pilot-scale water distribution system provided with chlorinated and UV-treated water also resulted in an accumulation and persistence of these bacteria over the experimental period of 38 days (Långmark et al., 2005b). Replication of *L. pneumophila* was not observed in our experiments and other studies mentioned above which were all conducted at temperatures of ≤ 20 °C. A possible reason may be low water temperatures, where growth of *L. pneumophila* is not expected to occur. These observations suggest that, under cold water conditions, drinking water biofilms can represent a reservoir of *L. pneumophila*. In case of a shift to higher temperatures multiplication of the bacteria can be expected; possibly, also the transition between the culturable and VBNC state may be influenced by water temperature. Future experiments have to be done in order to investigate these aspects of multiplication and the role of the VBNC state of *L. pneumophila* under warm water conditions on the relevant plumbing materials considered in the present study.

The prerequisite for *Legionella* colonisation of water systems seems to be the presence of other heterotrophic organisms, which establish the biofilms and thus provide the habitat for *Legionella* colonisation and facilitate interactions between bacteria and protozoa. A major mechanism of *L. pneumophila* growth in aquatic biofilms is supposed to be the replication within protozoan hosts which supply nutrients for *Legionella*e and protect them from external stresses (Lau and Ashbolt, 2009). Intracellular occurrence of *Legionella* in amoebae within biofilms on PE has been demonstrated *in situ* in domestic cold water plumbing systems (Kalmbach et al., 1997). In a number of studies, replication of *L. pneumophila* within biofilms of heterotrophic bacteria grown under static conditions or in flow-through systems was only ob-

served in the presence of amoebae such as *Hartmannella vermiformis* (Murga et al., 2001; Kuiper et al., 2004).

In the present study, amoebae of the genera *Hartmannella* and *Acanthamoeba* as well as other protozoa have been detected in drinking water biofilms on all new domestic plumbing materials using the FISH technique (see 4.6.). Thus, amoebae may be involved in the persistence of *L. pneumophila* in the biofilms of the present study. The aspect of bacteria-amoebae interaction is discussed later (5.5).

The incorporation of *L. pneumophila* into drinking water biofilms grown on domestic plumbing materials was not significantly influenced by material ageing due to treatment with NaOCl or ClO_2. There are no studies investigating the influence of material alterations on the incorporation of *L. pneumophila* into drinking water biofilms grown on these materials. From the results of the present work it can be concluded that material ageing by disinfectant treatment does not decisively influence the integration and survival of *L. pneumophila* in drinking water biofilms.

The concentrations of culturable *L. pneumophila* were generally higher compared to culturable *P. aeruginosa*. In only 7 % of the samples the concentrations of culturable *L. pneumophila* were < 1 cfu/cm² whereas they were > 100 cfu/cm² in 44 % of the samples. This might be due to the fact that most of the *L. pneumophila* inoculated to the drinking water biofilms were in a culturable state, whereas only 1.9 % of the *P. aeruginosa* cells in the inoculum were culturable. The difference between the number of *L. pneumophila* detected by culture and by FISH was not as high as in the case of *P. aeruginosa*. However, in some of the samples concentrations of FISH-positive *L. pneumophila* were 3 orders of magnitude higher than the concentrations of culturable *L. pneumophila* and in 85 % of the samples, the percentage of culturable *L. pneumophila* on FISH-positive *L. pneumophila* was < 25 % (< 1 in 42 % of the samples). In some cases, FISH-positive *L. pneumophila* were detected in biofilms despite the absence of culturable cells. This again indicates that part of the *L. pneumophila* population in biofilms may exist in a VBNC state and that cultural detection might underestimate the number of *L. pneumophila* present in a drinking water biofilm.

A significant drop in *L. pneumophila* culturability was observed on copper in contrast to the PE-X and EPDM materials. Sensitivity of *L. pneumophila* in copper-associated biofilms has also been found in other experimental systems. Copper temporarily limited the growth of *L. pneumophila* in biofilms of a model warm water system compared to PE-X during 250 days of exposure to warm water, but the levels of the bacteria were the same on both materials after 2 years (van der Kooij et al., 2005). Keevil (2002) found *L. pneumophila* to colonise mature biofilms on aged copper surfaces only in low culturable numbers. However, despite low culturability high

numbers of FISH-positive cells of *L. pneumophila* were still observed in our study, suggesting that copper may play a role in the transition of *L. pneumophila* to a VBNC state in biofilms on copper surfaces.

5.4.4. Integration and fate of *E. nimipressuralis* in drinking water biofilms

The coliform bacterium *E. nimipressuralis* was not detected culturally in any of the biofilms grown on new and aged plumbing materials. Although the role of biofilms in the persistence of coliform bacteria in drinking water systems as well as in the contamination of drinking water is largely unclear, there are studies showing the ability of coliform bacteria to survive in biofilms of drinking water systems. Kilb et al. (2003) demonstrated that biofilms grown on rubber-coated valves in a drinking water distribution system acted as a point source for recurring coliform detection in water. The coliform bacteria detected in the biofilms were predominantly *Citrobacter* spp., but also an *Enterobacter cloacae* was found. However, Wingender and Flemming (2004) rarely detected coliform bacteria in biofilm samples from drinking water distribution pipes in Germany. LeChevallier et al. (1987) only found coliform bacteria (5.1 cfu/5 cm²) in one coupon taken from a cast iron pipe (LeChevallier et al., 1987). Four of 19 biofilm samples from plumbing systems of sport facilities contained coliform bacteria (1 - 3 cfu/100mL; Bonadonna et al., 2009). In an investigation on water mains in France, 11 % of the water samples were positive for coliform bacteria, whereas only 3.5 % of the biofilms contained detectable coliform bacteria (Batté et al., 2006).

Many laboratory studies on the fate of coliforms introduced artificially into experimental systems indicate that these bacteria are able to integrate into established drinking water biofilms, but do not permanently colonise them. 12 h after the injection of coliform bacteria into a pilot drinking water distribution system coliforms could not be recovered from the water downstream of inoculation (McMath et al., 1999). In the same study, coliforms could be detected in low concentrations for up to 7 weeks in a situation simulating dead-end conditions, but were not found in the autochthonous biofilm grown on the pipewall after flushing the pipe (McMath et al., 1999). *Klebsiella pneumoniae* remained in biofilms on corroded iron coupons for 2 – 9 days depending on the concentration in the inoculum. Persistent colonisation of the surfaces was not observed (Szabo et al., 2006). In drinking water biofilms grown under high-shear turbulent-flow conditions *E. coli* were detectable by culture for only 4 days after spiking of the biofilms (Lehtola et al., 2007). Data on *E. nimipressuralis*, the coliform bacterium used in the present study is rare. This organism has neither been detected in biofilms of real distribution and plumbing systems and has

only been the subject of one seeding experiments using a pilot scale domestic plumbing system, in which it was also not detectable 24 h after inoculation of drinking water biofilms grown on EPDM, PE-Xb, PE-Xc and copper (Benölken et al., 2010).

5.4.5. Suitability of the experimental system

The experimental system was shown to be suitable for the generation of quasi-stable drinking water biofilms with relatively high cell numbers within 14 days. It thus allowed for the relative comparison of biofilm formation on different types and qualities of materials relevant in domestic plumbing.

Inoculation of 14 d-old biofilms grown on coupons of domestic plumbing materials and monitoring of the persistence of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* in the biofilms were performed in 100-mL stainless steel reactors under continuous flow-through with drinking water. The conditions during the study differed significantly from those prevailing in a real, full-scale domestic plumbing system. It is difficult to directly conclude from the data obtained in the reactor system to a complex plumbing system consisting of a mixture of different materials with varying flow conditions, temperatures and stagnation periods. However, the results of the present work allow general propositions regarding the formation of drinking water biofilms grown on materials utilised in domestic plumbing and the integration and persistence of hygienically relevant bacteria in these biofilms.

The seeding experiments of the present study were performed on young biofilms over a time period of about four weeks similar to the observation periods of several weeks reported in comparable studies (Lehtola et al., 2005, 2007; Declerck et al. 2009; Gião et al., 2009a, 2009b). This experimental period was suitable for monitoring the fate of *P. aeruginosa* and *L. pneumophila* in drinking water biofilms. On several occasions, *P. aeruginosa* and *L. pneumophila* were detected over the whole period of investigation by culture or FISH or by both. Considering dynamics of biofilm composition during biofilm ageing (Deines et al., 2010) experiments over longer periods of months or years will be necessary to show possible long-term persistence of *P. aeruginosa* and *L. pneumophila* in a culturable or unculturable form.

With the exception of EPDM 2, all synthetic polymeric plumbing materials tested in the present study complied with the specific physical and chemical requirements (Anonymous, 1985; UBA, 2008) as well as with the microbiological specifications (DVGW, 2007), that are the basis for their approval for use in drinking water systems in Germany. The evaluation of materials used in contact with drinking water is usually based on the determination of biomass formed on the surface either measured by the volume of slime formed on a material surface (Germany, W 270; DVGW,

2007), the ATP content of a biofilm and the bacteria in the bulk water (The Netherlands, biofilm production potential test; van der Kooij and Veenandaal, 2001) or the oxygen demand of a biofilm (United Kingdom; British standard 6929; Colbourne, 1985; Moorman, 2006). The potential of hygienically relevant bacteria to integrate into biofilms formed on the respective material or to directly adhere to the surface is not taken into account. The fact that biofilm formation on the state-of-the-art EPDM (EPDM 1) and PE-X materials (PE-Xb and PE-Xc) was significantly lower compared to EPDM 2, which did not meet any of the German requirements for materials in contact with drinking water, demonstrates that the use of materials fulfilling the respective regulations decreases the risk of massive biofilm formation in a domestic plumbing system. Nevertheless it was shown that *P. aeruginosa* and *L. pneumophila* entering a system can survive in biofilms on all synthetic polymers regardless of the concentration of general biomass for at least 28 d even on materials approved for use in contact with drinking water.

5.4.6. Evaluation of methods for the detection of potentially pathogenic bacteria in drinking water biofilms

The concentrations of *P. aeruginosa* and *L. pneumophila* in drinking water biofilms detected using FISH were often orders of magnitude higher than the concentrations of culturable *P. aeruginosa* and *L. pneumophila*. It is not known whether all bacteria detected by FISH using oligonucleotide probes which are targeted at intact rRNA are still viable. But based on the assumption that detection of rRNA with fluorescent oligonucleotide probes indicated viability, the low ratio of culturable cells to FISH-positive cells suggests that *P. aeruginosa* and *L. pneumophila* may occur in a VBNC state in drinking water biofilms in the present study. Up to now this phenomenon has not been described for *P. aeruginosa* whereas for *L. pneumophila* the observations of the present study correlate well with other investigations (Lehtola et al., 2005, Långmark et al., 2005b; Gião et al., 2009b). In a study performed by Lehtola et al. (2007, investigating the fate of *L. pneumophila* in drinking water biofilms under high-shear conditions in a Propella reactor the number of the bacteria detected with culture methods highly underestimates the number of bacteria detected by FISH (Lehtola et al., 2007). Långmark et al. (2005b) found previously that the percentage of culturable *L. pneumophila* on FISH-positive *L. pneumophila* in a pilot distribution system varied between 0.002 % and 65 % (Långmark et al., 2005b). In a heterotrophic drinking water biofilm grown in a chemostat at 35 °C *L. pneumophila* was not detected by culture although high numbers (up to 25 % of the total flora) were detected using FISH (Gião et al., 2009b). The transistion of *L. pneumophila* to a VBNC

state has been described (Hussong et al., 1987; Bej et al., 1991; Steinert et al., 1997; Allegra et al., 2008; Dusserre et al., 2008).

The hygienic relevance of bacteria in the VBNC state is still largely unclear. For some bacteria including *L. pneumophila* (Hussong et al., 1987) and *Shigella dysenteriae* it has been shown that they exhibit several virulence factors even in a non-culturable state. In addition to that, a change in environmental conditions, e.g. change in temperature, nutrient availability or the release of stress factors can cause a resuscitation of bacteria from the VBNC state back to a culturable state with a recovery of their virulence (Oliver et al., 2010). Copper-stressed and thus unculturable *P. aeruginosa* were shown to become culturable and infectious again upon release of the copper-stress (Dwidjosiswojo et al., 2010).

The results of the present work suggest that standard culture-based methods may have limitations for the evaluation of the hygienic risk of drinking water biofilms in terms of the presence of *P. aeruginosa* and *L. pneumophila*. Cultivation methods have proved value in drinking water monitoring and the prevention of water-borne diseases and epidemics. *P. aeruginosa* and *L. pneumophila* are emerging pathogens with special relevance in domestic plumbing systems. In many studies, detection of these bacteria with molecular biological methods based on PCR (Wellinghausen et al., 2001; Declerck et al., 2009; Felföldi et al., 2009) or on phylogenetic staining using fluorescent oligonucleotide probes (Långmark et al., 2005b; Lehtola et al., 2007; Gião et al., 2009b) has been shown to be more efficient compared to culture-based methods.

In addition to that, the present study showed that the parameters of the drinking water ordinance (colony count, coliform bacteria) do not indicate the presence of culturable *P. aeruginosa* or *L. pneumophila*. No correlation was observed between the concentrations of *P. aeruginosa*, *L. pneumophila* and the number of HPC bacteria and although the coliform bacterium used in the present study (*E. nimipressuralis*) was not detected in any of the biofilms, *P. aeruginosa* and *L. pneumophila* were detected by culture and FISH. Similar observations were made) in water samples from taps, boilers, water tanks and dental water units of a hospital (Petti et al., 2004; Felföldi et al., 2009) and in laboratory-grown drinking water biofilms (Lehtola et al., 2007). This demonstrates that monitoring the bacterial count alone is not enough to evaluate biological safety and possible health hazards in drinking water systems. This has also been demonstrated in other investigations (Lehtola et al., 2007; Felföldi et al., 2009).

5.5. Amoebae in drinking water biofilms grown on domestic plumbing materials

Free-living amoebae naturally present in source waters (Kuiper et al., 2006) can survive the process of drinking water purification (Hoffmann and Michel, 2001; Corsaro et al., 2010). Although the number and diversity of amoebae is significantly reduced during processing of the raw water, some species survive the treatment process and enter the water mains and domestic plumbing (Loret and Greub, 2010). Biofilms growing on the inner surfaces of distribution systems and domestic plumbing provide attractive niches for amoebae by fulfilling their nutritional requirements (Pedersen, 1990; Barker and Brown, 1994; Kalmbach et al., 1997; Khan, 2006). Amoebae grazing on biofilms can reduce biofilm thickness by 60 % (Huws et al., 2005). In drinking water biofilms free-living amoebae play an important role in the control of biomass as well as in the persistence and multiplication of certain bacteria. Especially for *Legionella*e, intracellular multiplication in and protection by amoebae has been described in detail (Lau and Ashbolt, 2009). Therefore, the presence of protozoa including amoebae in drinking water biofilms grown on domestic plumbing materials was investigated by FISH.

Amoebae of the genera *Hartmannella* and *Acanthamoeba* were present in drinking water biofilms grown on all tested plumbing materials before and after inoculation of the 14 d-old biofilms. Data on the occurrence of amoebae in biofilms grown on domestic plumbing materials are relatively rare. Amoebae of the genera that were detected in the present study, were recovered from several man-made aquatic systems, but only a few investigators sampled surfaces in order to analyse the prevalence of amoebae in biofilms. Amoebae, mainly of the genera *Naegleria*, *Echinamoeba*, *Acanthamoeba* and *Hartmannella* were detected in water and biofilms samples from different sites of water treatment plants (Thomas et al., 2008; Corsaro et al., 2010). In biofilm samples from cast iron, PVC and steel pipes of drinking water distribution systems up to 328 amoebae/cm² were detected; *Acanthamoeba* spp, *Echinamoeba* spp., *Hartmannella* spp., *Vannella* spp., *Vahlkampfia* spp., *Platyamoeba* spp. and *Saccamoeba* spp. were identified on the basis of morphological characteristics (Hoffmann and Michel, 2003). In a study conducted by Thomas et al. (2006), *H. vermiformis* and *A. polyphaga* were recovered from swab samples of taps and showers in a hospital. Kilvington et al. (2004) recovered free-living amoebae including *Acanthamoeba* spp. from biofilm samples of domestic taps in 89 % of the homes sampled; the high prevalence of amoebae was attributed to water storage cisterns where amoebae might proliferate due to the high food supply from biofilms growing at the inner walls of the storage tanks (Kilvington et al., 2004).

The presence of amoebae in drinking water biofilms has also been described in laboratory investigations (Rogers et al., 1994b, Loret et al., 2005). In a domestic water supply simulation unit consisting of galvanized steel and copper pipes that was operated with sand filtered river water for 2 weeks and subsequently with tap water for 1 month, a stable biofilm community with an amoeba population comprising *Hartmannella*, *Acanthamoeba* and *Vahlkampfia* spp. established (Loret et al., 2005). Rogers et al. (1994b) detected amoebae of the genus *Hartmannella* (*H. vermiformis* and *H. cantabrigiensis*) and *Verillifera bacillipedes* in drinking water biofilms grown on ethylene-propylene, latex and PVC by microscopical identification.

The high percentage of *Hartmannella* and *Acanthamoeba* spp. on the total number of protozoa detected by the *Eukarya* probe in the present study indicates that *Hartmannella* and *Acanthamoeba* are the dominant genera in drinking water biofilms grown on domestic plumbing materials in the present study. These genera were also found to predominate in drinking water environments in other studies (Paszko-Kolva et al., 1991; Michel et al., 1995; Thomas et al., 2006; Thomas, 2009).

In the present study, the concentrations of total protozoa, *Acanthamoeba* spp. and *Hartmannella* spp. and the proportion of the three genera were comparable in biofilms grown on the PE-X materials and on copper. Thus, copper did not inhibit attachment and growth of amoebae. A colonisation of copper by amoebae was also reported in other studies (Rogers et al., 1994b; Hoffmann and Michel, 2003). In flow-through reactors connected to drinking water distribution systems, amoebae of the genera *Acanthamoeba*, *Prothacanthamoeba*, *Hartmannella*, *Naegleria*, *Vannella*, *Vahlkampfia*, *Willaertia* and *Saccamoeba* were detected regularly over a period of 6 – 24 months in biofilms grown on coupons of stainless steel, copper, PE and PVC; a material dependent occurrence of amoebae was not observed (Hoffmann and Michel, 2003). In biofilms grown on EPDM 1, the concentrations were about one order of magnitude higher compared to copper and PE-X materials. A strong correlation of total cell count and the concentration of total protozoa, *Hartmannella* spp. and *Acanthamoeba* spp. was observed. As protozoa are known to live in association with biofilms where they feed on the various bacteria (Barker and Brown, 1994), the higher concentrations of biomass growing on EPDM 1 expectedly result in the presence of higher concentrations of protozoa. This was also described in an investigation of different water treatment plants, where most of the samples positive for amoebae originated from biofilms grown on granular activated carbon filters or from sediments (Corsaro et al., 2010).

The results of the present study confirm the observation that amoebae are ubiquitous in water distribution systems. The presence of amoebae is of hygienic rele-

vance because the amoebae themselves can constitute a threat to human health by causing amoebic keratitis (Khan, 2006) as well as life-threatening and often fatal infections of the central nervous system (Marciano-Cabral and Cabral, 2003). The ability of amoebae to form cysts upon exposure to harsh environmental conditions makes them highly resistant against a number of biocides (Barker et al., 1992; Cengiz et al., 2000; Beattie et al., 2003), including chlorine and chlorine dioxide (Loret et al., 2008). The potential of amoebae to harbour strict or opportunistic pathogens including *L. pneumophila* (Berk et al., 1998; Kuiper et al., 2004; Donlan et al., 2005), *P. aeruginosa* (Hwang et al., 2006), *M. avium* (Cirillo et al., 1997; Thomas et al., 2007) and *Parachlamydia acanthamoeba* (Greub et al., 2003) is of high hygienic and clinical relevance (Khan, 2006). The bacteria are able to infect and survive in association with different amoebae, and thus may by-pass disinfection treatment (Corsaro et al., 2010; Loret and Greub, 2010).

The presence of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* did not significantly influence the presence or concentrations of *Eukarya*, *Hartmannella* spp. and *Acanthamoeba* spp. in drinking water biofilms. This demonstrates that the autochthonous bacterial flora of the drinking water biofilm constituted a sufficient nutrient source for *Acanthamoebae* and *Hartmannellae* and that the amoebal populations in the mixed species biofilm were not affected by the addition of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis*.

In contrast to *P. aeruginosa*, the concentration of FISH-positive *L. pneumophila* in biofilms grown on domestic plumbing materials in the present study strongly correlated with the concentration of total protozoa, *Hartmannellae* and *Acanthamoebae* indicating that survival of *L. pneumophila* in the biofilms was associated with the presence of amoebae and other protozoa. Amoebae might protect *L. pneumophila* from the harsh environmental conditions of the drinking water environment. It was shown that *L. pneumophila* Lens could survive for at least 6 months in a poor medium in association with *A. castellanii* (Bouyer et al., 2007).

In several investigations it was observed that *L. pneumophila* in biofilm communities is only able to multiply in the presence of amoebae (Murga et al., 2001; Kuiper et al., 2004; Declerck et al., 2009). In the present study, no multiplication of *L. pneumophila* was detected, but this might be explained by the low temperatures used in the present study compared to that in literature data. Nevertheless, the observations of the present study suggest that amoebae may serve as a reservoir for *L. pneumophila* in cold water domestic plumbing systems.

Amoebae were shown to be involved in the resuscitation of bacteria from a viable but non-culturable state (Steinert et al., 1997; Hwang et al., 2006) and the passage of

L. pneumophila through amoebae can lead to enhanced resistance against disinfection and an increase in virulence and invasiveness of *L. pneumophila* (Cirillo et al. 1999; Brieland et al., 1997; Abu Kwaik et al., 1998; Barker et al., 1992; Donlan et al., 2005; Molmeret et al., 2005). *L. pneumophila* and other *Legionella*e probably are in complex interaction with all organisms inside a biofilm (Taylor et al., 2009), but the presence of amoebae and their prevention and eradication should definitely be taken into account in case of a *Legionella* contamination of a domestic plumbing system. Loret et al. (2008) suggest considering the hazard arising from the presence of amoebae and their possible interaction with bacteria in any risk assessment conducted in the framework of a water safety plan.

5.6. Interaction between *P. aeruginosa*, *L. pneumophila* and *A. castellanii*

It is well known that amoebae and hygienically relevant bacteria such as *Legionella* spp. can share drinking water biofilms as a habitat (see 5.5). In order to elucidate the interactions of *P. aeruginosa* and *L. pneumophila* with the amoeba *A. castellanii* under conditions that are less complex than a multi-species drinking water biofilm, in vitro studies involving two or all three organisms under defined conditions in the laboratory were performed.

The results of the co-cultivation of *P. aeruginosa*, *L. pneumophila* and *A. castellanii* under nutrient rich conditions at 30 °C showed, that *P. aeruginosa* was the dominant organism impairing growth and survival of both *L. pneumophila* and *A. castellanii*.

In the literature, a wide variety of observations on the interaction of *P. aeruginosa* and amoebae have been described. These include an amoebicidal behaviour (Weitere et al., 2005) involving the effector proteins ExoU, ExoS, ExoT and ExoY of the type III secretion system of *P. aeruginosa* (Matz et al. 2008; Abd et al. 2008), but also survival of *A. castellanii* for 48 h of co-incubation with different strains of *P. aeruginosa* in PYG at 35 °C (Cengiz et al., 2002) or a complete reduction of microcolonies of the alginate overproducing *P. aeruginosa* strain PDO300 by *A. polyphaga* in a medium containing 400 mg/L glucose (Weitere et al., 2005) have been demonstrated. A growth promoting effect of *Acanthamoebae* on *P. aeruginosa* in the domestic plumbing system of a hospital as well as the intracellular infection of *Acanthamoebae* by *P. aeruginosa* were shown by Michel et al. (1995). Simmons et al. (1998) reported increased adhesion of a wild-type *A. castellanii* to contact lenses that were previously covered by a *P. aeruginosa* biofilm. The great variation in the observations made on the interaction of *P. aeruginosa* and amoebae indicate that the inter-

play of these organisms is highly complex and dependent on a variety of factors. Further experiments are necessary to define the parameters influencing the interaction of *P. aeruginosa* and free-living amoebae in drinking water biofilms.

The suppression of *L. pneumophila* by *P. aeruginosa* observed in the present study has also been demonstrated before (Gomez-Lus et al., 1993; Kimura et al., 2009). Gomez-Lus and colleagues (1993) demonstrated that *P. aeruginosa* was active against *L. pneumophila* when co-cultivated on BCYEα agar. Kimura and co-workers (2009) showed that *P. aeruginosa* quorum sensing autoinducer 3-oxo-C_{12}-HSL has a suppressive impact on growth and biofilm formation of *Legionella* spp.. Cotuk et al. (2005) found that cultures of *P. aeruginosa* ATCC strain 9027 grown on plate count agar and added to *L. pneumophila* serogroup 1 (ATCC 33152) grown on BCYE plates inhibited *L. pneumophila*, whereas cell-free supernatants of *P. aeruginosa* were ineffective against *L. pneumophila*.

In cultures containing all three organisms, *L. pneumophila* and *A. castellanii* did not show any beneficial or antagonistic impact on each other. Neither did *A. castellanii* prevent killing of *L. pneumophila* by *P. aeruginosa*, nor did *L. pneumophila* decrease the amoebicidal activity of *P. aeruginosa*.

When co-cultured only with *A. castellanii, L. pneumophila* was shown to enter the amoebae within 24 h of incubation and to completely kill *A. castellanii* within 7 d. Intracellular multiplication and subsequent killing of amoebae due to lysis of the host induced by massive proliferation of ingested *L. pneumophila* has been demonstrated in several investigations (Cirillo et al. 1994; Lück and Steinert, 2006; Seno et al., 2006; Ohno et al., 2008). Intracellular growth of *L. pneumophila* with subsequent release of the bacteria may be of hygienic relevance in drinking water environments, because intracellularly grown *L. pneumophila* showed an increased ability to invade human monocytes compared to *L. pneumophila* grown in the absence of amoebae (Cirillo et al., 1994), which enhances the health risk for humans.

In co-cultures of *A. castellanii* and *L. pneumophila*, amoebal cysts were observed after 24 h of incubation some of them containing bacteria. Survival of *L. pneumophila* inside amoebal cysts may hinder the eradication of *L. pneumophila* in drinking water using disinfection with chlorine and chlorine dioxide (Loret et al., 2008).

Under nutrient-limited conditions (incubation in PAS), results obtained for the interaction of *P. aeruginosa*, *L. pneumophila*, and *A. castellanii* significantly differed from the results gained in the co-cultivation studies in the growth medium. No antagonistic effect of *P. aeruginosa* on *A. castellanii* was observed in co-cultures of the two organisms in PAS. This observation correlates well with the results of Pukatzki et al. (2002) who demonstrated that *P. aeruginosa* did not kill *A. castellanii* in co-

cultivation in PAS. However a growth-restricting effect of *P. aeruginosa* on *A. castellanii* was shown (Pukatzki et al., 2002). Contradictory to that co-incubation of *A. castellanii* and *P. aeruginosa* in non-nutrient saline resulted in an amoebicidal effect of *P. aeruginosa* on the amoebae (Qureshi et al., 1993). Enhanced proliferation of *A. castellanii* incubated with *P. aeruginosa* in PBS or Neff's saline at 28 °C was observed by de Moraes and Alfieri (2008).

P. aeruginosa showed a rapid increase in both culturable and FISH-positive cells in the presence of *A. castellanii* as well as in the presence of *A. castellanii* and *L. pneumophila* despite the absence of nutrients. The proliferation of *P. aeruginosa* may be attributed to the production of growth promoting substances by the amoeba or to *P. aeruginosa* feeding on a few lysed amoeba cells present in the culture.

When co-cultivated under non-growing conditions *P. aeruginosa* did not affect *L. pneumophila* and *A. castellanii* as significantly as it did under growing conditions indicating that the presence of nutrients is a prerequisite for the expression and action of *P. aeruginosa* virulence factors such as toxic pigments, extracellular enzymes and rhamnolipids (von Delden, 2004) or the type III secretion system (Qureshi et al., 1993). Further research is necessary to define the virulence traits involved in the bactericidal and amoebicidal action of *P. aeruginosa* and the appropriate conditions leading to their expression.

Co-cultivation of *A. castellanii* with *L. pneumophila* in PAS did not result in amoebal lysis. Cell numbers of *A. castellanii* remained constant over the whole period of the experiment. However, microscopic observation showed *L. pneumophila* to be internalised by amoebae already after 24 h of incubation. A reason for the survival of the amoebae might be the relatively short incubation time that was probably not sufficient for *L. pneumophila* to grow to concentrations high enough to induce lysis of the amoebae. Several laboratory studies have been performed on interactions between *L. pneumophila* and *A. castellanii* under nutrient-limited conditions. Moffat and co-workers (1991) showed that *L. pneumophila* was able to replicate within *A. castellanii* under nutrient-limited conditions in *A. castellanii* buffer at 37 °C within 24 h. Bouyer and co-workers (2007) demonstrated that *L. pneumophila* survived for at least 6 months in an oligotrophic medium in association with *A. castellanii.* Co-cultivation of *L. pneumophila* with *A. castellanii* can even result in resuscitation of viable but nonculturable *L. pneumophila* (Steinert et al., 1997).

The present study demonstrates that *P. aeruginosa*, *L. pneumophila* and *A. castellanii* interact in pairs and in combination of all three organisms. Previous studies as well as the present study have shown the interaction of the three organisms to be complex and highly dependent on a variety of factors. In the present study, the in-

fluence of nutrient availability has been elucidated, but other factors, e.g. temperature or the ratio of bacteria to amoebal cells (multiplicity of infection) have to be taken into consideration as well. Ohno et al. (2008) found that *L. pneumophila* invades *A. castellanii* and replicates inside the amoebae at temperatures above 25 °C. At temperatures below 20° C *L. pneumophila* was digested by *A. castellanii* and eliminated by the process of encystment (Ohno et al., 2008). Greub et al. (2003) proved a similar effect for the interaction of *Parachlamydia acanthamoeba* and *A. polyphaga*: at temperatures between 32 °C and 37 °C *P. acanthamoeba* was lytic for *A. polyphaga* and co-incubation at 25 °C - 30 °C resulted in the establishment of a symbiosis between the two organisms (Greub et al., 2003). Wang and Ahearn (1997) studied the influence of various bacteria on *A. castellanii* at different multiplicities of infection in buffered saline. At a density of bacteria to amoebae of 1:1 or 1:10, *P. aeruginosa* promoted growth and survival of *A. castellanii* whereas at a ratio of bacteria to amoebae of 100:1 or greater, *A. castellanii* were killed by *P. aeruginosa* (Wang and Ahearn, 1997). Further experiments are necessary to define the impact of additional factors such as virulence and developmental stage of the organisms.

The interactions between *P. aeruginosa*, *L. pneumophila* and *A. castellanii* are of high relevance as they can occupy the same ecological niche. They all occur in biofilms of natural and artificial environments such as surface waters, soil, drinking water distribution systems and domestic plumbing systems. In the present study, it was demonstrated that amoebae of the genera *Hartmannella* and *Acanthamoeba* were ubiquitous in drinking water biofilms formed on domestic plumbing materials in the presence and absence of *P. aeruginosa* and *L. pneumophila* (see 5.5). The various interactions of *P. aeruginosa*, *L. pneumophila* and *A. castellanii* discussed in the present chapter are probably also part of the various processes occurring in a drinking water biofilm. Although it is difficult to draw direct conclusions from a laboratory system consisting of two or three microorganism to complex mixed-population drinking water biofilms, general propositions can be made. In health concerns arising from *P. aeruginosa* or *L. pneumophila* present in drinking water systems, the interaction between the two organisms and especially the role of free-living amoebae has to be taken into account.

5.7. Copper sensitivity of *P. aeruginosa*

During the investigation of biofilm formation on domestic plumbing materials and the incorporation of *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* into these biofilms, several observations indicating an inhibiting effect of copper on the cul-

turability of microorganisms were made: (i) The fraction of culturable heterotrophic plate count bacteria on the total cell count was significantly lower in biofilms grown on copper compared to biofilms grown on EPDM and PE-X materials; (ii) The culturability and detectability of *P. aeruginosa* and *E. nimipressuralis*, respectively, significantly decreased after incubation in copper-containing drinking water for 24 h; (iii) *P. aeruginosa* integrated into biofilms grown in the presence of copper, but their culturability was extremely low (< 1 cfu/cm² in 82 % of the samples) compared to the concentration of *P. aeruginosa* detected using the culture-independent FISH; (iv) *P. aeruginosa* was not detected by culture or using FISH in biofilms grown on copper; (v) Recovery of *L. pneumophila* by culture from biofilms grown on copper was lower than from biofilms grown on EPDM and PE-X materials. Based on these observations and evidence from the literature (Teitzel and Parsek, 2003; Harrison et al., 2005; Teitzel et al., 2006; Santo et al., 2008; Elguindi et al., 2009) it was assumed that copper has an inhibiting effect on the autochthonous drinking water microflora as well as on *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis*. The influence of copper ions present in drinking water on bacterial survival was exemplarily investigated for *P. aeruginosa*.

5.7.1. Effect of copper on the culturability of *P. aeruginosa*

An antimicrobial effect of copper on *P. aeruginosa* (Teitzel and Parsek, 2003; Harrison et al., 2005; Teitzel et al., 2006; Elguindi et al., 2009) as well as on a number of Gram-negative bacteria, including *Escherichia coli* (Santo et al., 2008), *Acinetobacter baumannii* (Huang et al., 2008) and the plant pathogens *Erwinia amylovora* (Ordax et al., 2006) and *Agrobacterium tumefaciens* (Alexander et al., 1999) has been demonstrated in several studies. These studies were performed in growth media or mineral salt solutions, but not in real drinking water. In the present study, survival of the environmental strain *P. aeruginosa* AdS was determined in drinking water samples which differed in their copper content.

Incubation of the bacteria in the water samples (10^6 cells/mL) at 20 °C for 24 h resulted in a decrease of culturability with increasing copper concentrations of the waters, whereas total cell counts did not change. After incubation in drinking water collected in Duisburg containing 0.19 mg/L copper *P. aeruginosa* was not culturable anymore. Experiments in deionised water with or without added copper ($CuSO_4$), representing water without any substances which might possibly interfere with the biological effect of copper verified that the decrease of *P. aeruginosa* was in fact related to the presence of copper. A copper concentration of 1 µM resulted in a decrease of culturability by about three log units, while at concentrations ≥ 10 µM (≙

0.67 mg/L), the colony counts declined to below detection limit. This correlates well with a study of Huang et al. (2008) in which colony counts of *P. aeruginosa* incubated in deionised water containing copper concentrations of 0.1 – 0.8 mg/L were reduced by more than 99.999 % within 1.5 h. Inhibition concentrations of copper are usually reported to be higher (sometimes in the mM range) possibly due to the use of growth media (e.g. Luria-Bertani media with vitamin B1) and salt solutions, which contain components that may interact with copper ions, thus reducing the availability of copper (Teitzel and Parsek, 2003; Harrison et al., 2005; Santo et al., 2008). In the present study, bacteria were only exposed to media which did not (deionised water) or only contain low levels of such substances (drinking water samples, finished water).

In order to confirm the role of copper in the inactivation of *P. aeruginosa*, the chelator sodium diethyldithiocarbamate (DDTC) which was previously found suitable to neutralise copper (Harrison et al., 2005), was applied to copper-containing water samples. The experiments showed that due to the addition of DDTC (100 µM) to deionised water containing 1 µM copper, the culturability of *P. aeruginosa* was not affected whereas in test suspensions containing copper (1 µM) without DDTC, colony numbers were found to decrease by about four log units. Accordingly, survival of *P. aeruginosa* in drinking water and finished water was greatly improved due to DDTC addition (100 µM). A similar protective effect of metal chelators was reported by Santo et al. (2008); the survival of *E. coli* on copper surfaces could be improved using relatively high concentrations (mM range) of the chelators EDTA and bathocuproine disulfonate (BCS).

In the present study, using DDTC, a complete restoration of culturability was observed in deionised water supplemented with copper whereas the culturability in natural copper containing water samples was not completely restored. This indicates the presence of additional substances that impaired the culturability of *P. aeruginosa* apart from copper ions. However, the results confirm that copper ions are the major active agents involved in the inhibitory effect on *P. aeruginosa* in drinking water.

5.7.2. Viability of *P. aeruginosa* in deionised water and natural copper-containing drinking waters

The spread plate method which has been used for estimating the viable count only includes culturable bacteria that are able to initiate cell division at a sufficient rate to form visible colonies on agar media. This method is biased by probably unsuitable culture conditions such as temperature, composition of the medium, incuba-

tion time and by the physiological state of bacteria cells (McFeters, 1990). Therefore, the viability or activity of non-culturable cells was assessed using the LIVE/DEAD® kit that allows for differential quantification of viable cells, i.e. cells with an intact cytoplasmic membrane from cells with a damaged membrane defined as dead cells (Haugland, 2005).

The concentration of viable *P. aeruginosa* detected using the LIVE/DEAD kit was almost as high as the total cell count in natural copper containing waters and in deionised water supplemented with copper, even at high concentrations (100 µM) whereas the culturability decreased depending on the copper content present in the different water samples. These experiments demonstrate that, although copper inhibited culturability, it did not damage the cytoplasmic membrane of *P. aeruginosa.* It can be assumed that the fraction of *P. aeruginosa* that is unable to form colonies on nutrient agar is still viable indicating that part of the bacteria entered a VBNC state upon exposure to copper containing drinking waters or deionised water.

This result correlates with those obtained in several other studies. The exposure of *E. amylovora* to copper (5 µM) resulted in a significant decrease in colony counts whereas using the LIVE/DEAD kit a high fraction of cells (94.5 % of total cells) was found viable (Ordax et al., 2006). A similar observation was reported in a study investigating the viability of *R. leguminosarum* in the presence of copper in concentrations ranging from 5 µM to 0.5 mM (Alexander et al., 1999). Contrary results were described by Teitzel and Parsek (2003). They investigated the susceptibility of *P. aeruginosa* PAO1 in biofilms to copper and found that determined with the LIVE/DEAD kit > 99 % of cells on the biofilm surface were dead. This could be explained by the higher copper concentrations tested (1 mM) in their study and by the different strain, which might be more susceptible to copper than the environmental strain from a domestic plumbing system used in the present study (*P. aeruginosa* AdS).

5.7.3. Resuscitation of copper-stressed planktonic *P. aeruginosa*

The VBNC state can only be considered a significant means of survival if bacteria are able to regain metabolic activity when environmental conditions become more favourable and thus become culturable again. It has been reported that several bacteria including *P. aeruginosa* enter the VBNC state, but only few have been demonstrated to resuscitate to the culturable state upon release of stress factors (Oliver, 2005).

To investigate a possible resuscitation of non-culturable *P. aeruginosa* a suspension containing copper-stressed bacteria (total count 10^6 cells/mL, 10^3 cfu/mL culturable

P. aeruginosa) was incubated in deionised water containing the copper-neutralizing agent DDTC (100 µM) without addition of nutrients for a period of 7 d at 20 °C. During the period of 7 d of incubation in the presence of DDTC, total cell counts remained constant at 10^6 cells/mL, whereas colony counts increased, reaching a level equal to the initial cell numbers (10^6 cells/mL) after 7 d.

Reports of a similar case concerning *P. aeruginosa* were not found. In a study on the plant pathogen *E. amylovora* resuscitation of the bacteria from a copper-induced VBNC state was achieved by addition of EDTA or citric acid (Ordax et al., 2006). Resuscitation of several bacteria including *Vibrio vulnificus, V. cincinnatiensis* and *L. pneumophila* upon the release of stress factors such as low temperatures, heat shock and nutrient limitation has been demonstrated in various studies (Whitesides and Oliver, 1997; Steinert et al., 1997; Kell et al., 1998; Allegra et al., 2008; Zhong et al., 2009). The major problem concerning resuscitation is the difficulty in demonstrating that the increase in bacterial culturability after resuscitation was a result of true resuscitation of the VBNC cells and not because of the re-growth of a few culturable cells which were not detected before (Kell et al., 1998; Whitesides and Oliver, 1997; Oliver, 2010). In resuscitation experiments using DDTC solution in the present study, bacteria became again culturable whereas the total cell counts did not increase. This shows that re-growth of culturable cells did not occur. Thus, it can be assumed that VBNC cells were truly resuscitated.

5.7.4. Resuscitation of copper-stressed *P. aeruginosa* from drinking water biofilms

Experiments in copper containing drinking water and deionised water supplemented with copper showed that *P. aeruginosa* enters a non-culturable state in the presence of copper ions and becomes culturable again upon the addition of the copper chelator DDTC within 7 d. The incorporation of *P. aeruginosa* into drinking water biofilms resulted in low numbers of culturable *P. aeruginosa* in biofilms compared to the number of *P. aeruginosa* detected by the culture-independent FISH. As the drinking water used for generating biofilms originated from a copper plumbing system, copper was suspected to impair culturability of *P. aeruginosa* not only in suspension, but also in biofilms. The presence of copper ions (up to 46 µg/cm²) was detected in drinking water biofilms grown on EPDM 1, PE-Xb and PE-X c. In order to investigate whether resuscitation of non-culturable *P. aeruginosa* is also possible in biofilms, 14 d-old drinking water biofilms grown on coupons of EPDM 1 under constant flow conditions and inoculated with *P. aeruginosa*, *L. pneumophila* and *E. nimipressuralis* (10^6 cells/mL each) were statically incubated in the presence of 100

µM DDTC at 20 °C for 7 d. After 7 d of incubation with 100 µM DDTC, the concentration of culturable and FISH-positive *P. aeruginosa* increased by 4 and 2 orders of magnitude, respectively. The percentage of culturable *P. aeruginosa* on FISH-positive *P. aeruginosa* increased from 0.08 % to 8.2 %. The results show that non-culturable *P. aeruginosa* present in a drinking water biofilms can become culturable again upon the release of the copper stress. No significant change in total cell count and colony count was observed indicating that the increase in culturable *P. aeruginosa* detected can indeed be attributed to resuscitation.

Previous studies on the resuscitation of bacteria were conducted using pure culture bacterial suspensions with or without the addition of nutrients (Whitesides and Oliver, 1997; Ordax et al., 2007; Zhong et al., 2009). Comparable results for biofilms or biofilm suspensions were not recorded up to now.

Using the example of copper, the results of the present study imply that changes in the conditions of a water distribution system resulting in the release of stress factors may result in the resuscitation and subsequent proliferation of VBNC bacteria from a non-culturable state back to a state in which they are able to grow on routine media. This process might explain phenomena such as rapid re-colonisation of water systems by the pathogens immediately after cessation of a disinfection or recurring incidents of contamination. From a public health perspective this is of great importance, because potentially pathogenic bacteria in the VBNC state may retain or regain their virulence (Hussong et al., 1987; Rahman et al., 1996; Zhong et al., 2009) and can thus initiate infection when they undergo transition back to the culturable state. First experiments on resuscitated cells of the *P. aeruginosa* strain used in the present study have also demonstrated the cytotoxicity of these cells on hamster cells (Dwidjosiswojo et al., 2010). Thus, the VBNC state constitutes an infectious potential and a probable reservoir of undetected strict or opportunistic pathogens when present in biofilms of man-made water systems (Oliver, 2010).

In addition to that, the results presented in this work put into question the exclusive use of culture-based methods for the detection of hygienically relevant bacteria in drinking water. Improvements in detection, especially in sensitive areas, could be achieved by complementation of the standard cultivation methods with molecular techniques such as PCR and FISH. These tools have been successfully applied for the detection and quantification of hygienically relevant bacteria and amoebae in the present work as well as in previous studies (Stothard et al., 1999; Grimm et al., 2001; Khan et al., 2001; Schroeder et al., 2001; Kuiper et al., 2006; Declerck et al., 2007; Declerck et al., 2009; Feazel et al., 2009).

5.8. Outlook

The results of the present study show that *P. aeruginosa* and *L. pneumophila* are able to incorporate into and persist in drinking water biofilms relevant in domestic plumbing systems over a period of 43 days. The influence of different plumbing materials serving as a substrate has been elucidated. Other factors influencing the complex mechanisms of incorporation and persistence of these bacteria in mixed-species biofilms of domestic plumbing systems including water temperature, hydrodynamic conditions, nutrient availability or the presence of disinfectants probably are of importance and should be taken into account.

In addition to that, considering dynamics of biofilm composition during biofilm ageing experiments over longer periods of months or years will be necessary to show possible long-term persistence of *P. aeruginosa* and *L. pneumophila* in a culturable or unculturable form.

The interactions of the potentially pathogenic bacteria *P. aeruginosa*, *L. pneumophila* and the amoeba *A. castellanii* have been shown to be complex even under controlled conditions in the laboratory. Especially the interaction between *P. aeruginosa* and *A. castellanii* seems to be multifarious and highly dependent on the environmental factors. The various processes occurring in mixed population drinking water biofilms that might allow or prevent incorporation and persistence of hygienically relevant bacteria are still largely unclear. The interspecies relationships between potentially pathogenic bacteria and the autochthonous aquatic microorganisms involving the production of inhibitory substances, intercellular communication, competition for substrates or cooperation in the utilisation of carbon and energy sources are processes that determine the incorporation, survival and proliferation of biofilm inhabitants.

The results of this work suggest that *P. aeruginosa* and *L. pneumophila* can be present in drinking water biofilms in the VBNC state. Further research is necessary to explain the mechanisms taking place at the molecular level when bacteria are exposed to environmental stress factors. Similarly, the conditions and mechanisms leading to a reversible transition into the VBNC state and to resuscitation from a non-culturable back to a culturable state need to be elucidated to completely understand the significance of the VBNC state.

Copper has been identified as one factor inducing the VBNC state in *P. aeruginosa* in drinking water environments. A decline in culturability of *L. pneumophila* has also been observed in biofilms grown on copper in the present study and other investigations (Rogers et al., 1994a; van der Kooij et al., 2005; Mathys et al., 2008), but *L. pneumophila* does not seem to be as sensitive against copper as *P. aeruginosa*.

Other factors that are responsible for a transition of *P. aeruginosa* and *L. pneumophila* into the VBNC state in drinking water environments have to be determined. The hygienic relevance of *P. aeruginosa* and *L. pneumophila* in the VBNC state is still largely unclear. The infectivity of non-culturable *L. pneumophila* towards embryonated chick eggs has been demonstrated (Hussong et al., 1987), but it is not clear, if resuscitation was involved in this process. First experiments showed that copper-stressed and thus non-culturable *P. aeruginosa* are not cytotoxic on hamster cells, but resuscitation of the bacteria resulted in the recovery of cytotoxicity (Dwidjosiswojo et al., 2010).

Although molecular biological detection methods such as FISH and PCR have proved value for the detection of hygienically relevant bacteria in the present study and in previous investigations (Långmark et al., 2005b; Lehtola et al., 2007; Gião et al., 2009a, 2009b), the exact physiological state and the infectious risk emanating from a pathogen that is not culturable, but detected by culture-independent methods are still unclear. Further research on the molecular biological techniques is necessary for their implementation as complementary tools for the elucidation of contamination cases and the assessment of disinfection measures, especially in sensitive areas such as hospitals and other health care facilities.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Integration of hygienically relevant bacteria in drinking water biofilms grown on domestic plumbing materials"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im Februar 2011

(Miriam M. Moritz)