Influence of temperature on the survival of hygienically relevant bacteria in drinking water and drinking water biofilms

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„It always seems impossible until it's done."

Nelson Mandela

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Abbreviations

ACIA arctic climate impact assessment
AOC assimilable organic carbon
APS ammonium persulfate
ATP adenosine triphosphate
BMBF Bundesministerium für Bildung und Forschung (German minitry of education and research)
cfu colony forming units
DAPI 4',6-diamidino-2-phenylindole
DEPC diethylpyrocarbonate
dNTP deoxyribonucleotide triphosphate
DGGE denaturing gradient gel electrophoresis
DNA deoxyribonucleic acid
DSMZ Deutsche Stammsammlung von Mikroorganismen und Zellkulturen (German collection of microorganisms and cell cultures)
DVC direct viable count
DVGW Deutscher Verein des Gas- und Wasserfaches (German Gas and Water Association)
E.coli. Escherichia coli
EDTA ethylene diamine tetra-acetic acid
EEA European Environment Agency
ELR Emscher-Lippe Region
EPDM ethylene propylene diene monomer
EPS extracellular polymeric stubstances
FISH fluorescence *in situ* hybridization
GHG Greenhouse gases
HPC heterotrophic plate count
IPCC Intergovernmental Penal on Climate Change
KOH potassium hydroxide
K.pneumoniae Klebisella pneumoniae

LB Lenox broth
L. pneumophila. *Legionella pneumophila*
MPN most probable number
NA nutrient agar
NaCl sodium chloride
n.d. not detected
n.q. not quantified
p. a. per analysis
P. aeruginosa. *Pseudomonas aeruginosa*
PAGE polyacrylamide gel electrophoresis
PBS phosphate-buffered saline
RCP representative concentration pathways
PCR polymerase chain reaction
PE polyethylene
PI propidium iodide
RNA ribonucleic acid
RT room temperature
PBS phosphate buffered saline
PCR Polymerase chain reaction
SDS sodium dodecyl sulfate
TAE Tris/acetic acid/EDTA
TBE Tris/borate/EDTA
TE Tris/EDTA
TCC total cell count
TEMED N,N,N',N'-tetramethylethylenediamine
TrinkwV Trinkwasserverordnung (German Drinking Water Ordinance)
Tris Tris(hydroxymethyl)-aminomethane
VBNC viable but nonculturable
v/v volume per volume
WMO World Meteorological Organization
w/v weight per volume
YEB yeast extract broth

Abstract

Drinking water biofilms are ubiquitous and approximately over 95 % of the biomass within distribution systems is attached to surfaces. Predominantly, drinking water biofilms consist of autochthonous aquatic microorganisms of no relevance for human health. However, pathogens may occasionally enter and contaminate drinking water distribution systems and persist in these biofilms. Temperature is a critical factor for growth of microorganisms. As a consequence of climate change induced global warming, raw water and soil temperatures may increase resulting in elevated temperatures in drinking water distribution systems. Therfefore, this study focused on the influence of temperature on incorporation and persistence of pathogens in drinking water biofilms.

In laboratory experiments, biofilms were grown on coupons of elastomeric (EPDM) and plastic (polyethylene PE80) material in either oligotrophic or copiotrophic drinking water at temperatures in the range of 8 °C to 29 °C. Incorporation and persistence of the target organisms were monitored by standard cultural methods and by culture-independent fluorescence in situ hybridization (FISH). All target organisms incorporated into the established biofilms. Numbers of cultivable *E. coli* and *K. pneumoniae* were hardly affected by water temperatures. On both materials incorporation decreased at temperatures above 21 °C under oligotrophic conditions.The decrease of the numbers of both target organisms was more significant whit cultural methods than with FISH. Incorporation of cultivable *P. aeruginosa* increased at a first step at temperatures above 8 °C regardless of material and nutrient situation. Another increase was observed at temperatures above 21 °C. At temperatures above 8 °C incorporation of *L. pneumophila* was largely similar in the oligotrophic water,

whereas no cultivable *L. pneumophila* were detectable at water temperatures above 21 °C regardless of the nutrient situation. Persistence of *E. coli* and *K. pneumoniae* was significantly enhanced by nutrient addition but not by water tempertaure. With FISH, *E. coli* and *K. pneumoniae* were detectable over the entire period of the experiment under all conditions and cell densities were significantly higher than those detected by cultural methods indicating that these bacteria possibly have entered a viable but nonculturable (VBNC) state in drinking water biofilms. However, at 25 °C no culturable *E.coli* and *K. pneumoniae* were detectable independet of nutrient situation and coupon material. Culturable as well as nonculturable *P. aeruginosa* was detectable over the whole period of the experiment, regardless of water temperature and nutrient situation. Persistence of culturable *L. pneumophila* was significantly shortened by the addition of nutrients. On PE persistence was enhanced at temperatures above 21 °C under oligotrophic conditions.

In field experiments, biofilms were established in a real drinking water system to monitor possible impacts of drinking water temperature on drinking water quality. No distinct effect of water temperature was observed. However, drinking water biofilms showed higher cell densities on nutrient-leaking materials. In 18 % of the analyzed drinking water biofilms, hygienically relevant bacteria were detected by cultural methods. The number of colony forming units of coliform bacteria slightly increased with elevated temperatures (4 % in winter; 17 % in summer). With FISH, culturally not detectable hygienically relevant organisms were detected (*P. aeruginosa*, *E. coli*, *K. pneumoniae*, *L. pneumophila*), indicating their presence a VBNC-state. Moreover, the influence of temperature on the persistence of the target organisms possibly present in the VBNC state was strain dependent and did not follow a distinct pattern.

The study revealed that the influence of water temperature on incorporation and persistence of hygienically relevant bacteria was not distinct and species-dependent. It was demonstrated that water temperature cannot be taken into account as an isolated

parameter when monitoring drinking water quality but has to be considered as one factor in a system of complex interdependencies.

1 Introduction

1.1 Global climate

"Warming of the climate change system is unequivocal, as now evident from observations of increases in global average air and ocean temperatures, widespread melting of snow and ice, and rising global average sea level".

This is a citation of the synthesis report 2007 of the Intergovernmental Panel on Climate Change (IPCC). In this synthesis report 2007, the ICPP stated that eleven years in the time from 1995 to 2006 were among the twelve warmest years reported since 1850. Hartmann et al. (2013) described the last three decades in the period 1983 to 2012 to be likely the warmest 30 years in the Northern Hemisphere since 1400 years. In the summary report of 2008 of the Chartered Institute of Environmental Health (UK) Stanwell-Smith described 1998 to be the warmest year in the last thousand years. The World Meteorological Organization (WMO) defined the decade 2001-2010 the "decade of climate extremes" (WMO Summary Report 2013). With nine of the decade's years being among the ten warmest years on record, the decade was the warmest decade ever reported since 1850 when meteorological records began (Hartmann et al. 2013). The European Environmental Agency reported an increase in temperature of about 0.83 to 0.89 °C for the decade 2006 to 2015, which makes it to be the warmest decade since temperature is recorded (Jol 2017). Global surface temperatures have increased of about 0.4 °C to 0.8 °C since the late 19th century (Folland et al. 2001, Stanwell-Smith 2008, WMO Summary Report 2013). This elevation of temperatures predominantly occurred from 1910 to 1945 and since 1976. Since 1976, the increase in temperature was about 0.15 °C/ decade (Folland et al. 2007).

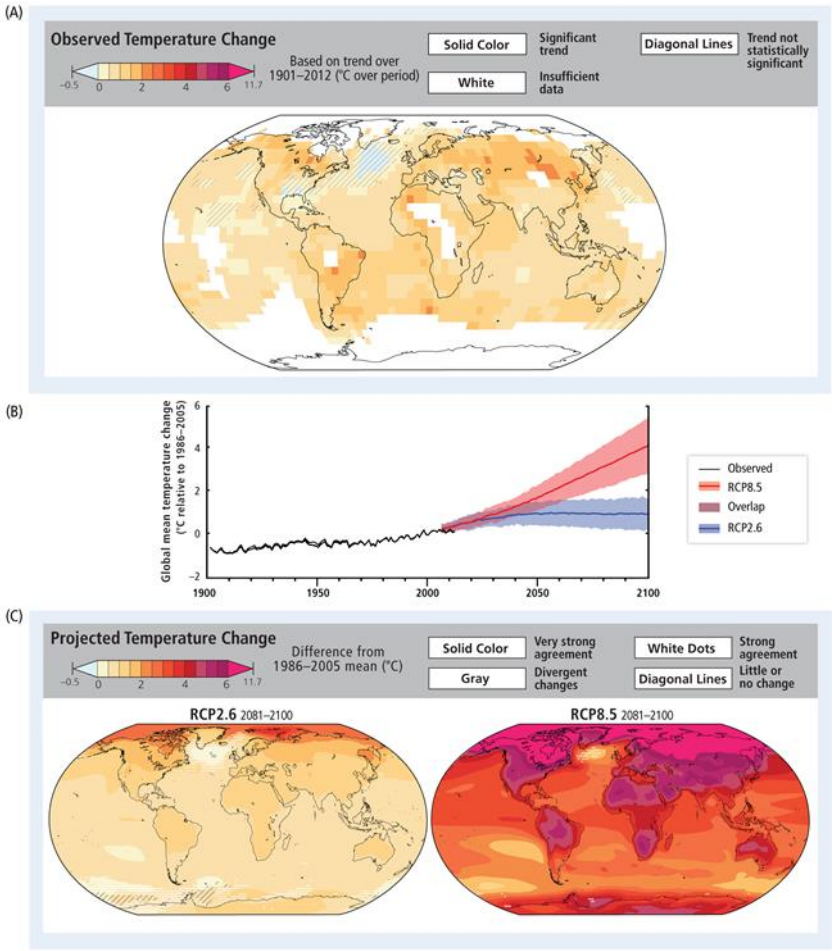

Figure 1: Observed and projected changes in annual average surface temperature (IPCC, 2014: Summary for policymakers. In: *Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Field, C.B., V.R. Barros, D.J. Dokken, K.J. Mach, M.D. Mastrandrea, T.E. Bilir, M. Chatterjee, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken, P.R. Mastrandrea, and L.L. White (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, pp. 1-32; Figure SPM.4; p 10)

The impacts of global warming underlie spatial variations. Europe will have to face other climate change- induced challenges than Africa or the Polar Regions.

Polar Regions

Consistent with the observed warming of the average global surface temperature, an extensive retreat of alpine and continental glaciers has occurred due to global warming in the 20th century. Even if there was no increase in temperature from now on, glaciers would continue shrinking (Vaughan et al. 2013). Relative to the period of 1986 to 2005, the retreat of the glaciers worldwide (excluding the Antarctic ice content) is expected to

be 15- 55 % for a scenario assuming stringent mitigation of greenhouse gases (GHG) and 35- 85 % for a scenario assuming very high GHG emissions (Hewitson et al. 2014). Greenland and arctic ice sheets have shrunk during the last two decades. Over the period 1979 to 2012, the loss of mass in the arctic was very likely in the range of 3.5 % to 4.1 %. In contrast to this, it is very likely that the sea ice extent in the Antarctic has increased of about 1.2 % to 1.8 % per decade in the same period (IPCC, 2014). However, the sea ice extent in the Antarctic underlies strong regional variations with regions loosing mass and regions with increasing ice extent. In addition, the perennial Arctic sea ice extent decreased during 1979 to 2012 of about 0.73 to 1.07 million km^2 per decade. Moreover, the average sea ice thickness diminished while the annual period of surface melt increased of about 5.7 (± 0.9) days per decade (very likely) (Vaughan et al. 2013). Anisimov and colleagues (2007) reported in the IPCC 4AR that in both polar regions components of the cryosphere and hydrology are already at present affected by climate change. An increase in green vegetation and biological productivity has occurred in some parts of the arctic. McBean et al. (2005) reported in the Arctic Climate Impact Assessment (ACIA) that in the period 1900 to 2003 the mean temperature in the Arctic increased by 0.09 °C/ decade.

Africa

Since the beginning of the 20th century, the near surface temperature over most parts of Africa has increased of about 0.5 °C (Niang et al. 2014). Depending on the models used for the prediction of future climate in Africa and the considered region in Africa an increase of the mean annual temperature in the range of 2 °C to 6 °C by the end of this century is assumed to be likely (Boko et al. 2007, Niang et al. 2014). Precipitation projections are inconsistent for Africa as there are several processes influencing precipitation. These include among others the hydrological cycle, dust aerosol concentration and sea-surface temperature anomalies. All these factors result in large inter-model variations for the prediction of future rainfall events in Africa. However,

projections of precipitation changes have been assessed. Depending on the model used for the prediction of rainfall over the African region, decreases of precipitation are very likely over the Mediterranean region of North Africa and over the South African regions for the second half of the century. Over Central and Eastern Africa, a contrary trend of increasing annual precipitation is likely. However, using other models for projections of precipitation assuming stringent mitigation of GHG, most of the African region will not be affected by changes in precipitation in the mid- and late 21st century (Niang et al. 2014).

Europe

Between the pre-industrial period and the decade of 2002 to 2011, decadal average temperature over European land areas warmed about 1.3 °C (± 0.11 °C). Across Europe, the Iberian Peninsula and northern Europe (especially Scandinavia) was affected by elevating temperatures most significantly (Kurnik 2017 b). In the last 500 years, the five warmest summers in Europe were all recorded in the period of 2002 to 2011. As described for past trends in global climate change, the number of warm days and nights in Europe increased while the number of cold days and nights decreased. The years 2014 and 2015 have been recorded to be the warmest years in Europe since recording began (Kurnik 2017 a). Regarding the annual precipitation in Europe, an increase of up to 70 mm per decade was observed for Northeastern Europe as well as Northwestern Europe since 1960 (Kurnik 2017 b). Moreover, decreasing precipitation up to 90 mm per decade was recorded in some regions of Southern Europe. Only middle Europe did not face significant changes in annual precipitation since 1960 (Kurnik 2017 b). In the future, an elevation of temperatures in the range of 1 °C to 5 °C is expected in Europe depending on the scenarios used for climate projections (Jacob et al., 2014,Figure 2).

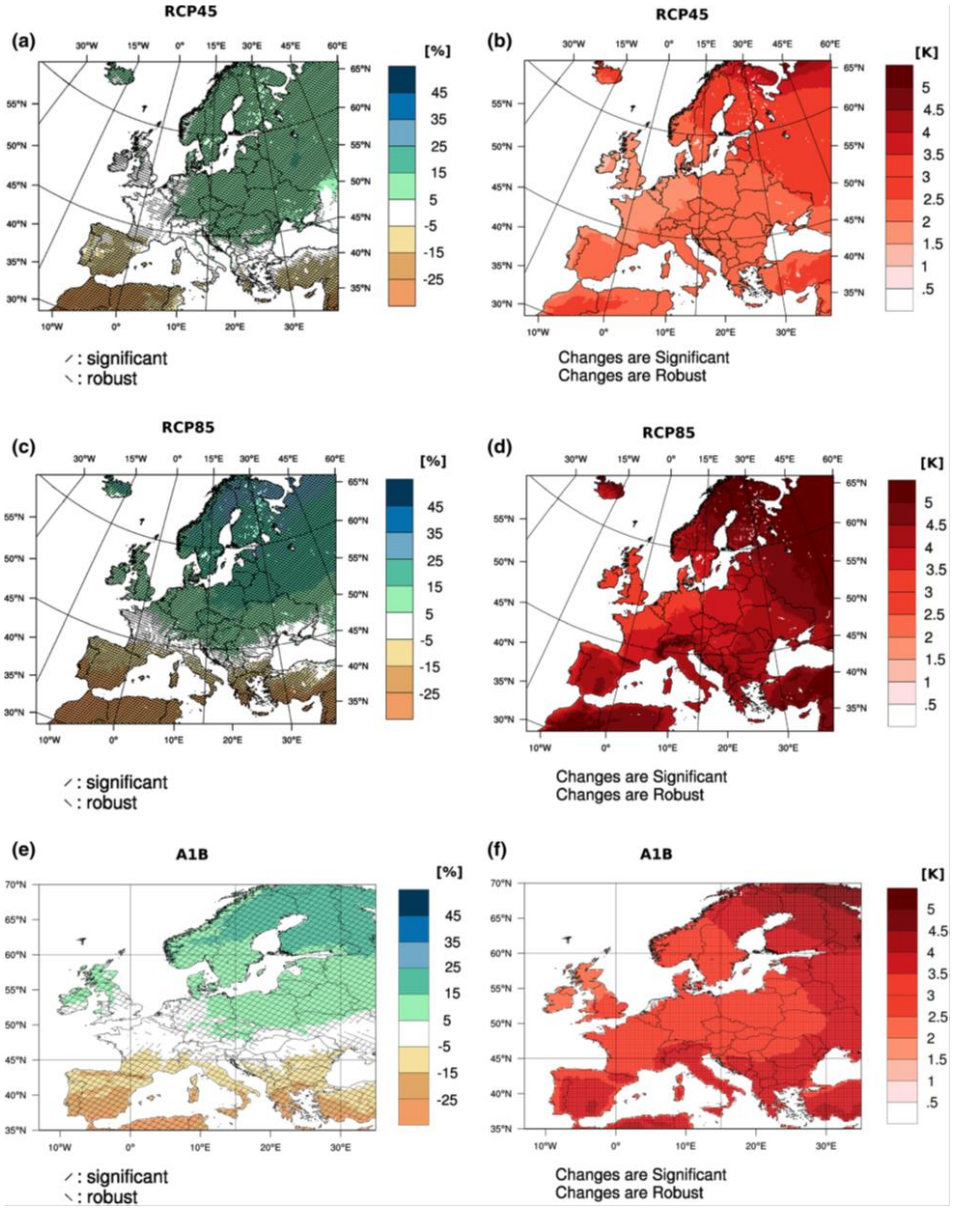

Figure 2: Projected changes of total annual precipitation (%) (left) and annual mean temperature [K] (right) for 2071–2100 compared to 1971–2000, for A1B (e, f), RCP8.5 (c, d) and RCP4.5 (a, b) scenarios (Jacob et al., 2014; Reg Environ Change (2014) 14:563–578).

In Eastern Europe warming will be most intense in the winter season while Western and Southern Europe will undergo the more significant warming during summer (Giorgi et al. 2004; Kjellström et al. 2004; Räisänen et al. 2004; Christensen and Christensen 2007). Similar projections were reported by Alcamo et al (2007). It is stated in their report that rising average temperatures result in an increasing frequency of extreme high temperatures while extreme low temperatures will occur less frequently. Moreover, they

described increasing nighttime temperatures for many regions across the world quenching the diurnal variation of temperatures. Giorgi and colleagues (2004) and Räisänen et al. (2004) reported that Northern and Central Europe will have to face more humid winters while South Europe will be affected by less precipitation in summer (Jacob et al. 2014). Even though the mean annual rainfall will decrease in some parts of Europe, the intensity of daily precipitation events will increase in mostly the whole European region (Giorgi et al. 2004; Kjellström 2004). Figure 3 depicts the projected changes in precipitation over Europe (Jacob et al., 2014) using the RCP8.5 scenario based on an increasing radiative forcing as well as the RCP4.5 scenario which is a medium stabilization scenario assuming stabilization of the radiative forcing shortly after 2100 (Van Vuuren et al., 2011). Projected seasonal changes in precipitation based on these emission scenarios both predict an increase in the mean annual precipitation in Northern Europe and a decrease of rainfall in the South is predicted (Jacob et al., 2014).

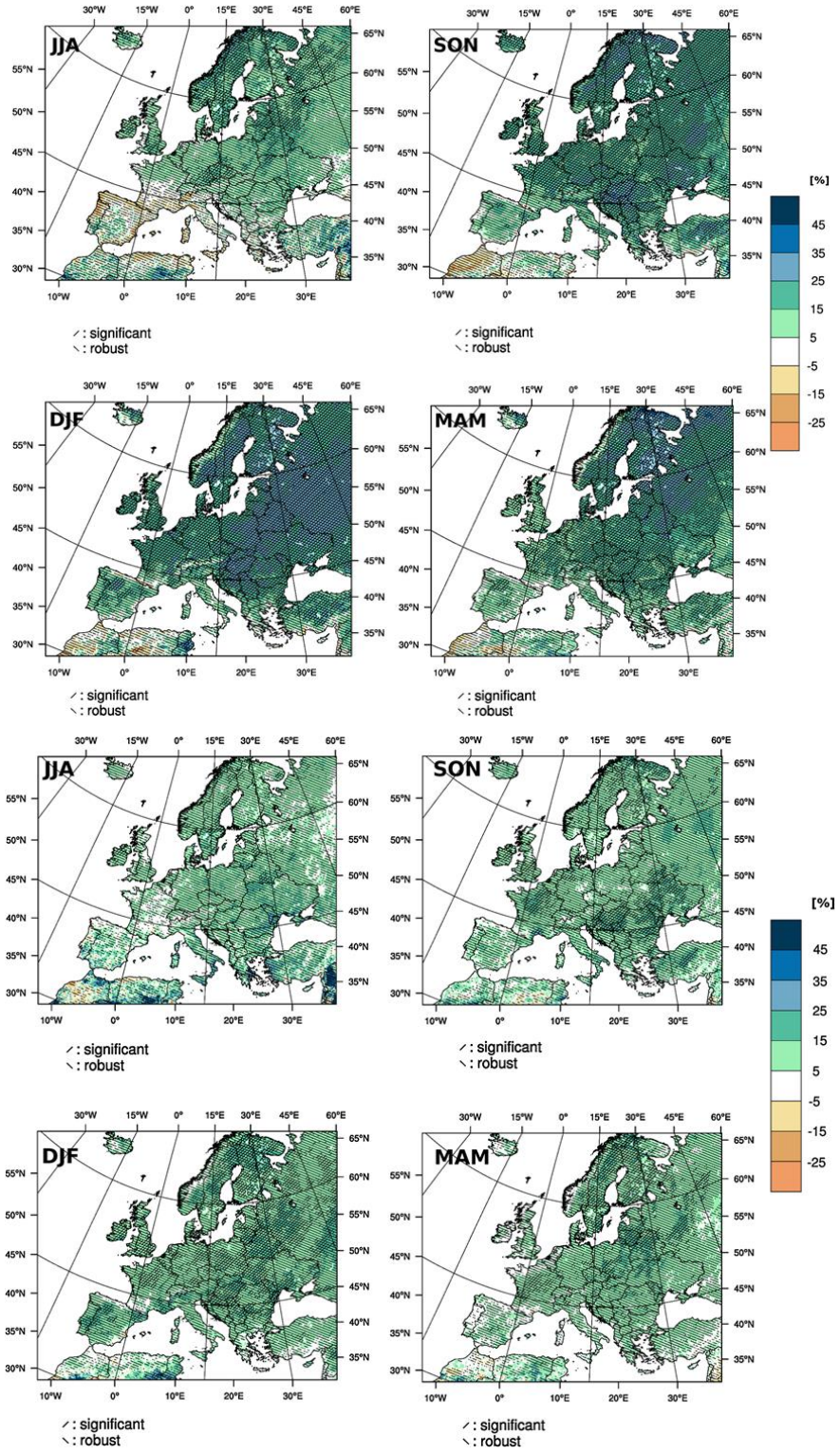

Figure 3: Projected seasonal changes of heavy precipitation (%) based on the RCP8.5 scenario for 2071–2100 compared to 1971–2000. Hatched areas indicate regions with robust and/or statistical significant change. (Jacob et al., 2014; Reg Environ Change (2014) 14:563–578)

On a smaller scale, Pfister et al. (2004) performed research on climate change, land use and runoff prediction in the Rhine-Meuse basins. They reported that an increase in annual rainfall totals is expected over the entire Rhine basin. Due to rising temperatures, increased snow-melt will occur in the Alpine region. Together with larger rainfall amounts in winter, this will result in a shift of flood events in the Upper Rhine from spring and summer to winter. Likewise, an increase in winter precipitation is expected for regions along the Meuse River. In contrast to that, summer precipitation will decrease. Different simulations result in the same trend: increasing runoff in winter and early spring and decreasing runoff in autumn.

1.2 The "dynaklim" project

As climate change is unequivocal and implications are already evident by now, it is very important to evaluate effects of climate change on a regional level. Therefore, the collaborative project "dynaklim" has been initiated. "dynaklim" is an acronym for "*Dynamic adaptation of regional processes to the effects of climate change in the Emscher-Lippe Region (ELR) in North Rhine-Westphalia, Germany*". It is one of seven projects within the programm "*Sustainable Development of Climate Change in Regions* (KLIMZUG)" funded by the German Ministry of Education and Research (BMBF). Focuses of the "dynaklim" project are possible impacts of predicted climate changes induced by global warming on the regional water balance and the development of adaptation strategies for environment, population, and economy in the ELR.

1.2.1 Contribution of the present study within the "dynaklim" project

The current study was focusing on the examination of the impact of climate change induced global warming on drinking water quality. Elevating temperatures may have an impact on surface waters and upper soil layers resulting in rising temperature within the

drinking water distribution system and finally in warmer drinking water (Meuleman et al, 2007; Blokker and Pieterse-Quirijns, 2013). The evaluation of possible promotion of integration, persistence, growth and regrowth of hygienically relevant bacteria due to elevating temperatures in drinking water biofilms was aim of this study.

1.3 Climate change in the Emscher-Lippe Region

Many approaches have been pursued to gain reliable information about the future weather situation and the consequences of climate change induced global warming in the ELR. Quirmbach and colleagues (2012) reported that in the last decade (2001- 2010) an increase in the mean temperature compared to a reference period (1961- 1990) of about 0.8 °C occurred. The increase in temperature calculated by using different climate models was estimated to be 1 °C for the "near" future (2021-2050), which is already reached, yet. For the "far" future (2071-2100), the increase in the mean temperature is predicted to be 3°C. There will be an above-average number of summer days (≥ 25 °C) and hot days (≥ 30 °C) while the number of frost days (T min < 0 °C) and ice days (T max < 0 °C) will decrease, indicating more intense and more often occurring dry periods. In another publication, Quirmbach et al. (2012b) evaluated the influence of climate change on precipitation data in North Rhine- Westphalia resulting from three different regional climate models. Projections were similar for all three models predicting an increase in dry summers associated with an increase in more humid winters (16- 20 % more precipitation in the "far" future (2071- 2100) compared to the reference period (1961- 1990). However, temporal development and significance of the predicted weather changes in the ELR are ambiguous for the different climate models. The precipitation during the winter months is only significant in the "far" future for run 1 of the CLM model and the WETTREG model. When taking into consideration run 2 of the CLM, which differs from run 1 of the model in the starting time and some

boundary conditions, and the STAR2 model for the evaluation of future weather conditions, a significant impact of the predicted trends can already be detected by 2050.

1.3.1 What will the weather be in the ELR?

To evaluate the possible impacts of climate change on a regional level, Quirmbach and colleagues (2013) discussed different possible moderate as well as extreme climate scenarios in the ELR. Moreover, they reconsidered the challenges the water sector in the region will have to cope with for all possible scenarios in the ELR. In their considerations Quirmbach et al. (2013) also took into account the Ruhr basin as it mainly serves as a raw water source for drinking water production. Projections were made for the near future (2021-2050) and the reference period were the three decades from 1961 to 1990. Socio-economic scenarios were considered until 2030 and the reference year was 2010. Extreme years were considered as extreme scenarios.

Moderate changings in the ELR

The first scenario describes moderate weather changes in the near future following the trends of the climate model data. The mean increase in temperature will be 1 °C. In average there will be six additional summer days, four hot days and one tropical night more compared to the reference time (1961- 1990). In addition, there will be 19 frost days and 8 ice days less per year. An insignificant increase in the annual amount of precipitation of 4 % and even a slight decrease of precipitation in the summer months will occur. The average number of dry days per year will stay constant. However, there will be a shift of dry days from winter to summer. Twice as much dry periods with at least 21 dry days compared to the reference period will occur. Heavy rainfall events will be recognized four to five times a year instead of three to four times in the reference time. The consequences for the water sector in the ELR will be mostly problems with

capacity overload of the sewage system and floodings of rivers due to heavy rainfall events.

Desert-like ELR

The assumption of a desert-like ELR was based on two extreme years chosen from the climate change model data and describes a hot and dry ELR. Winter and spring will be dry and there will already be summer-like temperatures in spring with often more than 25 °C and even days with more than 40 °C at the end of May. Precipitation will be very scarce with only 27 mm per month in May, June, and July. Without appropriate countermeasures and a lack of water-sensitive urban development, many parts of the inner city will be very hot due to the sealed areas. The water sector will have to face sporadic shortages in drinking water availability as groundwater reservoirs cannot be refilled and contributing streams of the Emscher will temporarily run dry. Another problem will be the stagnation of waste water in the sewage system going along with odor problems and problems with the effectiveness of waste water treatment plants.

The drowning ELR

For this scenario, one extreme year was chosen from the climate model data. Here, the impacts of significant increase in precipitation and the frequent occurrence of heavy rainfall events are described. The ELR will have to deal with significant increase in precipitation. There will only be short dry periods of one to three days. The consequences will be dramatic floodings of cities, contamination of groundwater due to floodings of abandoned agricultural lands and overloads of sewage systems. This will result in increasing infection risks and cause severe problems the water sector will have to cope with.

The four described scenarios simulate possible future weather conditions in the ELR based on different climate model data. As mentioned, these scenarios are either following the trends predicted by the model data or extreme climate changes based on

extreme years chosen from the model data. No matter which scenario will happen in the future, fact is, the water sector has to adapt to a changing water balance. And this is where "dynaklim" comes into consideration as it is dealing with the development of those important adaptation processes.

1.4 Climate change and infectious diseases

Climate change is expected to influence not only surface water availability, but also water quality including physico-chemical parameters (pH, temperature dissolved oxygen…), nutrients, pollutants (e.g. pesticides, metals), and pathogenic microorganisms (Delpla et al., 2009). Climate change has a direct influence on the occurrence of waterborne diseases due to impacts on temperature and precipitation. Droughts may increase the load of pathogens in effluents and even in countries with treated water the increase in extreme weather events may lead to interferences in water works and sewage treatment resulting in elevated risks of waterborne diseases outbreaks (Portier et al., 2010). An increase in extreme weather events such as droughts, heat waves and heavy rainfall events accompanied by floods is expected globally as a consequence of global warming (IPCC, 2014). Cann et al. (2013) conducted a systematic review to investigate the occurrence of waterborne diseases after such water-related weather events. They included 83 papers in their research. Despite some limitations the review suffered from, Cann et al. (2013) suggested that outbreaks of waterborne diseases were associated with extreme weather events and predominantly linked to heavy rainfall events. When investigating the influence of rainfall on the occurrence of waterborne diseases in Taiwan during the period of 1994 to 2008, Chen et al. (2012) observed a positive correlation between precipitation and outbreaks of waterborne diseases such as bacillary dysentery and dengue fever. Hunter (2003) also reported that various studies described waterborne outbreaks of infectious diseases, which were preceded by heavy rainfalls. One severe case he described in his article was the outbreak of *E. coli* O157:H7 in

Canada in 2000 after heavy rainfall and flooding had occurred. Over 1000 people were affected by this outbreak of whom 65 had to be treated at hospitals and six died. Schijven and Husman (2005) investigated the influence of climate change on the outbreaks of waterborne diseases in the Netherlands. They observed an inverse correlation between increasing water temperature and pathogen concentrations. Higher surface water temperatures resulted in enhanced inactivation of pathogens whereas increased precipitation and therefore an increase in the flow rate led to peak concentrations of pathogens in surface waters significantly higher than the average level. The World Health Organization (2014) evaluated the possible influence of climate change on the risk of different causes of death. Assuming the base-case socioeconomic scenario, a number of 48 000 additional deaths of children aged under 15 years caused by diarrheal disease will occur by 2030 worldwide. In 2050, 33 000 additional deaths will be attributed to diarrheal disease due to climate change. For malaria the number of projected additional deaths in 2030 associated with climate change is even higher and expected to be 60 000. Greer et al (2008) stated in their article that a climate change-induced spreading of zoonotic and vector-borne diseases is likely to be expected. Due to changes in temperature and precipitation patterns, spatial distribution of pathogens may increase. Moreover, the increased transmission of zoonotic diseases due to increased temperatures, changing rainfall and increasing reservoir populations seems to be likely in the future. The northward spreading of the Lyme disease is projected in Canada by 2020. Europe may be affected by an increase in the incidence of Lyme disease and Leishmaniosis (Greer et al. 2008). Purse et al. (2005) reported an association between climate change and the emergence of bluetongue in Europe. They suggested that the spreading of the bluetongue virus across Southern and Central Europe since 1998 was driven by climate change in this region. The incidence of bluetongue significantly increased in areas where changes in maximum temperatures were remarkably. Kovats et al. (2003) investigated the influence of the El Niño Southern Oscillation (ENSO) on infectious diseases. They observed a distinct influence of this climate event on malaria

and cholera. Greer et al. (2008) also reported that elevated water temperatures promote the occurrence of cholera. The enhancing effect of warm water temperatures on the survival of *Vibrio cholera* was described in various studies (Huq et al. 1984; Hunter 2003; Huq et al. 2005). The EEA (2017) stated that the transmission of vector-borne diseases is sensitive to climate but also underlies a variety of other factors such as land use, public health capacities, vector control, trade, travel and many more. Nevertheless, expected effects of climate change on waterborne and vector-borne diseases are negligible in wealthy countries but may be far more threatening for poor societies (Bouzid et al., 2013; Hunter 2003).

1.5 The viable-but-nonculturable (VBNC)-state

In this study, the influence of temperature on the survival of hygienically relevant bacteria in drinking water and drinking water biofilms was investigated. To evaluate possible effects of temperature on pathogens in drinking water determination of various target organisms was conducted using cultural and culture-independent methods.

Cultivability of bacteria is the gold standard when evaluating viability. For this reason, viability and survival of microorganisms are commonly determined by culture-dependent methods (Hammes et al., 2010). However, it is known that many hygienically relevant microorganisms can undergo a transition into a viable-but-nonculturable state (VBNC) (Oliver, 2010). In this state, microorganisms are still viable but evade culture-dependent detection methods. On a conference ("How dead is dead?" Berlin, May 2013; http://www.hdid-conference.de/general-information/review-2013/) the situation for hygienically relevant bacteria was defined as: "A bacterial cell in the VBNC-state may be defined as one which fails to grow at the routine bacteriological cultivation conditions under which it would normally grow, but which is in fact alive and has still metabolic activity (after Oliver, 2010)". Xu et al. first described this survival strategy in 1982. Unfavorable conditions such as temperatures outside the suitable range required for cell

growth, starvation, and heavy metals, oxygen concentrations, etc. may induce the VBNC-state in microorganisms (Oliver 2010, Li et al., 2014, Pinto et al., 2015). Once the stress is removed, VBNC-cells can resuscitate from this state and regain culturability. Bédard et al., (2014) demonstrated the recovery of *P. aeruginosa* in the VBNC-state after copper stress and chlorine stress. The ability of *P. aeruginosa* to resuscitate from the VBNC-state and regain cytotoxicity after copper stress was removed was demonstrated earlier by Dwidjosiswojo et al., (2011). Table 1 summarizes the differences between culturable cells, cells in the VBNC-state, and dead cells (Li et al., 2014).

Table 1: Differences between culturable cells, cells in the VBNC-state and dead cells (Li et al., 2014)

	Culturable cells	**VBNC-state**	**Dead cells**
Cell morphology	Under stress: dwarfing and rounding	Reduced cell size; dwarfing and rounding	-
Gene expression	Active	Active	Inactive
Nutrient uptake	Active	Active	Inactive
Membrane integrity	Intact membranes	Intact membranes	Damaged membranes
Metabolic activity	Active	Lowered activity	Inactive
Resistance properties		Enhanced resistance compared to culturable cells	-

Numerous pathogenic bacteria have been shown to enter the VBNC-state (Oliver, 2010). The underestimation of such pathogens in drinking water due to their presence in the VBNC-state and their ability to resuscitate and maintain virulence pose a risk to human health (Li et al., 2014) and need to be evaluated appropriately (Pinto et al., 2015). Therefore, the detection of these cells is an important key when providing safe drinking water, even though it is not mentioned in the German drinking water ordinance. In

principle, the number of VBNC-cells within a sample can be determined by the comparison of the number of viable cells with the number of culturable cells (Li et al., 2014). Several viability markers can be investigated to distinguish between viable and dead cells (Figure 4).

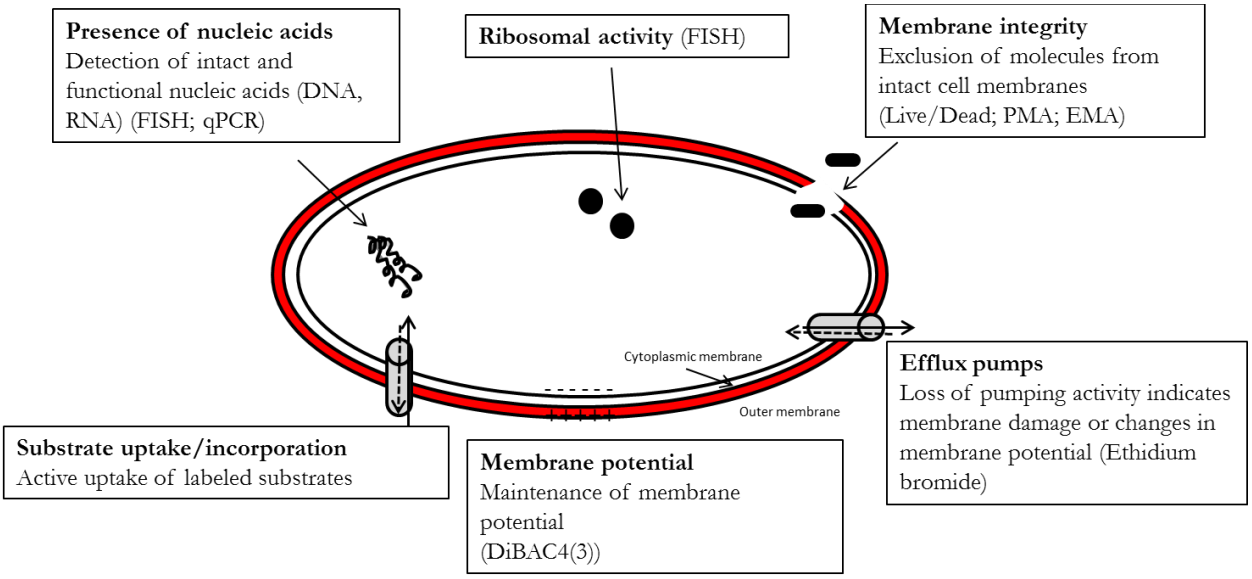

Figure 4: Cellular processes for the assessment of viability (after Hammes et al., 2010).

The investigation of membrane integrity is conducted frequently to evaluate the viability of bacteria. A common method for the general distinction between cells showing membrane integrity and cells having a damaged cell membrane is the LIVE/DEAD® BacLight™ assay (Oliver, 2010). The green fluorescent dye SYTO®9 penetrates cells with intact as well as damaged cell membranes whereas the red fluorescent dye propidium iodide only enters cells with damaged cell membranes. Using an epifluorescence microscope or a flow cytometer it can be distinguished between viable and dead cells as injured cells appear red and cells with intact membranes appear green (Rochelle et al., 2011). However, most often it is necessary to determine the viability of specific target organisms. Thus, methods allowing for the specific detection of the viability of those target organisms have to be applied. One common method is the

fluorescence in situ hybridization (FISH). This method is based on the detection of ribosomal RNA (rRNA) and thus demonstrates metabolic activity of the cell. Oligonucleotide probes, which are labelled with a fluorescent dye specifically, bind to a complementary target sequence of the rRNA inside the target cells (Rompré et al., 2002). Counterstaining of the sample with DAPI, which binds covalently to DNA of all cells enables the distinction between viable and dead cells. Using an epifluorescence microscope stained cells can be visualized. However, detection of rRNA of recently died or injured cells can lead to false positive results (Cenciarini et al., 2008, Rochelle et al., 2011). Moreover, the rRNA content within stressed cells might be too low for a sufficient level of fluorescence and therefore false negative results may occur (Rompré et al. 2002, Garcia-Armisen et al., 2004). Villarino et al. (2000) demonstrated that cells still showed cellular integrity after UV-killing and were recognized using different fluorescent markers such as FISH and DAPI. As it is not always possible to distinguish between viable and injured or dead cells by using FISH, this method can be combined with the direct viable count (DVC) method to improve the detection of viable cells. The DVC method includes the incubation of the sample in a special revivification medium containing nutrients and antibiotics prior to the standard FISH procedure and therefore refers to the metabolic activity of the target cells as nutrients are utilized. The nutrients enable growth of the target organisms, but the antibiotics prevent division of growing cells by inhibiting the Gyrase activity. Therefore, substrate responsive cells appear larger under the microscope and are considered viable (Buchrieser and Kaspar, 1993, Garcia-Armisen et al., 2004, Cenciarini et al., 2008). Another method, which is often used to detect VBNC-cells, is the combination of DNA-intercalating dyes with Polymerase chain reaction (PCR). The most commonly applied dyes are propidium monoazide (PMA) and ethdium monoazide (EMA). EMA penetrates cells with intact cell membranes and its azide group intercalates with the DNA of the cell. Due to exposure to light, it covalently binds to the DNA and prevents amplification during the PCR (Nocker et al., 2006, Rochelle et al., 2011). However, EMA is not entirely suitable as a

viability assay because it is assumed to not be strictly impermeable to intact cell membranes. It was shown to not penetrate the injured cells of some species but it entered cells with intact cell membranes of other bacterial species (Nocker and Camper, 2006.) The use of PMA, which is structurally comparable with EMA, might be favorable as PMA has a lower ability to enter intact cells due to its higher charge compared to EMA (Nocker et al., 2006, Rochelle et al., 2011, Seinige et al., 2014). However, even though PCR- based methods are suitable to detect even single cells in a complex sample, they are also limited by inhibitory compounds such as humic acids or iron. Moreover, the detection of DNA from injured or dead cells or cells in the VBNC state can result in false positive results (Wellinghausen et al., 2001, Lehtola et al., 2007).

1.6 Influence of temperature on bacteria in drinking water distribution systems

In the last decade (2001- 2010), the mean temperature in the ELR increased about 0.8 °C and believing different climate models, this trend will continue (Quirmbach et al., 2012). In course of the climate change induced global warming temperatures of surface waters and upper soil layers are expected to rise. This may lead to elevating temperatures in the drinking water distribution system as pipes are often located in upper soil layers and surface water is very often used as raw water for the processing of drinking water (Meuleman et al, 2007; Blokker and Pieterse-Quirijns, 2013). For this reason, the elucidation of the influence of temperature on microorganisms, especially those of hygienic relevance, in drinking water and drinking water biofilms is of great importance.

1.6.1 Influence of temperature on hygienically relevant microorganisms in drinking water distribution systems

Bacteria can survive a broad range of temperatures, differing significantly from their optimal growth temperature (Figure 5). Many studies investigated survival of various bacteria at different temperatures. Table 2 gives an overview about temperatures tolerated by the hygienically relevant bacteria, which were focus of this study.

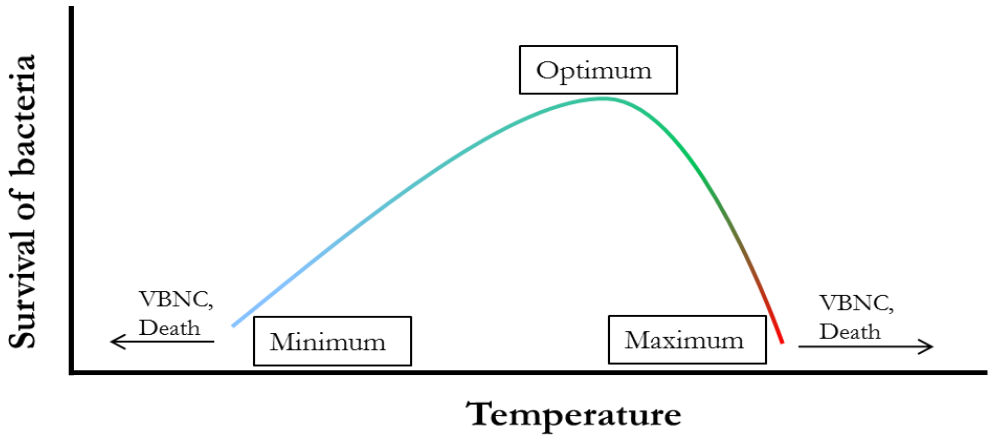

Figure 5: Cardinal temperatures for growth of bacteria (dynaklim publication No 52, 2014, modified)

Table 2: Cardinal temperatures of hygienically relevant bacteria (dynaklim publication No 52, 2014, modified)

Organism	Minimal Temperature (°C)	Optimal Temperature (°C)	Maximal Temperature (°C)	Literature
Escherichia coli	8 - 10	39	48	Leclerc et al., 2001; Madison und Martinko, 2013
Klebsiella pneumoniae	10	36-38	46	Leclerc et al., 2001
Pseudomonas aeruginosa	-20	37	45	Elliot, 1963; Kropinski et al., 1987, Khan et al., 2010
Legionella pneumophila	12	32-35	45	Wadowsky et al., 1985; Söderberg et al., 2004; Konishi et al., 2006; van der Kooij, 2014

In the following chapters, the influence of temperature on *E. coli* and other coliforms, *Legionella* spp, and *P. aeruginosa* is described in more detail. .*E. coli* was chosen for this study because it is traditionally considered to be an indicator for fecal contamination of drinking water and of great hygienically relevance if present in drinking water. *Legionella* spp. and *P. aeruginosa* are considered as water-related emerging pathogens of great relevance for human health as they can cause a variety of infections.

Escherichia coli and other coliform bacteria

Coliforms, especially *E. coli*, are worldwide used as indicator organisms for faecal and other contamination of drinking water and are an important key when monitoring water quality (Anderson et al., 2005). The broad temperature range tolerated by *E. coli* and

various coliform bacteria was reported by Leclerc et al. (2001). For *E. coli* growth was detected at temperatures within a range from 10 °C to 47 °C and for *K. pneumoniae* the temperature range was within 10 °C to 46 °C. For both organisms optimal growth was observed at 38 °C. Fass et al. (1996) demonstrated that *E. coli* was able to incorporate into existing drinking water biofilms and multiply within at 20 °C. When investigating the regrowth of coliform bacteria in drinking water in 31 water systems in North America, LeChevallier et al. (1996) observed an enhanced occurrence of coliform bacteria at temperatures above 15 °C. Tiefenthaler et al. (2009) reported similar results. In their study, they investigated the presence of fecal indicator bacteria during dry weather in Southern California Streams. They found that total coliform densities significantly increased at stream temperatures above 10 °C. For the occurrence of *E. coli* and enterococci, this positive correlation with water temperature was weaker, but still significant. However, survival and persistence of *E. coli* and coliform bacteria seems to be promoted rather by lower temperatures than by warm temperatures. In 2004, Craig et al. found an inverse relationship between the survival of *E. coli* in recreational water and sediment and water temperature when they observed decreasing numbers of culturally detectable *E. coli* at rising temperatures. Silhan et al. (2006) investigated the survival of *E. coli* in used drinking water pipes depending on temperature and pipe material. Pipes of different materials (galvanised steel, cross-linked polyethylene, and copper) were filled with drinking water and incubated both at 15 °C and at 35 °C. The pipes were inoculated with *E. coli* creating an initial concentration of 10^3 cfu mL^{-1} *E. coli* within each pipe. Cultural detection of *E. coli* revealed that survival of *E. coli* was promoted by the colder temperature (15 °C) rather than by the warmer temperature. However, *E. coli* was not detected in any of the biofilms regardless of pipe material and temperature. When investigating the factors influencing the survival of enterotoxigenic *E. coli* in sterile filtrated seawater over a period of eight weeks, Hernroth and colleagues (2010) observed higher viable counts at the end of the experiments at 8 °C compared to 18 °C. Arana and colleagues (2010) presented coherent results when they compared

the effect of temperature and starvation upon survival strategies of *Pseudomonas fluorescens* CHA0 and *E. coli*. In their study they observed enhanced survival of *E. coli* under nutrient poor conditions at low temperatures and determined the optimal survival temperature to be 5 °C. At higher temperatures, culturability of *E. coli* decreased significantly. However, a reduction of bacteria with intact cytoplasmic membranes was only observed at 5 °C and 20 °C. At higher temperatures, (25 °C and 37 °C) membrane integrity was maintained indicating the presence of the bacteria in the VBNC- state. Juhna et al. (2007) investigated the presence of *E. coli* in drinking water biofilms using cultural and culture-independent methods. When analysing the biofilm from pipe samples they found *E. coli* in one pipe with cultural methods whereas all pipe samples were positive for *E. coli* with the PNA FISH method. In 56% of the coupon samples *E. coli* was detected with PNA FISH. However, no cultural *E. coli* were detected. Interestingly, the only distribution network in which E. coli was not found was the one network with temperatures above 15 °C and high nutrient level. Pinto et al. (2011) studied, amongst other stimuli, the influence of temperature on the resuscitation of *Escherichia coli* VBNC cells. They examined the induction of VBNC cells under low temperature conditions (4 °C) and under room temperature (RT) as well as the influence of different temperatures (4, 25, 30 °C, and RT) on resuscitation of the VBNC cells. They showed that temperature had an effect either on the induction of the VBNC-state and on the resuscitation of VBNC cells. VBNC induction occurred earlier if cells were incubated in the VBNC media at RT compared to 4 °C. Moreover, they found that VBNC cells produced at RT were not able to resuscitate and regain culturability. For the VBNC cells produced at 4 °C a temperature effect regarding resuscitation could be detected. The number of resuscitation events was higher at 37 °C compared to 30 °C and 25 °C.

Legionella spp.

Legionellae are Gram- negative, rod- shaped bacteria often forming long filamentous structures (Declerck, 2010). *Legionella* spp. are facultative pathogenic organisms. They were first recognized in 1976 when an outbreak of severe pneumonia occurred in Philadelphia (Neil and Berkelman, 2008). By now, over 50 species with 70 distinct serogroupes are identified. The major route of infection is the inhalation of contaminated aerosols. Within the genus *Legionella*, *Legionella pneumophila* serogroup 1 is of the greatest medical importance as it is causing the influenza-like Pontiac fever and the more severe Legionnaires disease infecting the lungs (Yu et al., 2002; Neil and Berkelman, 2008; Lau and Ashbolt, 2009). *Legionella* can ubiquitously be found in natural and man-made freshwater systems (Ristroph et al., 1981) and is a common biofilm inhabitant (Lau and Ashbolt, 2009). In drinking water, *L. pneumophila* survives and multiplies intracellularly in protozoa, predominantly in amoeba (Neil and Berkelman, 2008; Moritz et al., 2010; Abdel–Nour et al., 2013). The broad range of temperature *L. pneumophila* can survive was reported by Fliermans et al. in 1981 when *L. pneumophila* was isolated from surface water with a temperature range from 5 °C to 63 °C. In a study, Wadowsky et al. (1985) determined the doubling times for *L. pneumophila* at different temperatures. 32 °C and 35 °C were most suitable for growth indicated by the shortest doubling times (16.8 h). At 37 °C and 25 °C doubling times were significantly higher with 28.8 h and 36.1 h. However, growth was even detected at 45°C. Variations of optimal growth temperatures occurred species-dependent and were influenced by other microorganisms. The antagonistic and synergistic influences of other species on the incorporation of *L. pneumophila* into existing biofilms was also described by Stewart et al. (2012). Although *L. pneumophila* is considered a warm water organism, several studies exist that indicate its ability to survive in drinking water even at lower temperatures. Arvand et al. (2011) reported that *L. pneumophila* and other *Legionella* species were culturally detected in drinking water installations of four different medical institutions in Germany in cold water already at 7 °C. Söderberg et al. (2004) demonstrated, that

L. pneumophila replicates at temperatures below 20 °C. The PilD prepilin peptidase promotes amongst other the type II secretion system which is essential for intracellular growth of *L. pneumophila* and considered to promote growth at low temperatures. Lethola et al. (2007) showed that *L. pneumophila* was culturally detectable in drinking water biofilms at 15 °C for at least 14 d and no significant decrease in culturability was detectable over the period of the experiment (14 d). In addition to cultural methods, FISH was also applied to investigate the survival of *L. pneumophila* in the drinking water biofilms. Numbers of the bacteria within the biofilms were 21 times higher using the culture-independent method compared to the culturally derived data. This corresponds to the results of a study performed by Långmark et al (2005). When they investigated the accumulation and fate of different microorganisms in drinking water biofilms, they also found significantly higher numbers of *L. pneumophila* using FISH. The occurrence of *Legionella* in the VBNC-state in drinking water was also described by Wullings and van der Kooij (2006). *Legionella* were detected at temperatures below 15 °C in high concentrations in different water works using PCR whereas no positive results were obtained culturally. However, *L. pneumophila* was not found in these systems. Wang et al (2012) reported similar results. They investigated amongst other pathogens the occurrence of *Legionella* spp. in two drinking water distribution systems in the USA. Using cultural methods, *Legionella* spp. was only detected in one out of 56 water samples while the frequency of detection using qPCR was significantly higher with 100% for the one distribution system and 69% for the other. However, positive results for *L. pneumophila* were lower (20 % and 15 %). Parthuisot et al. (2010) quantified *Legionella* spp. at different sites in a French river using cultural methods as well as qPCR for detection. Only about 21 % of all samples were positive for *Legionella* spp. using the cultural method for detection. However, all of the tested colonies were identified as *L. pneumophila*. Using the culture-independent qPCR, *Legionella* spp. was detected in all samples. It was not possible to detect *L. pneumophila* with the qPCR as numbers were below the quantification limit. Parthuisot et al. (2010) additionally investigated seasonal

effects on the occurrence of Legionella in the river water. They observed seasonal influences only at one of the investigated sites. Fingerprints of the *Legionella* populations in winter and fall differed from those in spring and summer.

Legionella spp. in biofilms

Temperature does not only influence the growth of *Legionella* in drinking water, but also biofilm formation, incorporation and persistence in biofilms. Rogers et al. (1994a) investigated the influence of temperature and plumbing material on biofilm formation and growth of *L. pneumophila*. They demonstrated that *L. pneumophila* was capable of incorporating into biofilms over a temperature range from 20 °C to 50 °C. However, the highest abundance was observed at 40 °C. Nevertheless, *L. pneumophila* remained culturable in biofilms on plastic materials even at 50 °C indicating that biofilms protect the organisms against high temperatures. Konishi et al. (2006) compared biofilm formation of *L. pneumophila* at different temperatures (35 °C, 42 °C, 45°C). They observed a sticky, granulated biofilm at 35 °C while at 42 °C a heterogeneous, multi-layered biofilm was established. At 45 °C no biofilm formation was observed. Moreover, Konishi et al (2006) developed three categories regarding the multiplication of the investigated strains. At temperatures < 43.1 °C almost all investigated clones multiplied (range I), at temperatures within the range of 43.1°C to 44.1°C 9.1% to 40.9% of the tested clones did not show multiplication (range II), and above 44.1 °C more than 88.9 % of the investigated clones were not able to multiply (range III).

Legionella spp. associated with amoeba

Temperature can also have an indirect effect on *Legionella*. Ohno et al. (2008) investigated the temperature-dependent parasitic relationship between *L. pneumophila* and *Acanthamoeba castellanii*. At 25 °C intracellular multiplication of *L. pneumophila* within *A. castellanii* was observed while at 15 °C and 20 °C the number of *L. pneumophila*

decreased possibly due to active digestion by the amoebae. Buse and Ashbolt (2011) examined the dependence of growth of *L. pneumophila* on host and temperature. They co-cultured different amoebae (*Acanthamoeba polyphaga*, *Negleria fowleri*, *Hartmanella veriformis*) with five different strains of *L. pneumophila*. Two of the five tested *L. pneumophila* strains grew well at 30 °C, 32 °C, 37 °C when co-cultured with *A. polyphaga*. However, with *N. fowleri* as a host, both strains proliferated even at 24 °C. None of the two strains showed growth in presence of *H. veriformis*. The third strain used in the study did not show growth in presence of any of the tested amoeba hosts except at 32 °C when co-cultivated with *H. veriformis* and *N. fowleri*. Similar results were obtained with the fourth strain, which did not grow at any of the temperatures regardless of the amoeba host. The fifth strain also revealed host and temperature- dependency in the experiments. These results demonstrate the how critical temperature is for the survival of *L. pneumophila* in aqueous systems. Ridenour et al. (2003) identified a gene (*lvhB2*) that is involved in host cell infection by *L. pneumophila*. At 30 °C, *lvhB2* mutants showed a diminished efficiency of adhesion, infection and intracellular replication compared to the wild-tpye strain. This was not the case at 37 °C indicating that this gene is important for host cell infection of *L. pneumophila* grown at lower temperatures.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, aerobic and non-spore forming bacterium (Madigan et al., 2006). Among *Pseudomonas*, *P. aeruginosa* is of the highest clinical relevance being an opportunistic pathogen causing a variety of nosocomial infections in immunocompromised people and people suffering from burn wounds. Moreover, it infects the urinary tract, the respiratory tract, the gastrointestinal tract, and causes ear infections mostly due to contact with contaminated water (Wingender et al., 2009; Mena and Gerba, 2009). People at a high risk of infection are those suffering from cystic fibrosis, whereas healthy people are hardly affected by *P. aeruginosa* (Anaissie et al. 2002, Stover et al., 2000). *P. aeruginosa* can ubiquitously be found in the environment and

is able to multiply in drinking water systems (Stover et al., 2000; Mena and Gerba, 2009, Wingender et al., 2009). The ability of *P. aeruginosa* to integrate and persist within drinking water biofilms at temperatures significantly lower than its optimum growth temperature was demonstrated by Bressler et al. (2009) and Moritz et al. (2010). *P. aeruginosa* tolerates a broad range of temperatures and was detected at 9 °C (Elliot, 1963) as well as at 45 °C (Kropinski et al., 1987). Haynes (1951) determined the growth of 138 *P. aeruginosa* strains at different temperatures. All tested strains grew well at 30 °C, 57 grew well at 41 °C and 43 at 36 °C. Kropinski et al (1987) determined the doubling time of *P. aeruginosa* in tryptic soy broth at five different temperatures (10 °C, 15 °C, 25 °C, 35 °C, and 45 °C). At 35 °C *P. aeruginosa* required the shortest time for doubling (54 min), followed by 25 °C and 45 °C with a doubling time of 96 and 98 minutes. At 15 °C doubling required 228 min and at 10 °C the doubling time was estimated to be about two days. No growth occurred at 4°C or 50 °C. In a report of the Health Council of the Netherlands (2001) the occurrence of high concentrations of *P. aeruginosa* at temperatures above 18 °C in bathing lakes was described. Van der Kooij et al. (1982) investigated growth of different *P. aeruginosa* strain in tap water supplemented with different substrates. They demonstrated the ability of *P. aeruginosa* to grow at 15 °C even when low concentrations of substrates were available. Khan et al. (2010) determined the percentage of culturability of marine, freshwater and clinical *P. aeruginosa* after incubation at four different temperatures: -20 °C, 4 °C, 25 °C, and 37 °C in distilled water and artificial sea water for 24 h. The clinical as well as the freshwater strain showed maximum growth at 25 °C, the marine strain at 37 °C. The marine strain was the only strain with increasing culturability at temperatures below 25 °C. However, this was only the case in the artificial sea water. All tested strains remained culturable even after 24 h of incubation at – 20 °C.

Other water related microorganisms

In various studies the influence of temperature on the survival of microorganisms was investigated. Fields of research were the influence of temperature on the culturability, VBNC-state, resuscitation, gene expression and many more.

Torvinen et al. (2007) investigated the influence of different parameters on the survival of *Mycobacterium avium* in drinking water biofilms showing that rather temperature than nutrient availability or water flow velocity influenced survival of *M. avium* in drinking water biofilms. This was not consistent with the results of an earlier study performed by Le Dantec et al. (2002). They investigated the occurrence of atypical Mycobacteria in two drinking water distribution systems in France. They did not observe a correlation between the occurrence of Mycobacteria and temperature. Detection frequencies were similar at 8 °C and at 22 °C. Trevors and colleagues (2012) suggested that global warming may influence microbial gene expression as a response to changing climate conditions. Saleh-Lakha et al. (2009) performed research on the effect of temperature on the denitrification gene expression in *Pseudomonas mandelii*. They determined gene expression at 10 °C, 20 °C, and 30 °C. At 10 °C gene expression was delayed compared to 20 °C and 30 °C. However, maximum gene expression was reached at all three temperatures after 8 h without a significant difference between the three temperature treatments. Moreover, the number of transcripts was significantly higher for the 10 °C treatment after 24. In various studies temperature was shown to influence the transition of bacteria into the VBNC-state and vice versa. Numerous studies have proven *Vibrio vulnificus* to enter the VBNC-state as a response to exposition into cold water (Oliver et al., 1995, Weichart et al. 1992, Nilsson et al., 1991). Also, the temperature-upshift-induced resuscitation of *V. vulnificus* has been reported in many studies (Smith and Oliver, 2006; Whitesides and Oliver 1997; Nilsson et al., 1991). Nilsson et al. (1991) demonstrated in their study the ability of low temperature induced VBNC cells of *V. vulnificus* to resuscitate after they were subjected to room temperature without exposing them into a different medium. After 27 d of incubation at 5 °C in artificial

seawater (ASW) *V. vulnificus* cells entered the VBNC-state. Afterwards, samples of the VBNC-suspension were exposed to room temperature and cells regained culturability already after two days of incubation. After three days the concentration of culturable cells was comparable to the concentration in the original cultures. The same effect could be observed also after two cycles of nonculturability and resuscitation indicating that the increase of culturable cells from below the detection limit to the original concentration prior to VBNC induction can more likely be referred to resuscitation than to regrowth of residual culturable cells. They justified this suggestion by the fact that any residual nutrients transferred from the initial growth medium to the salt solution used in the experiment should have been utilized during the first round of resuscitation. Imazaki and Nakaho (2009) studied the temperature- upshift- mediated revival from the sodium- pyruvate- recoverable VBNC-state induced by low temperature in *Ralstonia solanacearum*. They demonstrated that cells incubated at 5 °C entered a VBNC-state. They also tested the induction of the VBNC-state when incubated at 25 °C, respectively. The induction of the VBNC-state also occurred at this temperature. However, induction of the VBNC-state required more time at the higher temperature. In addition, Imazaki and Nakaho (2009) investigated whether VBNC cells could be revived by temperature upshift. They observed that cells entering VBNC-state at 5 °C regained culturability at 25 °C. Moreover, they found that revived cells remained virulent. Goudot et al. (2012) investigated the growth dynamic of the amoeba *Naegleria fowleri* in a microbial freshwater biofilm. In their study they observed the densitiy of *N. fowleri* biofilms on glass slides at 32 °C and 42 °C. They found that at 32 °C no growth of the amoeba could be detected, but at 42 °C it was able to proliferate in freshwater biofilms. However, other studies demonstrated growth of pathogenic *N. fowleri* at lower temperatures (Maclean et al. 2004, Marciano-Cabral, 1988) indicating that additional factors influence growth of the amoeba.

1.6.2 Drinking water biofilms

Bacteria in drinking water may be present as free living (planktonic) cells but predominantly exist surface-associated in biofilms (O'Toole et al., 2000; Donlan, 2002; Flemming and Wingender 2010, Wingender and Flemming 2011). Biofilms are microbial communities which are irreversibly attached to surfaces embedded in a self-produced extracellular polymeric substances (EPS) matrix (O'Toole et al., 2000; Donlan, 2002; Flemming et al., 2016). Of the total bacteria in drinking water distributions systems the majority is attached to surfaces while only a small fraction is present in the water phase (Servais 1992, Flemming et al., 2002, Lehtola et al., 2007, Wingender and Flemming 2011). Over 95 % of the biomass within distribution systems is assumed to be attached to surfaces (Flemming et al., 2002). Mostly, colonization of inner surfaces in drinking water distribution systems appears in form of single cells or microcolonies, but in some cases dense multi-layered biofilms develop (Wingender and Flemming 2004). Biofilm development includes different stages (Figure 6) beginning with the formation of a conditioning layer consisting of biomolecules, such as humic substances, proteins and polysaccharides, which are present in non-ultrapure water. This step is followed by the reversible and irreversible attachment of organisms to the substratum. In the next stage, formation of microcolonies occurs. Depending on nutrient availability, surface material, and environmental conditions microcolonies evolve into macrocolonies and mature biofilms develop. (O'Toole et al., 2000; Dunne 2002; Stoodley et al., 2002; Hall-Stoodley et al. 2004). Detachment may occur active by release of matrix- degrading enzymes (Stoodley et al., 2002) or passive by shear forces (Donlan 2002).

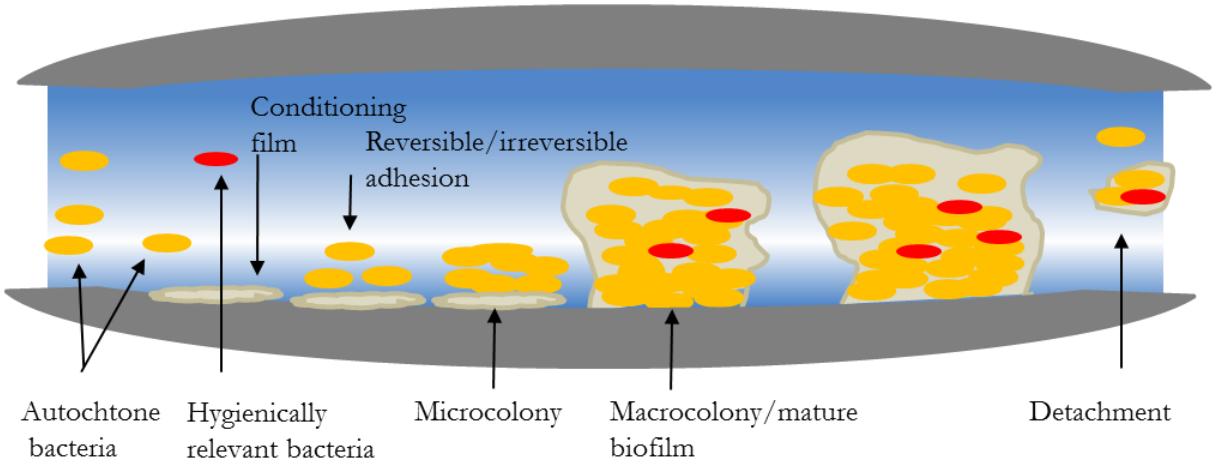

Figure 6: Biofilm formation in a drinking water distribution pipe (after Stoodley et al.,2002). Autochtonous bacteria (yellow) adhere to a conditioning film at the inner surface of the pipe. Microcolonies evolve into macrocolonies and finally into mature biofilms. The last step within the biofilm cycle is detachmentof single cells or flocs. Hygienically relevant bacteria which are capable of integrating into drinking water biofilms under certain conditions are shown as red cells.

As mentioned earlier, microorganisms in biofilms are embedded in a matrix of self-produced extrapolymeric substances (EPS) (Donlan, 2002; Flemming et al., 2002; Hall-Stoodley et al. 2004; Wingender and Flemming, 2011). EPS consist of polysaccharides, proteins, extracellular DNA, and lipids and promote the mechanical stability of biofilms and contribute to cohesion and organization of biofilm communities (Flemming and Wingender, 2010, Flemming 2016). The formation of those biofilm consortia provides beneficial conditions for microorganisms such as improved nutrient availability, exchange of genetic information, and protection from various environmental stresses such as UV radiation, chlorination, dehydration, and antibiotics allowing for a fast adaption to environmental changes (Davey and O'Toole, 2000; Hall-Stoodley et al., 2004, Flemming and Wingender 2010, Flemming 2016). Biofilms are ubiquitous and can be found on all surfaces in drinking water distribution systems (Kilb et al., 2003; Wingender and Flemming 2004; Flemming et al., 2016). Predominantly, drinking water biofilms consist of autochthonous aquatic microorganisms, which are not of hygienic relevance. Wingender and Flemming (2004) investigated the contamination potential of drinking water distribution network biofilms. Therefore, one biofilm reactor was

installed at the outlet of a waterworks and another biofilm reactor was installed within a drinking water distribution system. Coupons of different materials (stainless steel, copper, polyethylene (PE), and polyvinyl chloride (PVC)) were inserted into the biofilm reactors. After 6, 12, 18, and 24 months the coupons were sampled, biofilms were removed and investigated using microbiological methods. Neither *E. coli*, nor *P. aeruginosa* or *Legionella* spp. were found in their study and coliform bacteria were only rarely observed. This corresponds to the results of a study by Långmark et al. (2005). When investigating biofilms in an urban water distribution system in Sweden, they were not able to detect opportunistic bacterial pathogens within the analyzed distribution system. The results of these studies indicate that biofilms in distribution systems do not commonly serve as a habitat for pathogens. However, in some cases pathogens may enter and contaminate drinking water distribution systems (Szewzyk et al., 2000, Flemming et al., 2002, Parsek and Singh, 2003). Numerous studies have demonstrated the potential of hygienically relevant bacteria to integrate into drinking water biofilms. LeChevallier et al. (1987) performed biofilm investigations at a drinking water utility in New Jersey. Background of their study was the occurrence of coliform bacteria in the distribution system while treatment plant effluents were always negative for those bacteria. They found coliform bacteria in distribution system biofilms as well as in the water column. Murga et al. (2001) demonstrated the ability of *Legionella pneumophila* to persist and survive within biofilms even in the absence of *Hartmanella veriformis* which is required for multiplication. Kilb et al. (2003) observed the presence of coliforms in biofilms originating from rubber-coated valves exposed in a drinking water distribution system. The basis of their study was the repeated occurrence of coliforms in drinking water above the legal limit of 0 coliforms/100 mL. On 15 of 21 investigated valves coliform bacteria were detected. In 11 of 12 cases coliforms could be detected in the biofilm as well as in the water phase suggesting that colonized areas within the drinking water distribution system may pose a contamination source to drinking water. Emtiazi et al. (2004) observed *L. pneumophila* and *P. aeruginosa* (sporadically) in biofilms isolated

from different sites in a waterworks. However, samples were negative for Enterococci. Lethola et al. (2007) proved the potential of various pathogenic bacteria to integrate and persist in drinking water biofilms for several weeks.

Several studies revealed that biofilm formation is influenced by temperature. Donlan et al. (1994) showed in their study, that biofilm formation on iron cast substrata was promoted by elevated temperatures. They investigated the biofilm formation in cold drinking water (4 °C to 15 °C) and in warm drinking water (15 °C to 25 °C) by determination of the HPC. In the warm water, the highest numbers of the HPC were detected already after 30 days of exposition while in the cold water an increase was still detectable until 80 days of exposition. Moreover, colony counts were significantly higher in the warm water. However, the authors could not exclude an effect of monochloramine used for disinfection, which was present in higher concentrations at lower temperatures. Hallam et al (2001) investigated the potential for biofilm growth in water distribution systems. Among other parameters they focused on the influence of temperature on biofilm formation. They concluded that decreasing temperatures resulted in lower biofilm formation and suggested that there was 50 % less biofilm formation at 7 °C compared to 17 °C. Stephanovic et al. (2002) evaluated biofilm formation by *Salmonella* spp. at different temperatures (22 °C, 30 °C, 37 °C). They demonstrated that biofilm formation after 24 h was highest at 30 °C. After 48 h, the highest rate of biofilm formation occurred at 22 °C. Patil et al. (2010) also investigated biofilm formation at different temperatures by measuring the bioelectrocatalytic oxidation current in an electrochemical cell. Biofilms derived from waste water were electrochemically active within a temperature range from 5 °C to 45 °C. Maximum biofilm formation at 15 °C required 40 days, whereas at 35 °C 3.5 days were needed for maximum biofilm formation. Moreover, they observed, that the temperature during biofilm establishment influenced the electrochemical activity of the biofilms at the operation temperature of the electrochemical cells. Biofilms grown at lower temperatures showed higher physiological activity at low operation temperatures than

those established at higher temperatures and vice versa. Silhan et al. (2006) showed a positive relationship between temperature and biofilm formation when they investigated the formation of biofilms in cold (15 °C) and warm (35 °C) drinking water on various materials (steel, galvanised steel, polyethylen (PE), crosslinked polyethylene (PEX) and copper) under static conditions by determination of the HPC and the ATP content. Both the HPC and the ATP content were always higher at 35 °C than at 15 °C. This trend was more significant on the plastic materials than on the metals. Ndiongue et al. (2005) studied the effect of temperature and biodegradable organic matter (BOM) on the control of biofilm using free chlorine. In absence of additional BOM, biofilms established at 6 °C and 12 °C required more time to reach the steady state than biofilms at 18 °C. Moreover, the HPC was slightly higher at the warmer temperature. Piao et al. (2006) investigated the temperature-regulated formation of mycelial mat-like biofilms by *L. pneumophila* at three different temperatures (25 °C, 37 °C, and 42 °C) and on different materials (glass, polystyrene, and polypropylene). They observed a strong dependency of biofilm formation on temperature and surface material.

Biofilm diversity

The influence of temperature and other parameters such as exposition time or plumbing material on biofilm population can also be investigated on a molecular level by denaturing gradient gel gelelectrophoresis (DGGE). This method allows for the analysis of a bacterial community in a biofilm based on the separation of 16S rDNA fragments in acrylamide containing gels. It was first described by Muyzer et al. (1993). Separation of the 16S rDNA fragments is based on their base-pair sequences, which results in different migration patterns of the fragments in the gels. De Vet et al (2009) used this method for the genetic characterization of microbial diversity on sand filters for drinking water production and ground water sources. In 2010, Yu et al. revealed an influence of pipe material on bacterial communities in biofilms on water distribution pipes using DGGE. Roeder et al (2010) also observed an influence of the pipe material

on biofilm diversity when generating fingerprints of drinking water biofilms using DGGE. Moreover, they detected not only an influence of the pipe material, but also an effect of temperature on the microbial diversity of the biofilms. At different water temperatures (12 °C and 37 °C) biofilm populations differed from each other on the same material.

The great variety of studies regarding the influence of temperature on pathogenic organisms in natural aqueous systems as well as man-made systems and the previous considerations on expected temperature changes due to climate-change induced global warming show the great importance of water temperature regarding the maintenance of safe drinking water.

2 Materials

2.1 Bacterial test strains

P. aeruginosa AdS was isolated from the residual water within a tap water fixture of a contaminated domestic plumbing system of a school. The culture was stored on nutrient agar at 4 °C. For permanent preservation, a cryogenic culture was kept on glass beads (Cryobank system, Mast Diagnostica GmbH, Germany) at -70 °C.
L. pneumophila AdS (serogroup 1) was isolated from a biofilm in a tap water fixture of a contaminated domestic plumbing system of a school. The culture was stored on BCYE agar (Oxoid) at 4 °C. For permanent preservation, a cryogenic culture was kept on glass beads (Cryobank system, Mast Diagnostica GmbH, Germany) at -70 °C.
E. coli ATCC 25922 and *K. pneumoniae* ATCC 13883 were purchased from the DSMZ (German collection of microorganisms and cell cultures).

2.2 Cultivation media

Preparation of media:

All media were dissolved in deionized water and autoclaved at 121 °C for 20 min or alternatively filter sterilized (pore size 0.2 µm). The preparation was done according to the manufacturer's instructions. All solid growth media were poured in proportions 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, deionzed water ad to 1000 mL.

CASO agar (Merck)

Composition in g/L: Pepton from casein 17.0; Pepton from soymeal 3.0; D(+)-Glucose monohydrate 2.5; Sodium chloride 5.0; di-Potassium hydrogen phosphate 2.5.

Pseudomonas CN agar (Heipha)

Commercial agar plates, ready-to-use

Composition in g/L: peptone from gelatin 16, casein hydrolysate 10, magnesium chloride 1.4, potassium sulfate 10, cetrimid 0.2, agar 14, 10 ml glycerine

LB-Bouillon (Lennox) Carl Roth GmbH

Composition in g/L: yeast extract 5 , NaCl 5 , Bacto™ Trypton 10 , deionzed water ad to 1000 mL.

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.

Preparation: 20 g/ L of the commercially available granulate were dissolved in 1 L of deionized water

R2A agar (OXOID)

Composition in g/L: yeast extract 0.5, proteose peptone 0.5, casein hydrolysate 0.5, glucose 0.5, starch 0.5, di-potassium phosphate 0.3, magnesium sulphate 0.024, sodium pyruvate 0.3, agar 15.0 / pH 7.2 ± 0.2 at 25 °C

Preparation: 18.1 g/ L of the commercially available granulate were dissolved in 1 L of deionized water

Yeast Extract Broth (Ristroph et al.1980)

Composition in g/L: Yeast extract 10, L-cysteine-hydrochloric monohydrate 0.4, Iron(III)- pyrophosphate 0.25

The pH was adjusted to 6.9 with 1 M KOH and the medium was sterile filtered (0.2 μm).

2.3 Chemicals

Acetone (Analytical reagent grade; Fisher Chemicals)
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)
4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma)
DNA Ladder, (MassRuler DNA Ladder Mix 100 – 10.000 bp; Thermo Scientific)
Di-Sodiumhydrophosphate-dihydrate (, Na_2HPO_4 x 2 H_2O)
Ethanol Rotipuran (Roth)
Ethidium bromide (10 mg/ml) (BioRad)
Formaldehyde solution, ~ 36 % in H2O (Fluka, 47630)
Formamide deionised, ≥ 99.5%, p.a (Roth, P040.1)
GelRed (Biotium)
Glycerol (C3H8O3) (KMF Laborchemie Handels GmbH)
Hydrochloric acid 36 % (Sigma)
Iron(III)- pyrophosphate 0.25
L-cysteine-hydrochloric monohydrate (Fluka; Ultra ≥ 99.0 % RT)
Loading Dye TriTrack (Fermentas)
Na4EDTA x 2 H2O (Sigma)
N-laurylsarcosine
N,N,N',N'-Tetramethylethylenediamine (TEMED; Bio-Rad, 161-0800)
Potassiumchloride (KCl) (Merck)
Potassium-dihydrogenphosphate(KH_2PO_4) (Merck)
Paraformaldehyde (Merck, 1.04005)
Rotipuran Water (Roth, p.a., ACS)
SDS (Sodium Dodecyl sulfate; ≥ 98.5 %, for molecular biology; Riedel de Haen)
Sodium chloride (NaCl) [VWR PROLABO®]
Thiourea GR for analysis (Merck, 1.07979)

Urea for microbiology (SIGMA, 51456)
Water for Molecular Biology (Roth, DPEC treated)
Yeast extract (Merck Millipore Corporation)

2.4 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)
Black polycarbonate membrane filters, pore size 0.2 μm (Millipore)
Cell density meter model 40 (Fisher Scientific)
Cooling centrifuge, Sorvall® RC26PLUS (Sorvall)
Centrifuge Sorvall® (RC-5B; SS-34 Rotor)
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®)
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

Epoxy-coated 8-well diagnostic slides (Thermo Scientific)
Fluorimeter SFM 25 (BIO-TEK KONTRON Instrumente)
Gel documentation system Universal Hood II (Bio-Rad)
Gradient Delivery System Model 475 (Bio-Rad)
Hybridisation oven (Thermo electron cooperation)
Hybridisation oven (HB-1000 Hybridizer ;UVP)
Imaging Densitometer GS-710
Incubator (30 °C): Kelvitron® t (Heraeus)
Incubator (36 °C): Kelvitron® t (Heraeus)
Incubator (36 °C) MEMMERT
Laser scanner (Molecular imager FX pro plus Bio-Rad)
Light microscope; Leica DM LS, (Leica Microsystems)
Mastercycler ep gradient S (Eppendorf)
Membrane filters black, MicroPlus-31 ST, 0.45 μm pore size, □ 50 mm, mixed cellulose ester (Whatman)
pH meter WTW (ph 549 ELP) MultiCal®
pH meter WTW (Microprocessor pH 535 MultiCal®)

Phase contrast microscope; Leica DM LS, (Leica Microsystems)
Plate Reader (Infinite Pro M200; Tecan)
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)
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)
UV-light bulb, 254 nm (Faust)

2.5 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)

Colilert-18 Quanti-Tray®/2000 system (IDEXX)

PowerBiofilm® DNA Isolation Kit (Mobio)

Quant-iT™ PicoGreen® dsDNA Reagent Kit (Invitrogen, P7589)

2.6 Buffers and solutions

1 % Agarose solution

1 g agarose (Certified™ low melt; Bio-Rad) were dissolved in 100 mL 1 x TAE-buffer 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.

Ammonium persulphate (APS) solution, 10 %

0.1 g APS were dissolved in 1 mL deionized water

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

12.5 mg DAPI were dissolved in 27 ml formaldehyde (37 %) and 473 ml deionized water and filtered through a cellulose acetate filter (pore size 0.2 µm).

0 % denaturant solution in 7.5 % acrylamide

Composition in mL/ 100mL: 40 % acrylamide 18.8 mL, 50 x TAE-buffer 2.0, Rotipuran water 79.2.

The components were mixed and stored at room temperature in the dark.

100 % denaturant solution in 7.5 % acrylamide

Composition/100 mL: 40 % acrylamide 18.8 mL, 50 x TAE-buffer 2.0 mL, formamide 40.0 mL, urea 42 g, Rotipuran water ad 100 mL. Components were warmed in Rotipuran water at 50 °C until all ingredients were dissolved. Once the solution reached room

temperature, it was filled up to 100 mL with Rotipuran water and stored for four weeks at room temperature in the dark.

DNA-Ladder

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

dNTP Mix

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

EDTA solution, 0.25 M

104.5 g Na4EDTA x 2 H2O were dissolved in 1L of Rotipuran water and autoclaved for 20 min at 121 °C.

ES-solution (pH 8,0)

20.81 g EDTA (final concentration 0.5 M) and 0.5 g N-laurylsarcosine (final concentration 0.5 %) were dissolved in 100 mL deionised water. The pH was adjusted to 8.0 with 25 % HCl and the solution was filter-sterilized (0.2 µm).

Ethidium bromide solution

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

0.9 % NaCl-solution

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

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.

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

Paraformaldehyde solution (4 %)

Composition in g/l: 4 g paraformaldehyde (Merck) were dissolved PBS (pH 7.2) and stirred with a magnetic stirrer at 50 °C until dissolution. The solution was filled up to 100 mL and sterile filtered (pore size 0.2 µm).

Particle-free deionized water

Deionized water was filtered through a cellulose acetate filter (pore size 0.2 µm) and then autoclaved t 120 °C for 20 min.

Phosphate- buffered saline (PBS)

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

For the preparation Rotipuran water (Fa. Roth T143.1) was used. The pH was 7.2 ± 0.2.

SE-solution (pH 8,0):

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 8.0 with 1 M HCl and autoclaved for 20 min at 121 °C.

SDS solution, 10 %

10 g SDS were filled up to 100 mL with Rotipuran water and filter sterilized (0.2 µm)

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

20 mL of the commercial concentrate (50 x, Bio-Rad) were diluted in 980 ml deionized water. Final composition in mM: Tris 40, acetic acid 20, EDTA 1, pH 8.3.

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

TBE (Tris/borate/EDTA) buffer 10x

Commercial concentrate (Roth, 3061.2).

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

TE-buffer (pH 8,0)

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 8.0 with 1 M HCl and autoclaved at for 20 min 121 °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.

1 M Tris-buffer pH 7.2/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 7.2, pH 7.6, or pH 8.0 ± 0.2 with 7 M hydrochloric acid. The solution was autoclaved for 20 min at 121 °C.

2.7 Plumbing materials

The two different materials used for the coupons were polyethylene (PE) and ethylene-propylen-dien–monomer (EPDM) rubber. Both materials complied with the physical and chemical specifications of the recommendations of the German commission for drinking water (KTW) for plastic or rubber materials in contact with drinking water (Umweltbundesamt 2008, Anonymous, 1985) as well as the microbiological specifications of the German Gas and Water Association ((DVGW) Code of Practice W270 (DVGW, 2007).

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3 Methods

3.1 Field experiments

Coupons of three different materials (EPDM, PE80/100 stainless steel) were inserted in biofilm reactors which were exposed in the drinking water distribution system as a bypass. In eight different climatopes the influence of drinking water temperature on hygienically relevant bacteria in drinking water systems was investigated.

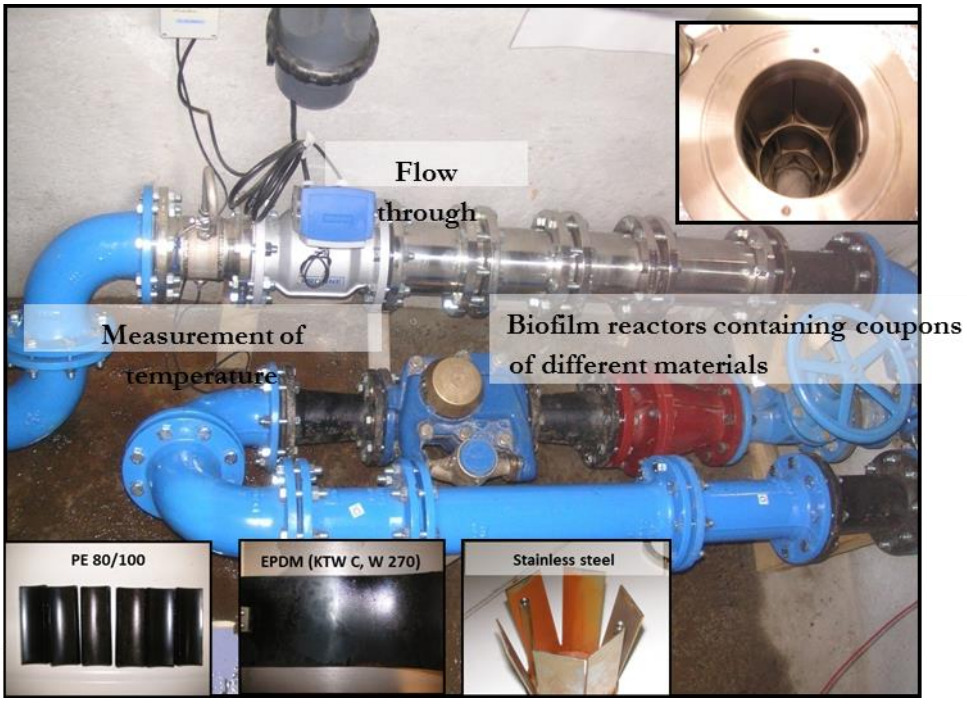


Figure 7: Biofilm reactor inserted in the drinking water distribution system as a bypass order to investigate the influence of temperature on hygienically relevant bacteria in drinking water biofilms.

3.2 Laboratory experiments

3.2.1 Preparation of bacteria

For cultivation of *P. aeruginosa* AdS, *E. coli*, and *K.pneumoniae* 20 ml of 10-fold diluted YEB-medium were inoculated with a single colony pre-grown on nutrient-agar (Merck) for 24 h at 36 °C and incubated at 30 °C for 24 h with shaking at 180 rpm. For cultivation of *L. pneumophila* AdS 20 mL of 5-fold diluted YEB medium were inoculated with a single colony pre-grown on BCYEα-agar for 72 h at 36 °C and incubated at 30 °C for 96 h with shaking at 180 rpm. Cells from the liquid culture were harvested by centrifugation (4000 rpm, 15 min, 10 °C) and washed twice with sterile filtered drinking water from a copper-free pipe. After washing, 20 ml of the sterile filtered drinking water from the copper-free pipe were inoculated with the harvested cells. The concentration of bacteria in the cultures was 2 x 10^8 cells/mL. Incubation was performed for 24 h at room temperature (approximately 22 °C) with shaking at 180 rpm in order to adapt the bacteria to drinking water conditions.

3.2.2 Preparation of the biofilms

Drinking water biofilms were grown in modified RotoTorque annular reactors iwith 12 flush-mounted rectangular slides of two different plumbing materials (EPDM and PE80) for 14 days. The reactors were perfused continuously with drinking water at 20 mL/min at water temperatures in the range of 8 °C to 29 °C (5,5 µg Acetat-C/L, oligotrophic conditions). Two reactors were additionally supplemented with 1:1000 diluted tryptic soy broth (150 µg Acetat-C/L, copiotrophic conditions). Prior to perfusing the annular reactors, the drinking water was collected in a small storage tank to properly adjust the temperature. Drinking water temperature was directly measured in the annular reactors by means of temperature sensors (Krone) at 5-min intervals. The

influent to the reactors was UV-disinfected drinking water from a surface water treatment plant.

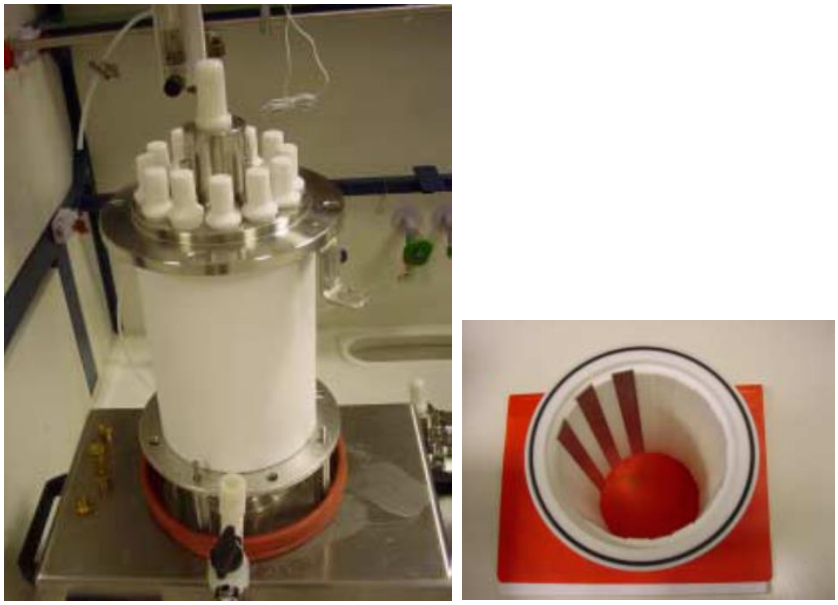


Figure 8: Annular reactor for the establishment of drinking water biofilms in order to investigate the influence of temperature on hygienically relevant bacteria.

3.2.3 Inoculation of the biofilm reactors

After two weeks of biofilm formation, the annular reactors were inoculated with a mixed culture of the laboratory strains *E. coli* and *K. pneumoniae*, and separately with a mixture of the environmental strains *P. aeruginosa* AdS and *L. pneumophila* AdS. The final concentration of the bacteria in the annular reactors was 10^6 cells/mL. Pumps were switched off prior to inoculation and rotors were turned off 5-10 min afterwards. After static incubation for 20 h pumps and rotors were turned on and after 30 min (equates the complete exchange of water in the annular reactors) samples were taken.

3.2.4 Sampling of drinking water biofilms and drinking water

Biofilm suspensions as well as influent and effluents have been analyzed. Samples were taken prior to inoculation, as well as 1, 7, 14, 21, and 28 days subsequently. Coupons of each material (EPDM and PE80) were taken out of the annular reactors and biomass

was scrapped off the plumbing materials using a teflon scraper and suspended in 20 mL DEPC treated water (Roth). Homogenization of the biofilm suspension was done by using syringes with different injection needles (0.6 mm and 0.4 mm). For every coupon taken out of the annular reactors a stainless steel (V2A) coupon was inserted into the reactor. 1 L of the influent and each reactor effluent was sampled in sterile glass bottles (Schott).

3.3 Microbiological analysis

3.3.1 Determination of total cell count

The total cell count in the biofilm suspensions and the drinking water (influent and effluents) was determined by staining with the fluorochrome 4, 6-diamidino-2-phenylindole (DAPI) and enumeration of cells under an epifluorescence microscope.

3.3.2 Determination of the heterotrophic plate count

The heterotrophic plate count (HPC, Reasoner and Geldreich, 1985) was determined on R2A medium. Undiluted, as well as decimal diluted biofilm and drinking water samples were spread on R2A medium. After incubation at 20 °C for 7 days, colonies were enumerated. Results are given as colony-forming units (cfu)/mL or cfu/cm^2.

3.3.3 Determination of culturable *P. aeruginosa*

P. aeruginosa was quantified according to the standard DIN EN ISO 16266. For the biofilm samples, serial dilutions of the biofilm suspensions were spread plated on cetrimid agar. For determination of *P. aeruginosa* in influent and effluents of the annular reactors, volumes up to 100 mL were filtered through 47 mm cellulose ester membrane filters with a pore size of 0.45 µm. Filters were transferred onto cetrimid agar. Plates

were incubated at 36 °C for 2 days and colonies enumerated. Results are given as cfu/mL or cfu/cm^2.

3.3.4 Determination of culturable *Legionella* spp.

L. pneumophila was quantified according to the standard ISO 11731 and ISO 11731 part two. For the biofilm samples, serial dilutions of the biofilm suspensions were spread plated on GVPC agar after acid treatment. In the influent and the effluents of the annular reactors, *L. pneumophila* was determined by filtering up to 100 mL through 47 mm cellulose ester membrane filters with a pore size 0.45 µm. Plates were incubated at 36 °C for 10 days. Results are expressed as cfu/mL or cfu/cm^2.

3.3.5 Determination of culturable *E. coli* and *K. pneumoniae*

Quantification of *E. coli* and coliforms was performed using the Colilert-18 QuantyTray®/ 2000 system (IDEXX). One vial of the Colilert-18 reagent was dissolved in 100 mL of the diluted or undiluted biofilm and water samples and subsequently transferred into a Quanty-Tray®. The Tray was sealed and incubated at 36 °C for 19 h. Positive wells were enumerated. Results are expressed as MPN mL^{-1} or MPN cm^{-2}

3.4 FISH analysis

FISH of drinking water biofilm suspensions as well as drinking water (influent and effluents of the annular reactors in the laboratory experiments and biofilm reactors in the drinking water distribution system in the field experiments) was performed using the specific probes Psae 16S-182 (Wellinghausen et al., 2005) for detection of *P. aeruginosa*, LEGPNE 1 (Grimm et al., 1998) for detection of *L. pneumophila*, Leg 705

for detection of *Legionella* spp, Colinsitu (Regnault et al., 2000) for detection of *E. coli*, and Kpn (Esperanza-Torres et al., 2008) for detection of *K. pneumoniae*.

Table 3: Oligonucteotide probes used in the present study

	Sequence (5'-3')	**Target**	**Reference**
Psae16s 182	CCA CTT TCT CCC TCA GGA CG	*P. aeruginosa*	Wellinghausen 2005
LEGPNE1	ATC TGA CCG TCC CAG GTT	*L. pneumophila*	
LEG705	CTG GTG TTC CTT CCG ATC	*Legionella* spp.	Manz et al., 1995
Colinsitu	GAG ACT CAA GAT TGC CAG TAT CAG	*E. coli*	Regnault et al., 2000
Kpn	CCT ACA CAC CAG CGT GCC	*K. pneumoniae*	(Kempf et al. 2000; Esperanza-Torres, 2008)

Table 4: Hybridization buffers (final concentrations)

	Colinsitu	**Kpn**	**LEG705**	**LEGPNE1**	**PSAE 16S-182**
5M NaCl	0.9 M	0.9 M	0.9 M	0.9 M	0.9 M
1M Tris pH 7.0	-	20 mM	-	-	-
1M Tris PH 7.6	-	-	20 mM	20 mM	20 mM
1M Tris pH 8.0	20 mM		-	-	-
10%SDS	0.01 %	-	0.01 %	0.01 %	0.01 %
Formamide	25 %	20 %	30 %	25 %	40%
Rotipuran	ad 1mL	ad 1mL	ad 1mL	ad 1mL	ad 1mL

Table 5: Washing buffers (final concentrations)

	Colinsitu	Kpn	LEG705	LEGPNE1	PSAE 16S-182
5M NaCl	250 mM	0.9 M	225 mM	160 mM	56 mM
1M Tris pH 7.0	-	20 mM	-	-	-
1M Tris PH 7.6	-	-	20 mM	20 mM	20 mM
1M Tris pH 8.0	20 mM		-	-	-
10%SDS	0.01 %	0.01 %	0.01 %	0.01 %	0.01 %
0.25 M EDTA	-	-	-	5 mM	5 mM
Rotipuran	ad 25 mL	ad 25 mL	ad 25 mL	ad 25 mL	ad 25 mL

3.4.1 Laboratory experiments

Fixation. 1 mL of the biofilm samples was transferred into safe lock tubes (Eppendorf, 2 mL volume). By centrifugation (5 min, 4 °C) cells were harvested and the supernatant was decanted. The pellet was resuspended in 2 mL 4% (w/v) paraformaldehyde in PBS, pH 7.2 and subsequently incubated at 4 °C for 1 h. Afterwards the suspension was centrifuged again (5 min, 4 °C) and the supernatant was discarded. The pellet was resuspended in 1 mL PBS and centrifuged (5 min, 4 °C). After centrifugation, the pellet was resuspended in a mixture of PBS and ethanol absolute (1:1) and stored at -20 °C until further examination. For the drinking water samples in the laboratory experiments the maximum filterable volume of the drinking water was filtered through an acetate filter (0.45 μm). The filter was reduced to small pieces, transferred into 10 mL sterile deionized water and shaken with maximum speed on a Vortex. Afterwards 2 ml of the suspension were transferred into a safe lock tube (Eppendorf, 2 mL volume) and centrifuged (5 min, 4 °C). The supernatant was decanted and again 2 mL of the suspension were transferred into the same safe lock tube and centrifuged (5 min, 4 °C).

The procedure was repeated until the 10 mL were pooled in one safe lock tube. The subsequent fixation was performed as described above.

Hybridization. 10 µL of the resuspended samples were pipetted onto epoxy-coated 8-well diagnostic slides (Thermo Scientific) and air-dried. After dehydration with three consecutive baths in ethanol (50%, 80%, and 96 %, 3 min. each) 10µL hybridization buffer containing 5 ng/L of the respective oligonucleotide probe were pipetted onto the dried samples on the glass slides. For the probes Psae 16S-182, LEGPNE1, LEG705, and Colinsitu hybridization was performed in a humid reaction chamber (Vermicon) at 46 °C for 90 min. For the probe Kpn hybridization was performed at 40°C, respectively. To remove unbound probes the glass slides were then transferred into the respective washing buffer at 46 °C for 15 min (40 °C for Kpn). Afterwards the slides were washed in deionized water and air-dried. Cells were counterstained by pipetting 10 µL DAPI (1 µg/mL) onto each well of the glass slide and incubating for 20 min at room temperature in the dark. After incubation the slides were washed with deionized water and air-dried. Enumeration of bacterial cells was performed using an epifluorescence microscope (Zeiss) at 1000-fold magnification. Therefore, citifluor was used as a mounting medium on the glass slides. 10 to 20 randomly selected fields of view were counted for each sample with the help of a counting grid (100 µm×100 µm).

3.4.2 Field experiments

Fixation. 6 mL of each biofilm sample were transferred into safe lock tubes (Eppendorf, 2 mL volume). By centrifugation (5 min, 4 °C) cells were harvested and the supernatant was decanted. The pellets were resuspended, pooled in 2 mL 4 % (w/v) paraformaldehyde in PBS, pH 7.2 and subsequently incubated at 4 °C for 1 h. The samples were then treated as the laboratory samples, respectively.

3.5 Denaturing Gradient Gel Electrophoresis (DGGE)

3.5.1 DNA Isolation

20 ml of each biofilm suspension from the field experiments and 6 ml of each biofilm suspension from the laboratory experiments, respectively, were centrifuged at 20.000 x g, 4 °C, for 20 min. The supernatant was discarded and the pellet was resuspended in 2 ml DEPC-water (Roth). The suspension was transferred into a 2 ml reaction tube (Eppendorf) and centrifuged at 16.000 x g, 4 °C, for 30 min. Again, the supernatant was discarded and the pellet was stored at – 20 °C until DNA was isolated. The DNA was isolated with the PowerBiofilm™ Isolation Kit (MO BIO) according to the manufacturer's instructions.

3.5.2 PCR for DGGE

Amplification of the 16S rDNA was performed by touchdown PCR

Table 6: PCR components and concentrations

	Concentration	**Final conc.**	**µl per well**
Tag-Master	-	-	10,0
PCR-Buffer	-	-	5,0
dNTP-Mix	10 mM	200 µM	1,0
Forward-Primer 27f_GC (Medlin et al., 1988, Murray et al.,1996**)**	10 µM	0,5 µM	2,5
Reverse-Primer 517r (Murray et al., 1996)	100 µM	0,5 µM	2,5
***Taq*Polymerase**	5 U/µl	2,5 U	0,5
DNA-Template	-	-	3,0
H_2O (PCR-clean)	-	-	25,5
Total volume	-	-	50,0

Table 7: PCR program

T [°C]	T [min]	
94	2:00	
94	1:00	30 cycles
59	1:00	
72	1:30	
72	5:00	
4	∞	

3.5.3 Agarose Gel electrophoresis

After isolation and amplification the 16S rDNA fragments from drinking water biofilms presence and size of the amplicons was analyzed by agarose gel electrophoresis using a 1 % agarose gel (50 mL of a 1 % Agarose solution in TAE buffer were filled into a gel tray and allowed to harden at room temperature). A mixture of 5 µL of the PCR products and 1 µl 6x loading buffer (Fermentas) was pipetted into each well of the gel. A DNA size ladder (Fermentas) was included in each run. 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 deionized water for 5 min. Analysis and documentation of the agarose gel was performed using a gel documentation system (Universal Hood II, Bio-Rad).

3.5.4 Determination of the DNA concentration within the drinking water biofilm samples

The DNA concentration of the PCR products was determined using the PicoGreen® assay. Therefore, calibration for high concentration samples was performed as described

in the manufacturer's instructions. (table 15). Determination of the DNA concentration within the analyzed biofilms was subsequently performed following the manufacturer's instructions using a Fluorimeter SFM 25 (BIO-TEK KONTRON Instrumente) or a plate reader (Tecan).

Table 8: Calibration for the determination of DNA concentrations within the drinking water biofilm samples

Volume (µl) TE-Buffer	**Volume (µl) DNA-stock solution (2 µg/ml)**	**Final concentration (ng/ml)**
0	1000	2000
900	100	200
990	10	20
999	1	2
1000	0	0 (blank)

3.5.5 DGGE

To encompass possible impacts of temperature on the biofilm population, genetic fingerprinting of drinking water biofilms was performed by denaturing gradient gel electrophoresis. For the separation of the different DNA isolates, a 7.5 % acrylamide gel with a gradient of 40 % to 60 % denaturation was prepared. For one gel (160 mm x 140 mm x 1 mm) 14 mL of each, a 40 % denaturing solution and a 60 % denaturing solution were prepared.

Table 9: Gel components for the DGGE

Component	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

Immediately after TEMED was added to the solutions, the gel was poured using a gradient delivery system (Bio-Rad) and allowed to harden at room temperature for about three to four hours. For a straight edge, the gel was covered with water-saturated 2-butanol. After hardening was completed, the remaining 2-butanol was washed off with TAE buffer and a collection gel was poured onto the gel for improved loading of the samples. The collection gel required 45 min of polymerization at room temperature. Afterwards, samples were mixed with 6x loading dye (Fermentas) and 250 ng DNA were loaded onto the gel. In some cases, the DNA content in the samples was too low to load 250 ng DNA onto the gel. Here, the maximum possible concentration was loaded onto the gel. 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 stained using GelRed (Biotium) following the manufacturer's instructions.

Analysis of banding pattern

In order to evaluate the diversity of the drinking water biofilms in the field experiments and the drinking water biofilms established in the annular reactors, the number of bands of each sample was counted. To compare the biofilm population of two samples the

similar bands of both samples were counted. Similarity of the bands was defined by the same distance of migration through the agarose gel.
Similarity of biofilm communities grown at different temperatures was calculated by means of the Sørensen Index (Cs; Sørensen, 1948):

Cs = 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 were expressed in percent.

3.5.6 Pulsed-field gel electrophoresis (PFGE)

PFGE of *P. aeruginosa* was performed following the descriptions of Head and Yu (2004). *P. aeruginosa* cultures were grown on nutrient agar (36 °C) for 24 h. Afterwards they 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 until the optical density at a wavelength of 600 nm of was 0.2. The culture was incubated at 36 °C for 2 h with agitation (180 rpm)followed by harvest of the cells 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. A volume of 82 µL of the suspension was then pipetted into plug molds (Bio-Rad), which were allowed to harden at 4 °C for 15-30 min and subsequently incubated in 800 µL of lysis buffer consisting of 780 µL SE solution and 20 µL proteinase K solution for 21 h at 50 °C for cell lysis. 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, 500 µL 1x NE buffer were pipetted onto the gel plugs and equilibrated on ice for 2 h. Afterwards 300 µL restriction buffer (2.3.5.) were added and the plugs were incubated on ice for 2.5 h and subsequently for 16 h at 37 °C. In the next step, 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 °. After 19 h, the switch time was changed from 5 – 30 s to 5 – 70 s. The variable speed pump was operated at 70 % speed. In each run, a marker (Lambda Ladder PFG Marker, New England BioLabs) was included in addition to the samples. For visualization of the bands, the gel was stained in ethidium bromide solution (1 µg/mL) for 60 min and then destained in deionised water for 20 min and analysis and documentation of the agarose gel was performed using a gel documentation system (Universal Hood II, Bio-Rad)

4 Results

The main goal of the present study was to investigate the influence of water temperature on the survival of hygienically relevant bacteria in drinking water and drinking water biofilms revealing possible challenges due to a global warming induced increase in temperature. Therefore field experiments as well as laboratory experiments were performed.

4.1 Field experiments

The influence of variations in drinking water temperature on the survival of hygienically relevant bacteria in drinking water and drinking water biofilms was investigated in a German drinking water distribution system. Coupons of three different materials (PE80/100, EPDM, stainless steel) were exposed in drinking water biofilm reactors installed in a public drinking water distribution system at eight different locations representing eight different climatopes (water works, city centre, park, forest, suburb, open land, industry 1, industry 2; Table 10). The plastic material PE and the elastomeric material EPDM met the microbiological specifications of the German Gas and Water Association (Anonymous, 2007) as well as the physical and chemical specifications of German recommendations for plastic or rubber materials (Anonymous, 1977, 1985) used in contact with drinking water. The plastic material is commonly used as pipe material in drinking water distributions systems in Germany. EPDM is used for rubber coated valves. Stainless steel was additionally chosen for the field studies because it is an inert material which in contrast to PE and EPDM does not release any nutrients. The

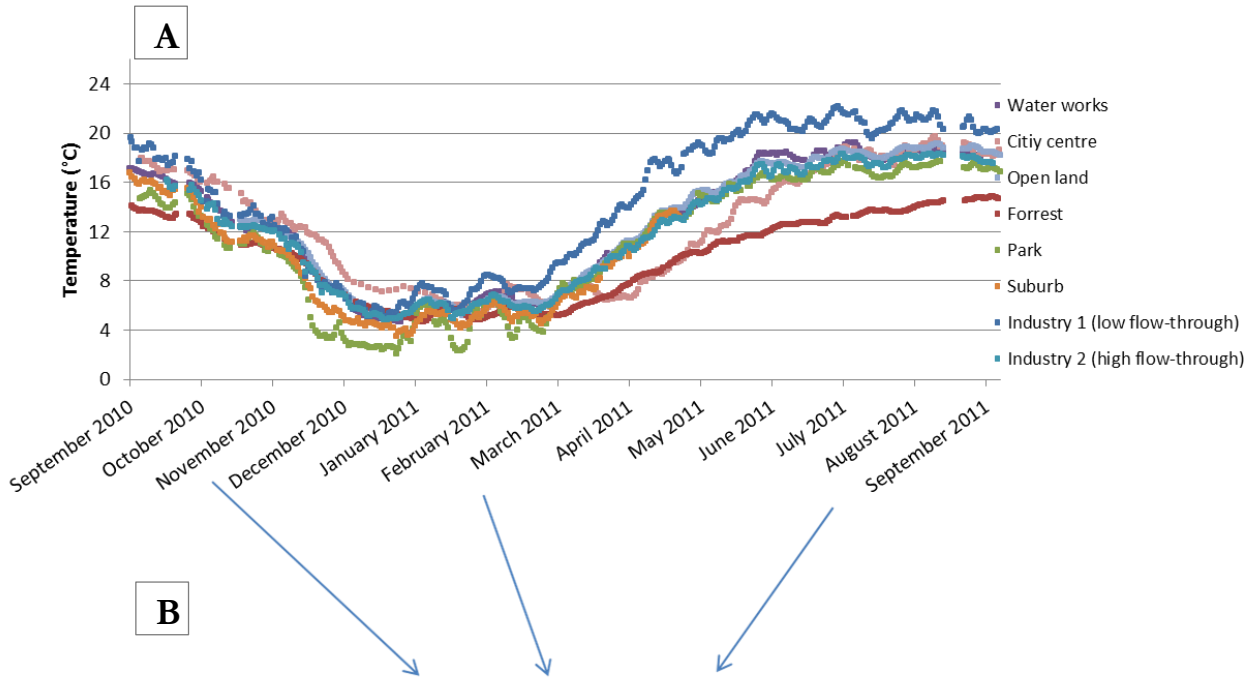
drinking water distribution system, in which the biofilm reactors were exposed, was supplied with drinking water from the water works Mülheim Styrum-Ost. The drinking water was artificially enriched surface water, which was UV treated after treatment prior to release into the distribution system. The AOC content of the drinking water after treatment was 5.5 µg/L acetate-C-equivalent and the molar C:N:P ($AOC:NO_3$-N:PO_4-P) ratio was 100:45.000:380. For bacterial growth a molar C:N:P ratio of 100:10:1 is optimal. Therefore, the present drinking water showed a significant C-limitation. This and the AOC below 10 µg/L acetate-C-equivalent indicated a biological stable drinking water which did not promote bacterial growth (Van der Kooij (1989)). In three different sampling campaigns (November 10 (autumn), March 11 (winter), September 11 (summer)), the coupons were removed and biofilms were scraped off and analyzed by various culture-based and culture-independent methods. In addition to the drinking water biofilms, influent and effluent of the drinking water biofilm reactors were sampled and analyzed for the same parameters.

Table 10: Climatopes in the field experiments (modified from Kuttler et al., 2012)

Climatope	**Characteristics**
City Centre	Highly sealed area
Park	Shady area surrounded by trees
Industry 1	Parking lot, no trees, low flow- through of the drinking water
Industry 2	Parking lot, Many buildings, trees, a channel in 40 m distance, high flow-through of the drinking water
Suburb	Partially shaded by trees, many one-family houses
Forest	Shady area surrounded by many trees, dead end pipe
Open land	Partially shaded by trees, along a street
Water works	Effluent of the day tank, treated drinking water

4.1.1 Drinking water temperature during the field experiments

During the exposure of the biofilm reactors in the drinking water distribution system the drinking water temperature was measured continuously (Figure 9). The maximum temperature in the drinking water distribution system during the complete time of the experiment was 23.3 °C in the climatope industry 1 (low flow-through) in July 2011. The minimum temperature was 2.1 °C in the climatope park in January 2011. During the three sampling campaigns the highest mean daily temperature in summer was 21 °C (climatope industry 1), in autumn 12.3 °C (climatope city centre), and in winter 9.6 °C (industry 1). Maximum differences in the drinking water temperature between the eight climatopes were up to 10 °C (between climatope industry 1 and climatope forest) during the complete period of the experiment.



B

Climatope	Average temperature (°C)		
	Autumn	Winter	Summer
Water works effluent	11.8	6.9	18.2
Open land	7.3	7.5	18.7
Forrest	10.6	6.0	14.9
Park	11.1	7.8	17.6
Industry 1	11.6	9.6	21.0
Industry 2	6.8	7.3	18.0
Suburb	11.1	6.7	18.4
City centre	12.3	8.6	18.8

Figure 9: Temperature profiles of the different climatopes (A) and mean daily temperatures in the different climatopes during the day of sampling (B).

4.1.1.1 Total cell counts and colony counts in the drinking water

Quantification of the total bacteria in the drinking water was conducted by determination of total cell counts using DAPI. In addition, the HPC was determined as a parameter for viable bacteria in drinking water. Moreover, total cell counts at 20 °C and 36 °C were determined according to the German Drinking Water Ordinance.

Independent of the drinking water temperature, the total cell counts were within a range of 3×10^4 to 1×10^6 cells mL^{-1} (Figure 10) in the influents of the reactors. In the effluents,

total cell counts varied between 10^5 cells mL^{-1} to 10^6 cells mL^{-1} regardless of the drinking water temperature. The HPC was about one to two log units higher in summer than in autumn. However, there was no significant difference between the numbers of the HPC in winter and summer, indicating that the exposition time might have the main influence on the numbers of the HPC and not the drinking water temperature. The proportion of HPC to total cell count was in the range of 0 % to 0.5 % in the influents. The HPC ranged from 0.45 to 4 x 10^3 cfu mL^{-1}. In the climatopes forest, suburb and industry 1 a trend of rising HPC associated with elevated temperatures was observed. Numbers were highest in summer in these climatopes. In the water works, the HPC was lowest in autumn and similar in winter and summer. In the other climatopes no correlation between temperature and the HPC was observed. The proportion of HPC to total cell count was in the range of 10^{-3} % to 2.4 % in the effluents.

There was no correlation detectable between the colony counts at 20 °C and 36 °C and the drinking water temperature. Numbers varied between 0 and 5 cfu mL^{-1} at 20 °C and between 0 and 25 cfu mL^{-1} at 36 °C and were below the limits of the German drinking water ordinance. Colony counts in the effluents were low during all sampling campaigns. At 20 °C, the numbers of the colony counts varied between 0 and 23 cfu mL^{-1} with one exception in the climatope open land in winter (186 cfu mL^{-1}). Colony counts at 36 °C were within a range of 0 to 14 cfu mL^{-1}.

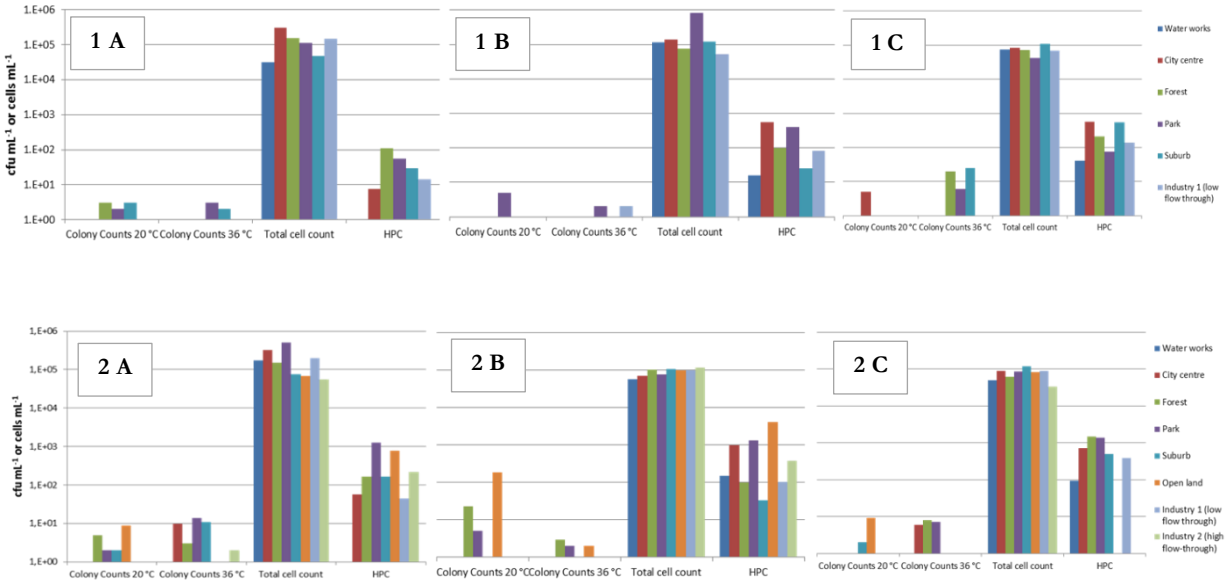


Figure 10: Total cell counts, colony counts 20 °C and 36 °C, and HPC of all climatopes and sampling campaigns; 1A: influent autumn, 1B: winter, 1C: summer, 2A: effluent autumn, 2B: effluent winter, 2C: effluent summer.

4.1.1.2 Hygienically relevant bacteria in the drinking water

In addition to the general parameters, the presence of hygienically relevant bacteria in drinking water and the influence of temperature variations on the occurrence of these organisms were investigated. Drinking water samples were analyzed for *E. coli*, coliform bacteria, *P. aeruginosa*, *Legionella* spp., Enterococci, and Aeromonads using cultural methods and FISH.

The occurrence of hygienically relevant bacteria in the analyzed drinking water did not depend on drinking water temperature. However, in 3 out of 24 samples hygienically relevant bacteria were detected culturally in very low numbers (Table 11). In one sample Aeromonads (*Aeromonas hydrophila/caviae*) and the coliform bacterium *Enterobacter amnigenus* were detected, in a second sample Enterococci were present and a third sample was positive for *Aeromonas hydrophila/caviae*. However, all samples were negative for any of the mentioned bacteria when sampling was repeated.

Table 11: Culturable hygienically relevant bacteria in the tested drinking water

Parameter	**Number of positive samples**	**Frequency of occurence (%)**	**cfu or MPN 100 mL^{-1}**
E. coli	0	0	-
P. aeruginosa	0	0	-
Legionella spp.	0	0	-
Coliform bacteria (without *E. coli*)	1	4,2	1
Aeromonads	2	8,3	1, respectively 2
Enterococci	1	4,2	3

The absence of colony forming bacteria does not necessarily mean that no bacteria were present but includes the possibility that some of them were in a viable-but-nonculturable (VBNC) state. The ability of microorganisms to enter the VBNC-state has been explained in the introduction (1.5. In order to detect also these bacteria the culturally independent FISH was applied for further characterization of the drinking water samples. Target organisms were *P. aeruginosa*, *Legionella* spp., *L. pneumophila*, and *E. coli*. In contrast to the culturally derived data, all target organisms could be detected with FISH (Table 12). However, it was observed, that elevated temperatures did not result in higher numbers of the FISH-positive target organisms. Conspicuously, the frequency of detection of all target organisms was lowest in summer (0% to 50 %) when the temperature was highest during the three sampling campaigns. The highest numbers of FISH-positive target organisms were detected in winter. 75 % to 100 % of all analyzed drinking water samples were positive for the target organisms in this sampling campaign (Table 12).

Table 12: Frequency of detection of hygienically relevant bacteria in the drinking water by FISH (n= 24).

	Frequency of detection (%)		
Organisms	**Autumn**	**Winter**	**Summer**
E. coli	50	75	50
P. aeruginosa	62.5	75	0
Legionella spp.	100	100	n. q.
L. pneumophila	75	100	12.5

n.q.: not quantified

Summary

Elevated drinking water temperatures did not result in an impairment of the biological stability of the analyzed drinking water (i.e., an increase of total cell counts, HPC, or colony counts according to the German Drinking Water Ordinance).

Colony counts according to the German Drinking Water Ordinance did not exceed the threshold of 100 cfu/mL, but remained within the single or low double-digit range. With culture-based methods, no temperature-related occurrence of hygienically relevant bacteria as observed. Using Fluorescence-*in-situ*-hybridization (FISH) as a culture-independent method, hygienically relevant bacteria, possibly in the VBNC-state, were detected in all analyzed drinking water samples. For all target organisms (*E. coli, P. aeruginosa, L. pnuemophila*) the frequency of detection in the drinking water was highest in the winter sampling campaign and lowest in the summer sampling campaign.

4.1.2 Drinking water biofilms

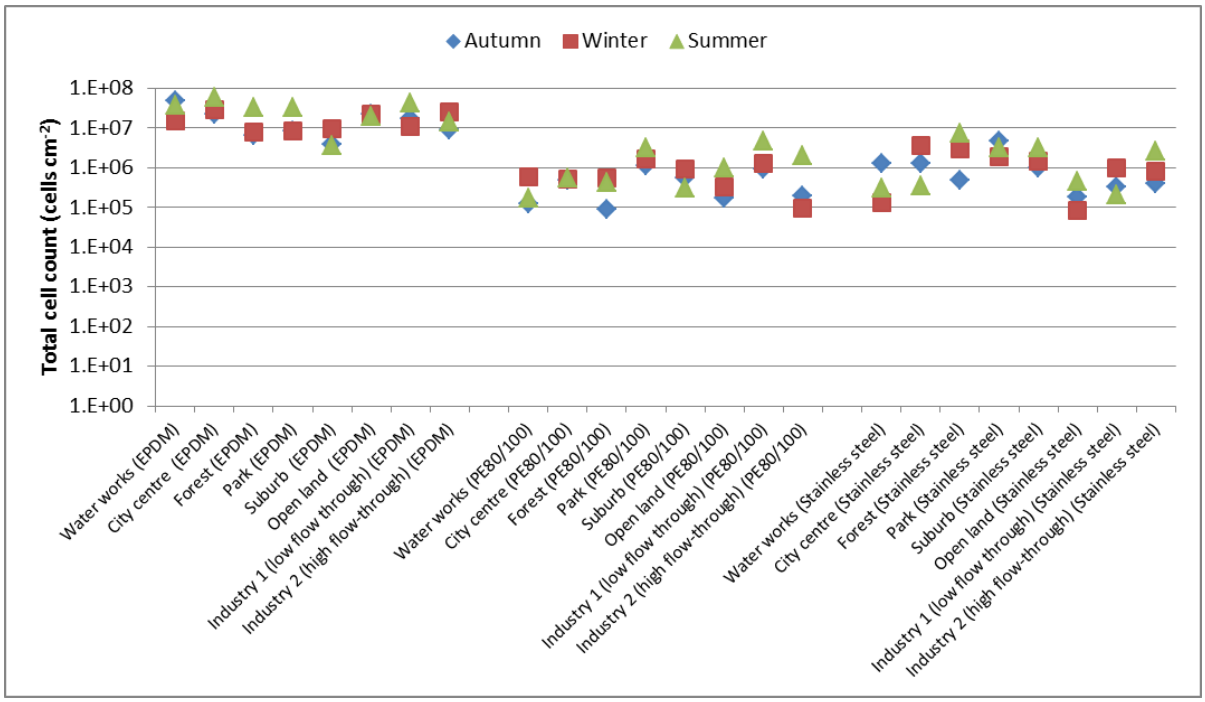
Biofilms are ubiquitous and can be found on all surfaces in drinking water distribution systems. The majority of all bacteria present in a drinking water distribution system is attached to surfaces (Wingender and Flemming 2011). For that reason, not only the water phase was analyzed in the field experiments, but also the biofilms established in

the biofilm reactors which were exposed for at least six months on various materials (EPDM, PE80/100, stainless steel, which were chosen for reasons indicated earlier).

4.1.2.1 Biofilm formation in the drinking water distribution system: total cell counts and colony counts

For a general characterization of the drinking water biofilms total cell counts, HPC and colony counts at 20 °C and 36 °C were determined.

The total cell counts of the different biofilms were only marginally influenced by the different seasons in which the temperature ranged from 6 °C to 21 °C in the three sampling campaigns (Figure 11). However, cell densities were influenced by the applied materials. Total cell counts were highest on EPDM (10^7 cells cm^{-2} to 10^8 cells cm^{-2}). On PE80/100 and stainless steel, numbers were about two to three log units lower and within a range of 9 x 10^4 cells mL^{-1} to 7 x 10^6 cells mL^{-1}. On PE80/100 and stainless steel, a trend of slightly rising total cell counts associated with elevated temperatures and increasing exposure time could be observed for various climatopes: total cell counts increased slightly of about 1 log-unit in the climatopes open land, park, industry 1, and industry 2. On stainless steel this trend was observed for the climatopes forest, industry 1, and suburb (Figure 11).



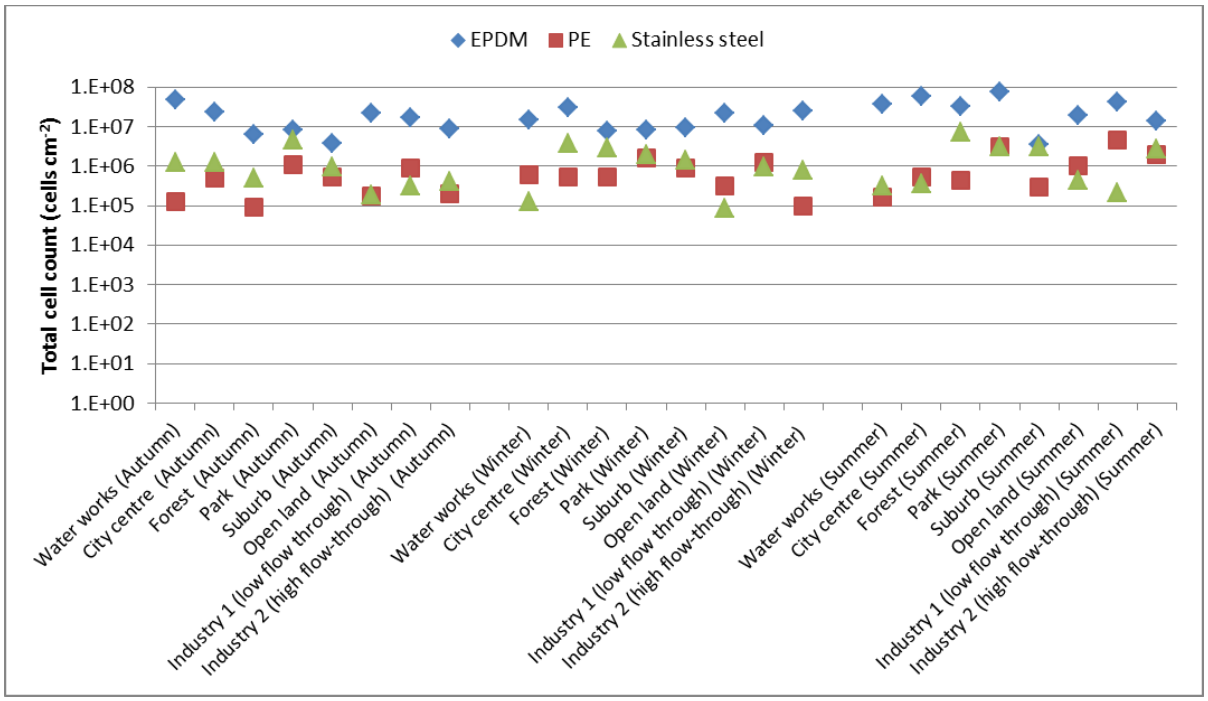


Figure 11: Total cell counts for EPDM (A), for PE80/100 (B), and for stainless steel (C).

As well as for the total cell counts, no temperature dependency was observed for the numbers of the HPC in the drinking water biofilms (Figure 11). Except for one sample in the climatope park and one sample in the water works, the HPC was in the range of 10^6 cfu cm^{-2} to 10^7 cfu cm^{-2} on EPDM during all three sampling campaigns. For biofilms

grown on PE80/100 and stainless steel, numbers of the HPC were about one to five log units below the HPC of the biofilms on EPDM. Thus, the material was the major factor influencing the numbers of the HPC, not the drinking water temperature. Taking into consideration the climatopes in particular, marginal variations in the number of the HPC between the three different sampling campaigns were observed for the cliamtopes water works and open land on EPDM. In both climatopes, the HPC decreased from autumn to winter and from winter to summer. Thus, the number of the HPC was lowest, when the temperature was highest and the exposure time was longest. In the other climatopes numbers were similar during all three sampling campaigns. On PE80/100 numbers of the HPC were within a range of 3.4×10^2 cfu cm^{-2} and 3.4×10^4 cfu cm^{-2}. The highest variations were observed between the sampling campaigns in autumn and winter in the climatope park. In autumn, the HPC was two log units higher than in winter. In the climatopes water works, city centre, forest, and industry 1 and 2, no influence of temperature and/or exposition time was observed and the HPC was similar for each climatope over the entire period of the experiment. On stainless steel, variations in the numbers of the HPC were higher than on EPDM and PE80/100 and ranged from about 1×10^1 cfu cm^{-2} to 2×10^5 cfu cm^{-2}. Only in the water works, the HPC remained similar during the three sampling campaigns. The other cliamtopes showed higher variations between the different sampling campaigns which could not be directly referred to variations in temperature.

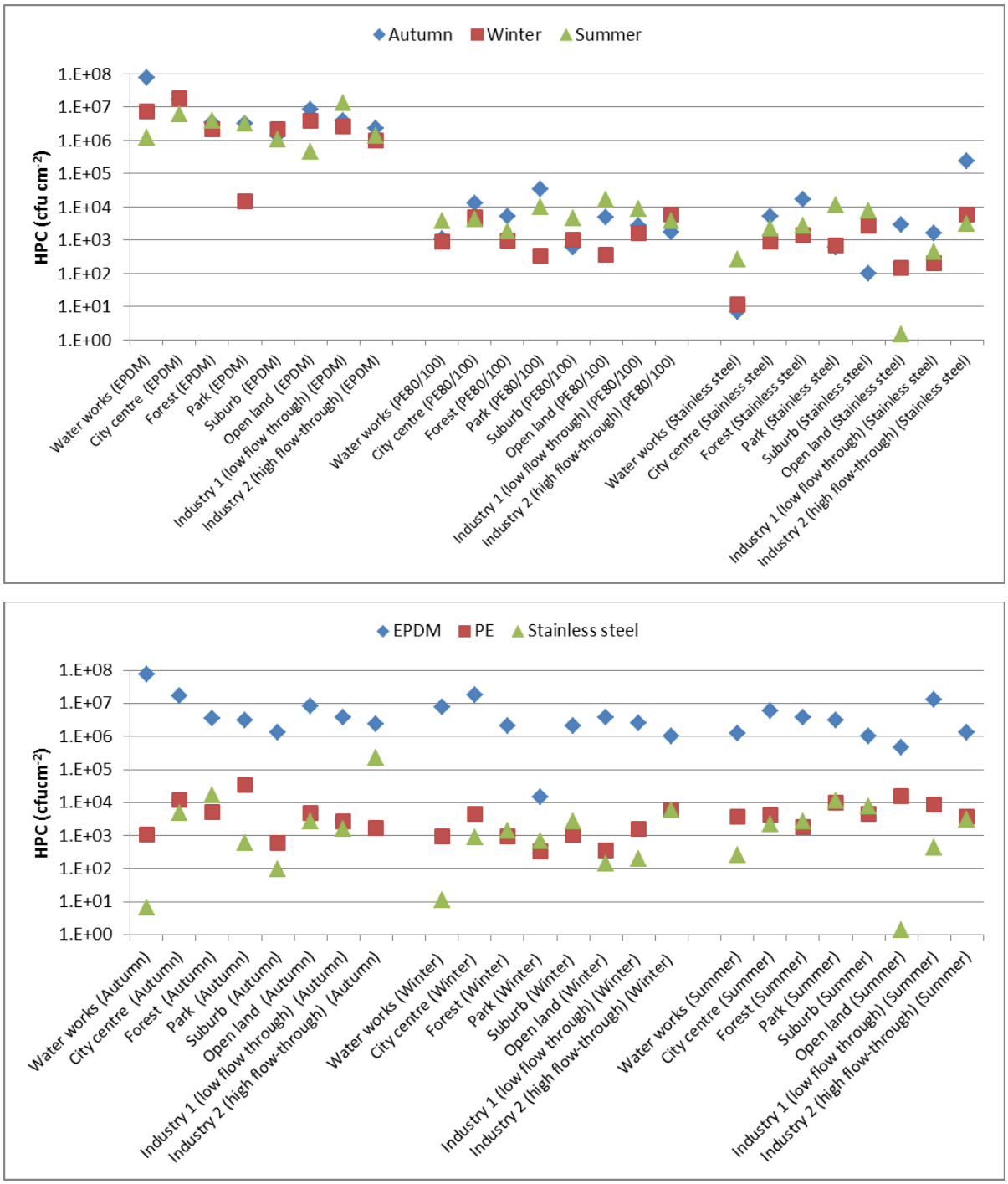


Figure 12: HPC for EPDM (A), for PE80/100 (B), and for stainless steel (C).

As well as the total cell count and the HCP, the colony counts at 20 °C did not show any significant temperature dependency (Figure 13). However, the number of cfu was higher on EPDM than on PE80/100 and stainless steel. A more detailed look at the

particular climatopes showed very high variations in the numbers of the colony counts at 20 °C on EPDM ranging from 1 x 10^1 to 2 x 10^5 cfu cm^{-2} (Figure 13 (A)). In the climatopes open land and industry 2, colony counts were similar for all three sampling campaigns. The other cliamtopes showed high variations in the numbers of the colony counts up to three log units. However, these variations could not be associated with the drinking water temperature. On PE80/100, numbers of the colony counts ranged from 0 to 5 x 10^3 cfu cm^{-2}. In the climatope open land, the number of colony forming units was similar during all sampling campaigns and varied between 1.6 x 10^1 cfu cm^{-2} and 8.5 x 10^1 cfu cm^{-2}. In four out of eight climatopes (city centre, suburb, park, and industry 1), colony counts were highest in summer. In the water works and the forest, the season with the highest number of colony forming units was autumn when the exposition time was shortest. In the climatope industry 2 the highest number of colony forming units was detected in winter. On stainless steel, the colony forming units were within a range from 0 to 5 x 10^2 cfu cm^{-2}. In the water works, open land, and industry 1, colony forming units were similar during all sampling campaigns and no temperature effect was observed. In the climatopes suburb, park and forest, colony counts decreased in winter and were highest in summer. In the other climatopes, colony counts varied without an indication for temperature influence.

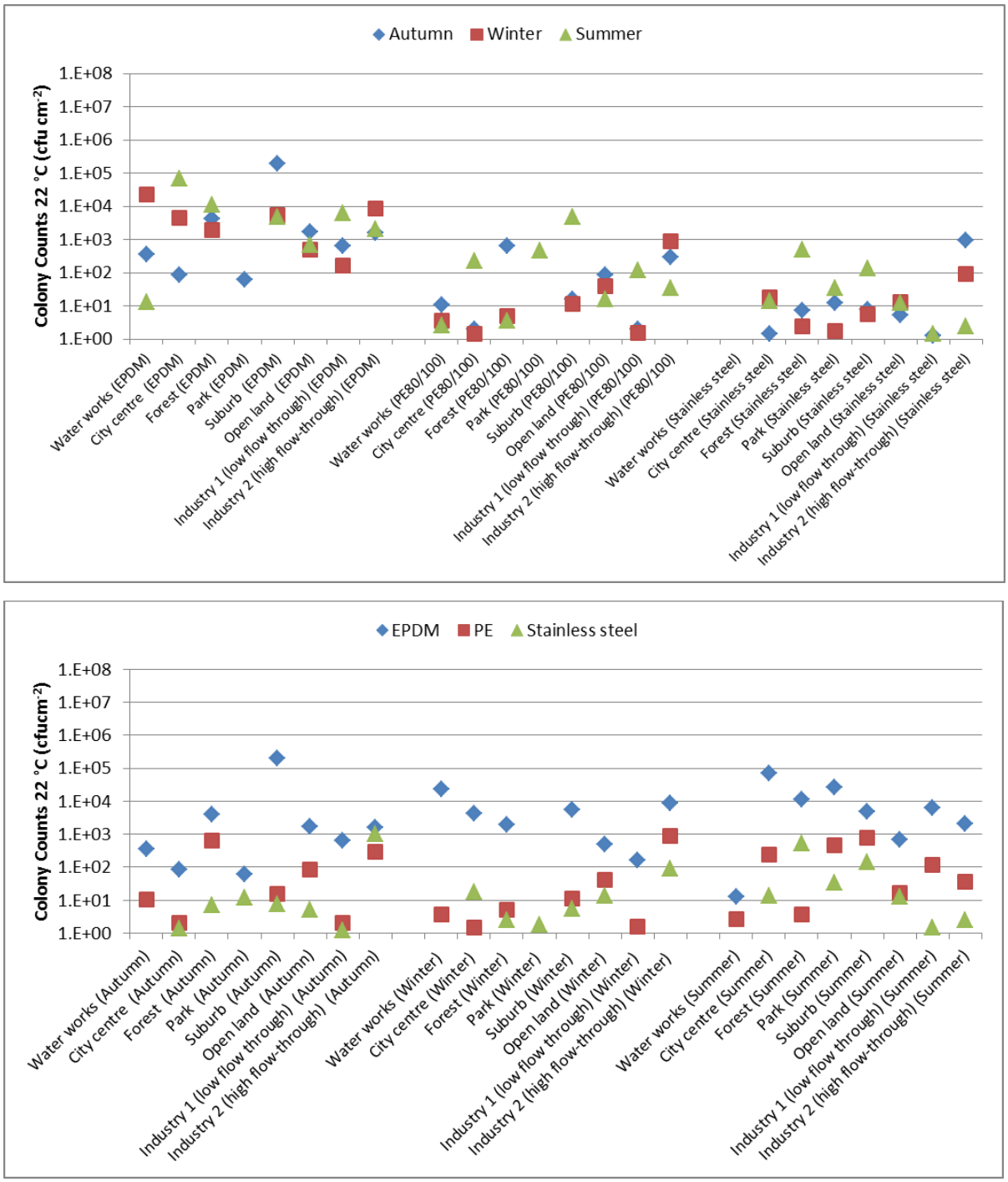


Figure 13: Colony Counts at 22 °C for all climatopes, sampling campaigns, and materials (A); for EPDM (B), for PE80/100 (C), and for stainless steel (D).

According to the German Drinking Water Ordinance, colony counts at 36 °C were determined in addition to the colony counts at 20 °C°. Variations in the numbers of colony forming units were highest on EPDM, followed by PE80/100 and stainless steel.

On EPDM, the number of colony forming units varied from 7 x 10^{-1} cfu cm^{-2} to 2 x 10^4 cfu cm^{-2}. Only in the climatope industry 2 colony counts were similar during all three sampling campaigns. In the climatopes city centre, forest, and park, colony counts were similar in autumn and winter and highest in summer. In the water works, the number of colony forming units was highest in autumn and decreased with increasing exposure time. Industry 1 was the only climatope in which colony counts increased with elevated temperatures. On PE80/100, colony counts were within the range of 0 cfu cm^{-2} to 2 x10^3 cfu cm^{-2}. In the climatopes water works, open land, and industry 2, the number of colony forming units was highest in sampling campaign 1 (autumn) and similar in winter and summer. In the climatopes city centre, park, suburb, and industry 1, colony counts at 36 °C were highest in summer and similar in autumn and winter. On stainless steel, the range of the colony counts was the same as for PE80/100. In the water works no colony forming units were detected in any of the sampling campaigns. In the climatopes open land, city centre, and suburb concentrations were similar for all sampling campaigns. In the climatopes forest and park numbers of colony forming units increased with increasing exposure time. Thus, colony counts were lowest in autumn and highest in summer. In the climatope industry 2, colony counts decreased with increasing exposure time and were lowest in summer and highest in autumn. The climatope industry 1 was the only climatope in which colony counts on stainless steel increased with increasing temperature and were highest in summer and lowest in winter.

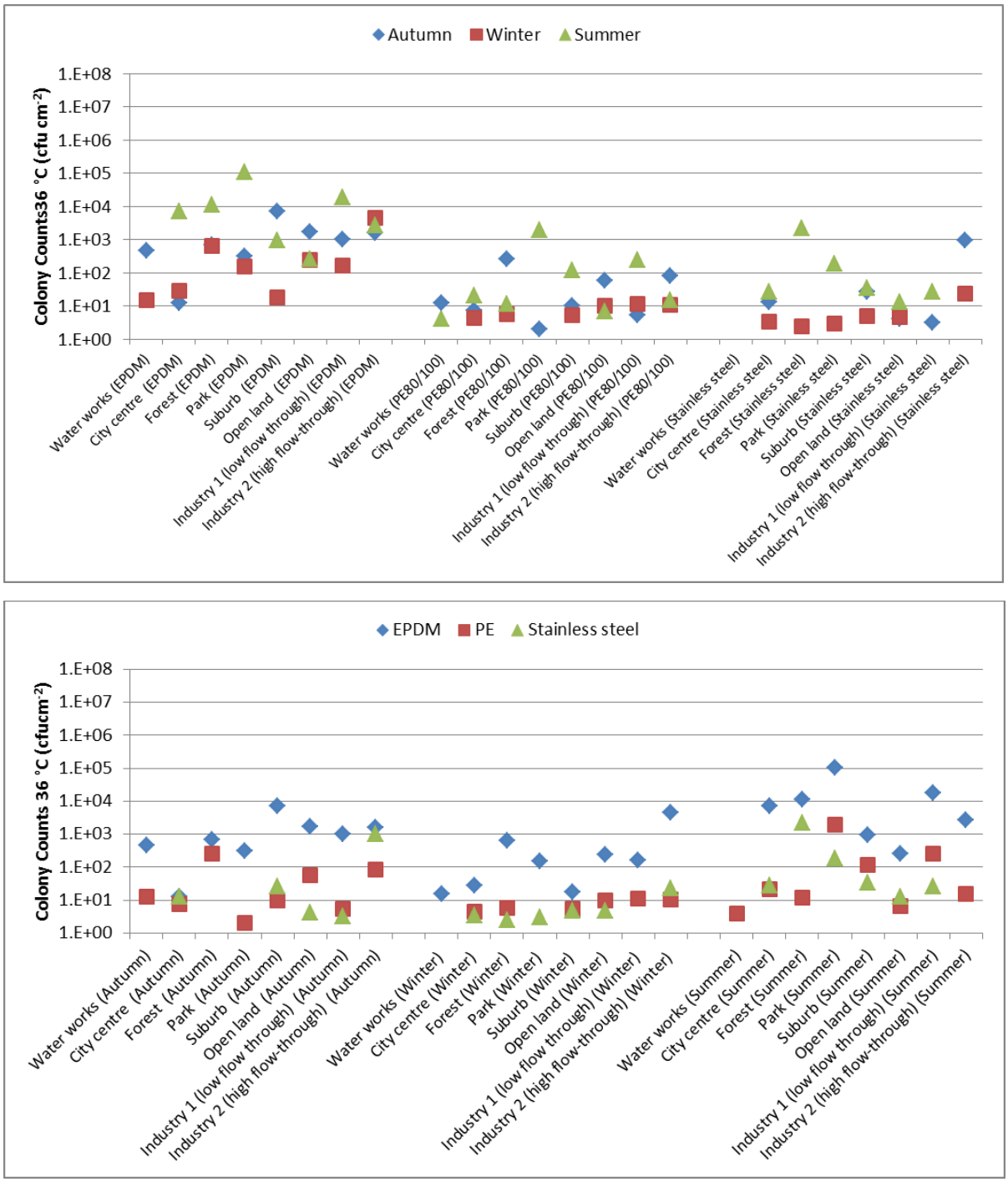


Figure 14: Colony Counts at 36 °C for EPDM (A), for PE80/100 (B), and for stainless steel (C).

4.1.2.2 Hygienically relevant bacteria in drinking water biofilms

In addition to total cell counts, HPC and colony counts at 20 °C and 36 °C, the occurrence of hygienically relevant bacteria in the drinking water biofilms was specifically analyzed by culture-based and culture independent methods. Target organisms were *E. coli* as an indicator for fecal contamination of drinking water, coliform bacteria and Enterococci as indicator for deficiencies in the process of drinking water treatment, and *P. aeruginosa*, *Legionella* spp., and Aeromonads being environmental pathogens of hygienically relevance.

Hygienically relevant bacteria were found in 18 % of all analyzed samples (n= 72). In seven samples, coliform bacteria (except *E. coli*) were detected, in three samples *Legionella* spp were present, in two samples *E. coli* and in one sample Enterococci were observed. Six of the 13 positive results originated from the climatope forest. As mentioned earlier (Table 10), this sampling site was a dead end pipe with low withdrawal of water.

Table 13: Hygienically relevant bacteria in the drinking water biofilms; cultural detection

Organism	**Cfu 100 mL^{-1}**
Coliform bacteria (*Citrobacter braakii/freundii, Enterobacter amnigenus, Enterobacter intermedius, Enterobacter chloacae*)	0.1 to 31 cfu/100 mL
E. coli	1 cfu/100 mL
Enterococci (*Enterococcus faecium*)	0.2 cfu/100 mL
L. pneumophila	3 to 29 cfu/100 mL

For the coliform bacteria, the number of positive samples, based on colony forming units, increased slightly with elevated temperatures (from 4 % in winter to 17 % in summer). The diversity of culturally detectable hygienically relevant bacteria was highest

in autumn when the exposition time was shortest. Here, four different species were found. In summer and winter only coliform bacteria could be detected (Figure 15).

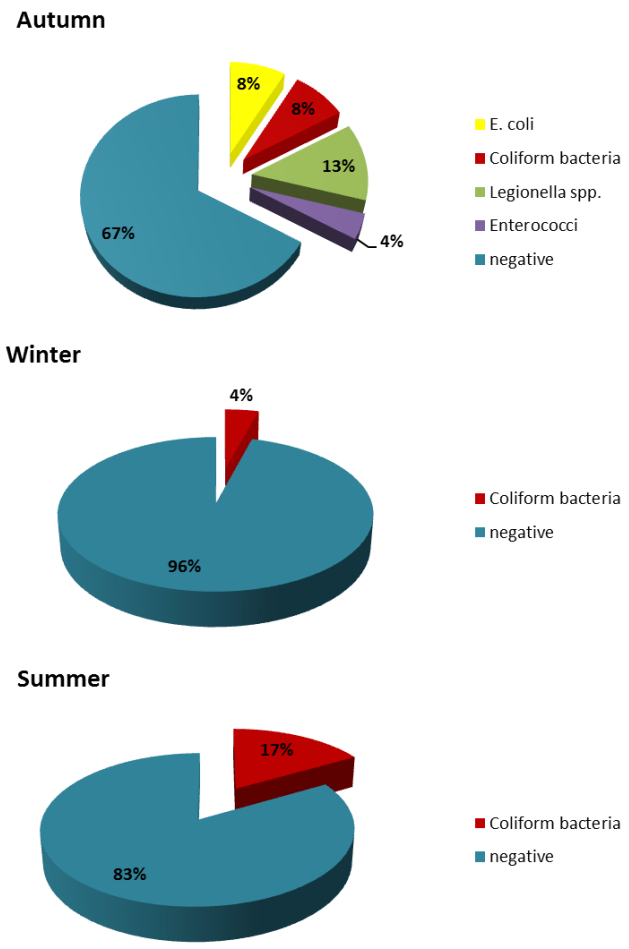


Figure 15: Season-dependent occurrence of hygienically relevant bacteria in drinking water biofilms.

To detect bacteria possibly present in the VBNC-state, FISH was performed in addition to the cultural methods. Target organisms were *E. coli*, *P. aeruginosa*, *Legionella* spp., and *L. pneumophila*. 24 biofilm samples per material (EPDM, PE80/100, stainless steel) were analyzed.

Independent of material and season, *Legionella* spp. was detected in almost all analyzed drinking water biofilms (97 %). FISH-positive *P. aeruginosa* were present in 74 % of all

analyzed samples. In 63 % of the drinking water biofilms *L. pneumophila* could be detected. *E. coli* occurred significantly less often compared with the other target organisms and was detected in one third of all samples. To encompass a possible influence of temperature on the occurrence of FISH-positive target organisms, the frequencies of detection were determined in dependency of season and material (Table 14). The occurrence of FISH-positive *E. coli* increased with elevated temperatures (25 % to 63 %) in biofilms on PE80/100 and with elevated exposition time (12.5 % to 87.5 %) on EPDM. Thus, detection frequencies were highest in summer on both materials. In contrast to *E. coli*, the presence of FISH-positive *P. aeruginosa* was lowest in summer either on PE80/100 and EPDM. In autumn, FISH-positive *P. aeruginosa* were found in all samples on both materials. *Legionella* spp. was found in 100 % of all analyzed samples with one exception (stainless steel, autumn). FISH-positive *L. pneumophila* were detected more frequently in winter than in autumn and summer on EPDM and stainless steel. On PE80/100, frequencies were the same during all sampling campaigns. Except for *L. pneumophila*, detection frequencies of the other target organisms were significantly lower on stainless steel (Table 14).

Table 14: occurrence of the target organisms in drinking water biofilms determined by FISH

Frequency of occurrence (%)						
Material	*E. coli*			*P. aeruginosa*		
	Autumn	Winter	Summer	Autumn	Winter	Summer
EPDM	12,5	75	87,5	100	87,5	62,5
PE80/100	37,5	25	62,5	100	100	75
Stainess steel	12,5	0	12,5	37,5	50	37,5
Frequency of occurrence (%)						
Material	*Legionella* spp.			*L. pneumophila*		
	Autumn	Winter	Summer	Autumn	Winter	Summer
EPDM	100	100	100	37,5	100	87,5
PE80/100	100	100	100	75	75	75
Stainless steel	75	100	100	37,5	57	12,5

In Figure 16, the number of FISH-positive *E. coli* within the drinking water biofilms either on different materials or in different seasons in dependence of the total cell count is depicted. In all seasons the number of FISH-positive *E. coli* slightly increased with increasing total cell counts. A positive correlation between the total cell count and the number of FISH-positive *E. coli* within the drinking water biofilms was also observed for the different materials. Moreover, a significant effect of the material on the total cell count and the number of FISH-positive *E. coli* was observed. On EPDM both parameters were (one to two log units higher compared to PE80/100 and stainless steel.

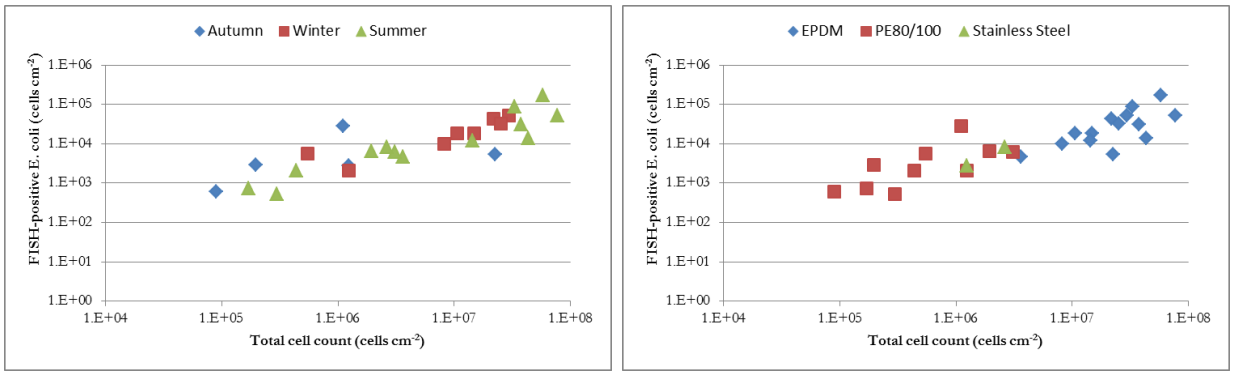


Figure 16: FISH-positive *E. coli* in the drinking water biofilms in dependence of a: season (autumn, winter, summer); b: material (PE80, EPDM, stainless steel).

For *Legionella* spp., the number of FISH-positive cells increased with increasing total cell counts in summer and in autumn. In winter, this trend was not as significant as in the other two seasons (Figure 17). Regarding the different materials, it was observed, that with increasing total cell counts the number of FISH-positive *Legionella* spp. increased on stainless steel and EPDM but not on PE80/100. A significant influence of the material was observed on numbers of FISH-positive *Legionella* spp. and total cell counts. On EPDM both parameters were higher compared to PE80/100 and stainless steel. Results were similar for *L. pneumophila* and for *P. aeruginosa*, respectively (Figure 18; Figure 19).

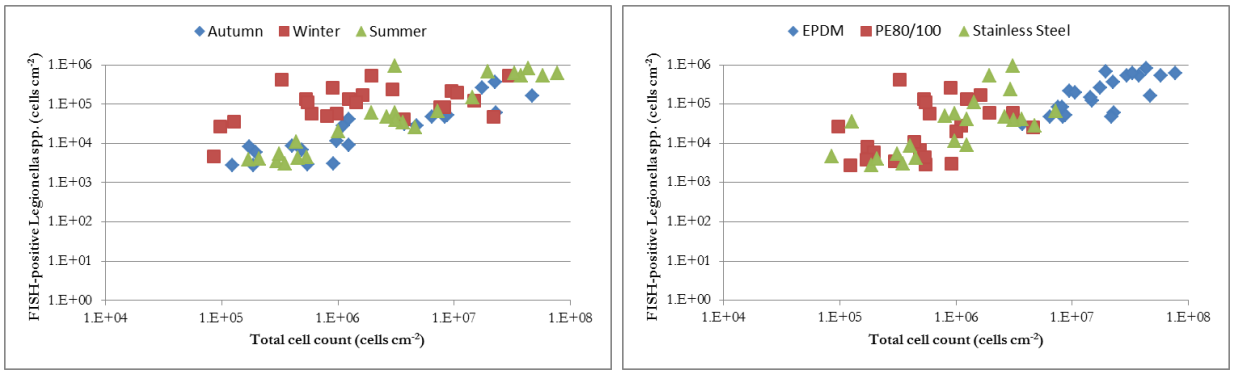


Figure 17: FISH-positive *Legionella* spp. in the drinking water biofilms in dependence of a: season (autumn, winter, summer); b: material (PE80, EPDM, stainless steel).

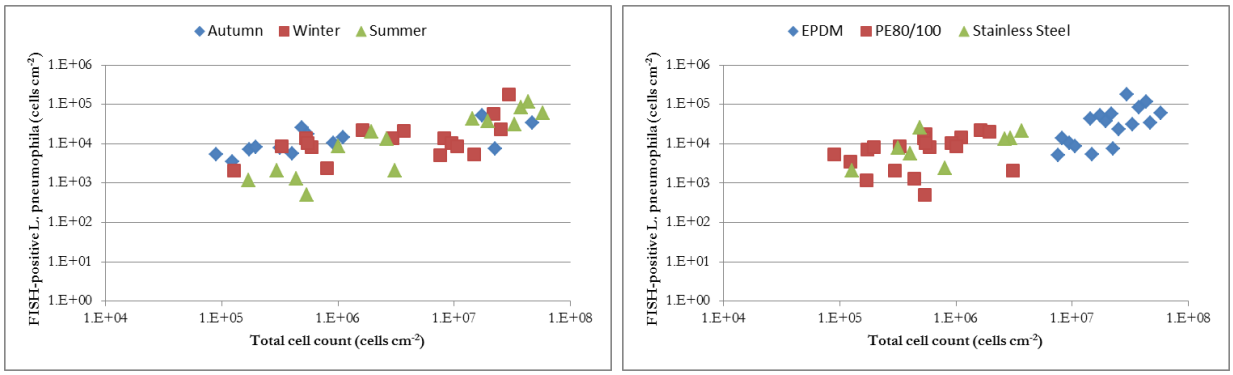


Figure 18: FISH-positive *L. pneumophila* in the drinking water biofilms in dependence of a: season (autumn, winter, summer); b: material (PE80, EPDM, stainless steel).

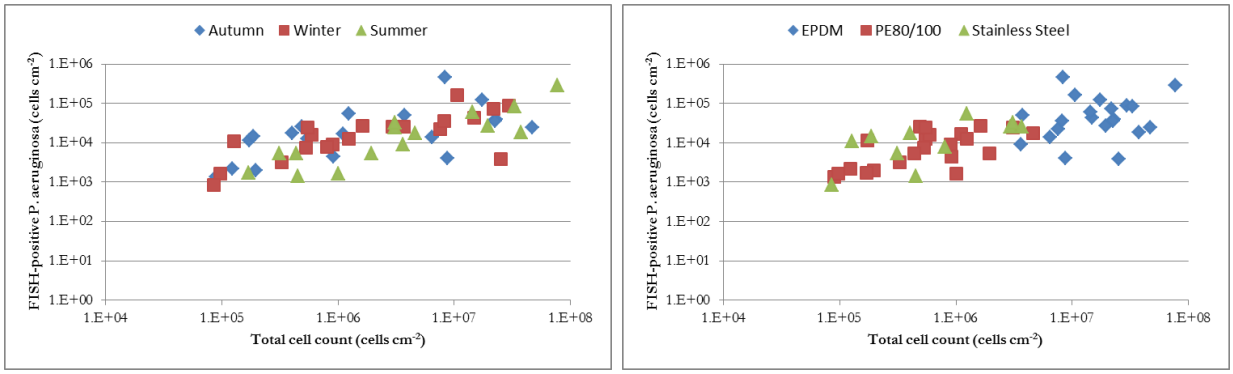


Figure 19: FISH-positive *P. aeruginosa* in the drinking water biofilms in dependence of a: season (autumn, winter, summer); b: material (PE80, EPDM, stainless steel).

It was also investigated if drinking water temperature influenced not only the detection freqency, but also the numbers of FISH-positive target organisms in the drinking water biofilms. Figure 20 shows the numbers of all target organisms for each material, season and climatope. The highest numbers of FISH-positive *E. coli* were detected on EPDM in each climatope. Numbers on PE80/100 and stainless steel were about 0.5 to 1.5 log units below. In five out of eight climatopes, the concentration of FISH-positive *E. coli* was highest in summer. On PE80/100 and stainless steel, this trend was not observed. No influence of drinking water temperature on the concentration of FISH-positive *P. aeruginosa* was detected. However, the material slightly influenced the number of FISH-positive *P. aeruginosa*. Higher numbers were found on EPDM than on PE80/100 and stainless steel in most climatopes which is not surprising as EPDM is known to be

a growth supporting material. For *Legionella* spp., a trend of increasing numbers of FISH-positive cells associated with elevated temperatures was observed on EPDM. In six out of eight climatopes, numbers were highest in summer. On PE80/100, most FISH-positive cells were detected in winter (seven out of eight climatopes). On stainless steel, no influence of drinking water temperature on the concentration of FISH-positive *Legionella* spp. was observed. For *L. pneumophila,* no influence of temperature on the number of FISH-positve cells was detected on any of the material. Concentrations were similar on EPDM and PE80/100.

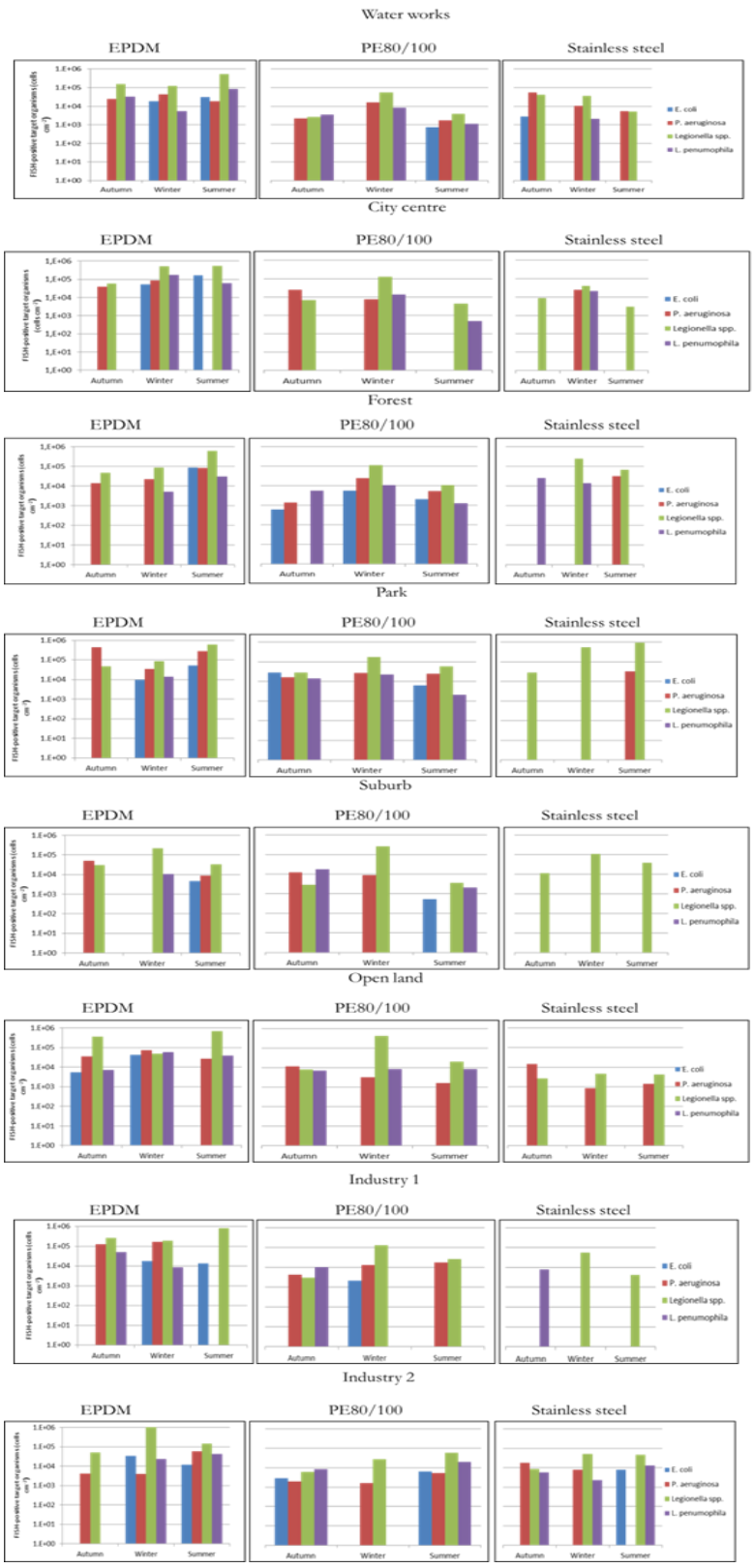


Figure 20: Numbers of FISH-positive *E. coli*, *P. aeruginosa*, *Legionella* spp., and *L.pneumophila* on various materials exposed to various climatopes.

4.1.2.3 Denaturing Gradient Gel Electrophoresis

To further characterize the influence of temperature on drinking water biofilms, population analysis of biofilms grown on EPDM was performed by means of denaturing gradient gel electrophoresis (DGGE). This method allows the characterization of the genotypic composition of a microbial community. Differentiation between the various organisms is based on the detection of the 16S rDNA which is ubiquitously present in bacteria but differs organism-dependent. The different 16S rDNA fragments were amplified by PCR followed by separation in gels based on their different denaturation behavior due to their different guanine/cytosine content. The generated band patterns were visualized using GelRed and represent the fingerprint of each microbial community. By comparison of the band patterns, similarities or shifts in the biofilm populations can be detected. In the field experiments, biofilms grown on EPDM in eight different climatopes (city centre, forest, open land, industry 1, industry 2, water works, suburb,park) in three sampling campaigns (autumn, winter, summer) were analyzed (Figure 21).

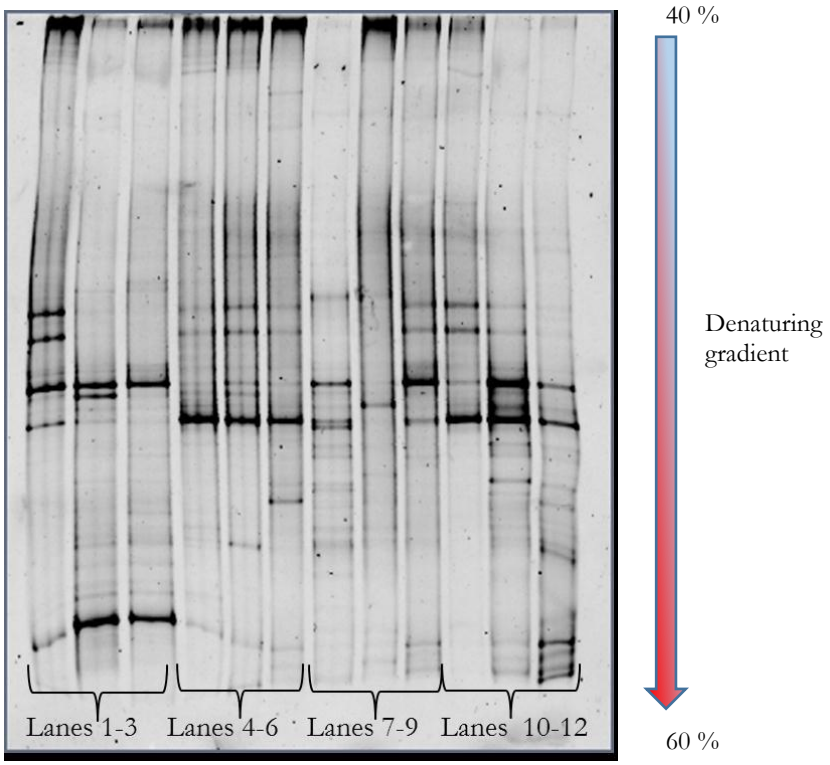


Figure 21: DGGE band patterns of drinking water biofilms isolated from different climatopes: Lanes 1-3: City centre (autumn, winter, summer); lanes 4-6: suburb (autumn, winter, summer); lanes 7-9: park (autumn, winter, summer); lanes 10-12: forest (autumn, winter, summer).

Taking into account each climatope in particular, the population analysis reveals similarities between 51 % and 98 %. The mean similarity of the biofilm populations was 76 % comparing summer and winter, 66 % comparing the biofilm populations between summer and autumn and 84 % of the biofilm populations were the same in autumn and winter (Table 16). The biofilm populations were most similar when both, the difference in temperature and the difference in exposure time were lowest. However, similarities of the biofilm populations were only 8 % less at the highest temperature difference, indicating, that temperature only marginally affects the biofilm community.

Table 15: Similarities of biofilm populations of the single climatopes in different seasons calculated using the Sørensen Index

Climatope	**Season**	**No. of bands**	**No. of similar bands between the seasons**		**Similarity in %**	
			Winter	Summer	Winter	Summer
Water works	Autumn	18	18	12	68	51
Water works	Winter	35	-	19	-	91
Water works	Summer	29	-	-	-	-
Open land	Autumn	25	21	21	58	84
Open land	Winter	47	-	29	-	81
Open land	Summer	25	-	-	-	-
Suburb	Autumn	38	26	21	78	59
Suburb	Winter	29	-	20	-	65
Suburb	Summer	33	-	-	-	-
City Centre	Autumn	33	22	22	70	71
City Centre	Winter	30	-	29	-	98
City Centre	Summer	29	-	-	-	-
Industry 2	Autumn	26	24	17	98	63
Industry 2	Winter	23	-	21	-	82
Industry 2	Summer	28	-	-	-	-
Industry 1	Autumn	25	21	16	79	64
Industry 1	Winter	28	-	25	-	94
Industry 1	Summer	25	-	-	-	-
Park	Autumn	27	18	17	72	64
Park	Winter	23	-	18	-	74
Park	Summer	26	-	-	-	-
Forest	Autumn	20	19	17	84	74
Forest	Winter	25	-	22	-	86
Forest	Summer	26	-	-	-	-

Table 16: Mean similarities of biofilm populations of the single climatopes in different seasons.

Climatope	**Difference in Temperature(°C)**	**Mean Similarity of the biofilm population (%)**
Summer/Winter	**10.7**	**76**
Summer/Autumn	**7.9**	**66**
Autumn/Winter	**2.9**	**84**

Taking into account all climatopes, no influence of temperature and/or exposition time was observed. Thus, the mean similarity of the biofilm populations was 46 % for each season comparison (Table 17). That means, biofilm populations in the same climatopes showed higher similarities than those between the various climatopes, although temperature differences were similar.

Table 17: Mean similarities of biofilm populations taking into account all climatopes

Climatope	**Difference Temperature(°C)**	**Mean Similarity of the biofilm population (%)**
Summer/Winter	10.5	46
Summer/Autumn	9.4	46
Autumn/Winter	3.1	46

Summary

Total cell counts, heterotrophic plate counts, and colony counts according to the German Drinking Water Ordinance were material dependent and showed higher cell densities on EPDM (EPDM> PE80/100> stainless steel). However, total cell counts of biofilms grown on EPDM, PE80/100, and stainless steel were mostly unaffected by temperature. In 18 % of the culturally analyzed drinking water biofilms, hygienically relevant bacteria were detected in low concentrations. For the coliform bacteria (except

E. coli) the trend of slightly increasing detection frequencies with elevated temperatures was observed.

With FISH, culturally not detectable hygienically relevant organisms were observed in all analyzed drinking water biofilms. The detection frequency of FISH-positive *E. coli* was temperature and material dependent and increased with elevated temperatures and on EPDM (EPDM> PE80/100 > stainless steel). The detection frequency of *P. aeruginosa* decreased with rising temperatures whereas the detection frequency of *Legionella* spp. was independent of season and material. However, the detection frequency of *L. pneumophila* was highest in winter. Therefore, the influence of temperature on the persistence of the target organisms possibly present in the VBNC state was strain dependent and did not follow a distinct pattern.

Similarities of the biofilm populations characterized by DGGE were higher within the single climatopes than those for all climatopes. Similarities of the biofilm populations of the particular climatopes were only marginally influenced by temperature and/or exposure time. Biofilm communities between the different climatopes did not show temperature dependency.

4.2 Laboratory experiments

In the laboratory experiments biofilms were grown on coupons of elastomeric (EPDM) and plastic (polyethylene PE80) material in modified RotoTorque annular reactors in at different temperatures (8 °C- 29 °C) in order to investigate the influence of temperature on the survival of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Legionella* spp., and *L. pneumophila* in drinking water biofilms. Continuous flow-through of the reactors was performed with UV-disinfected drinking water from a surface water treatment plant in Germany with low assimilable organic carbon (AOC: 6 µg/L acetate-C-equivalent), and, for nutrient rich conditions, water supplemented with tryptic soy broth (AOC: 150 µg/L acetate-C-equivalent). After 14 d of biofilm establishment, the reactors were inoculated with the

coliform indicator bacteria *E. coli* and *K. pneumoniae*, and separately with the environmental pathogens *P. aeruginosa* and *L. pneumophila* and operated for another 28 d.

4.2.1 Drinking water quality in influents and effluents

To investigate the influence of water temperature and biofilm formation on the quality of drinking water, the parameters total cell counts, colony counts regarding the German drinking water ordinance, and the HPC of the influents and effluents of the annular reactors were investigated in the laboratory experiments at various temperatures (8°C bis 29 °C). The water quality in the influents was not affected by water temperature. Total cell counts at all temperatures were comparable and ranged about 10^5 cells independent of the temperature. Moreover, water temperature influenced neither colony counts nor numbers of the HPC. Colony counts at 20 °C and 36 °C ranged from 0 to 10^1 cfu at 20 °C and 10^2 at 36 °C. The HPC varied between 1.5x 10^1 and 10^3. No distinct effect of temperature was observed.

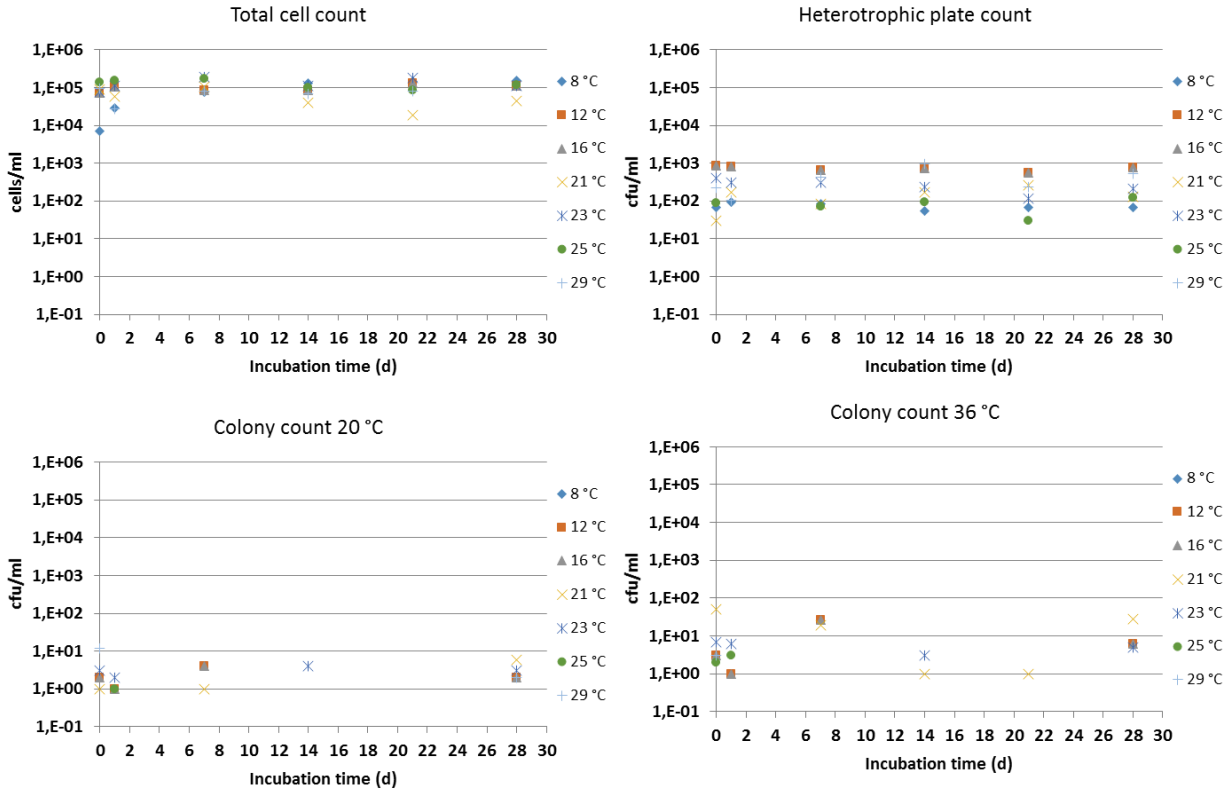


Figure 22: Total cell counts, HPC, and colony counts in the influent of the annular reactors

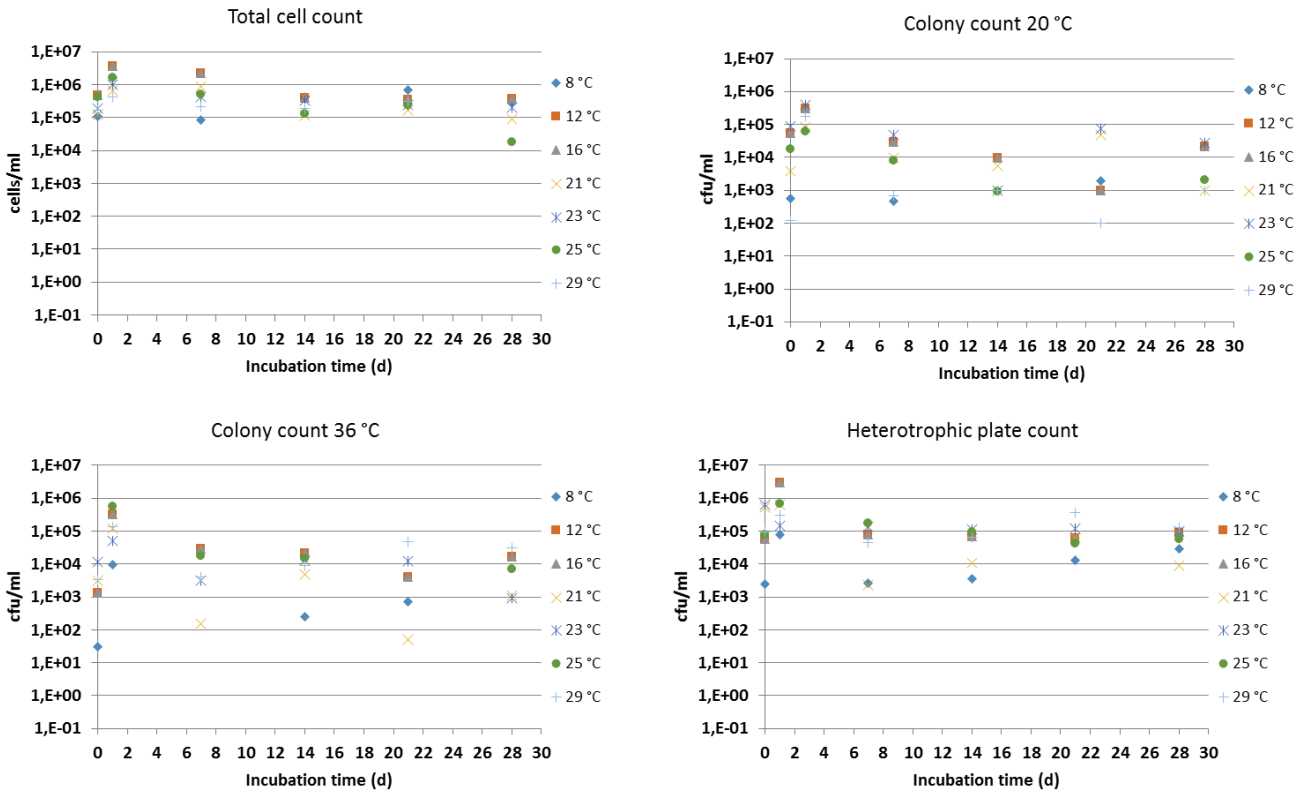


Figure 23: Total cell counts, HPC, and colony counts in the effluents of the annular reactors inoculated with *P. aeruginosa* and *L. pneumophila* under oligotrphic conditions.

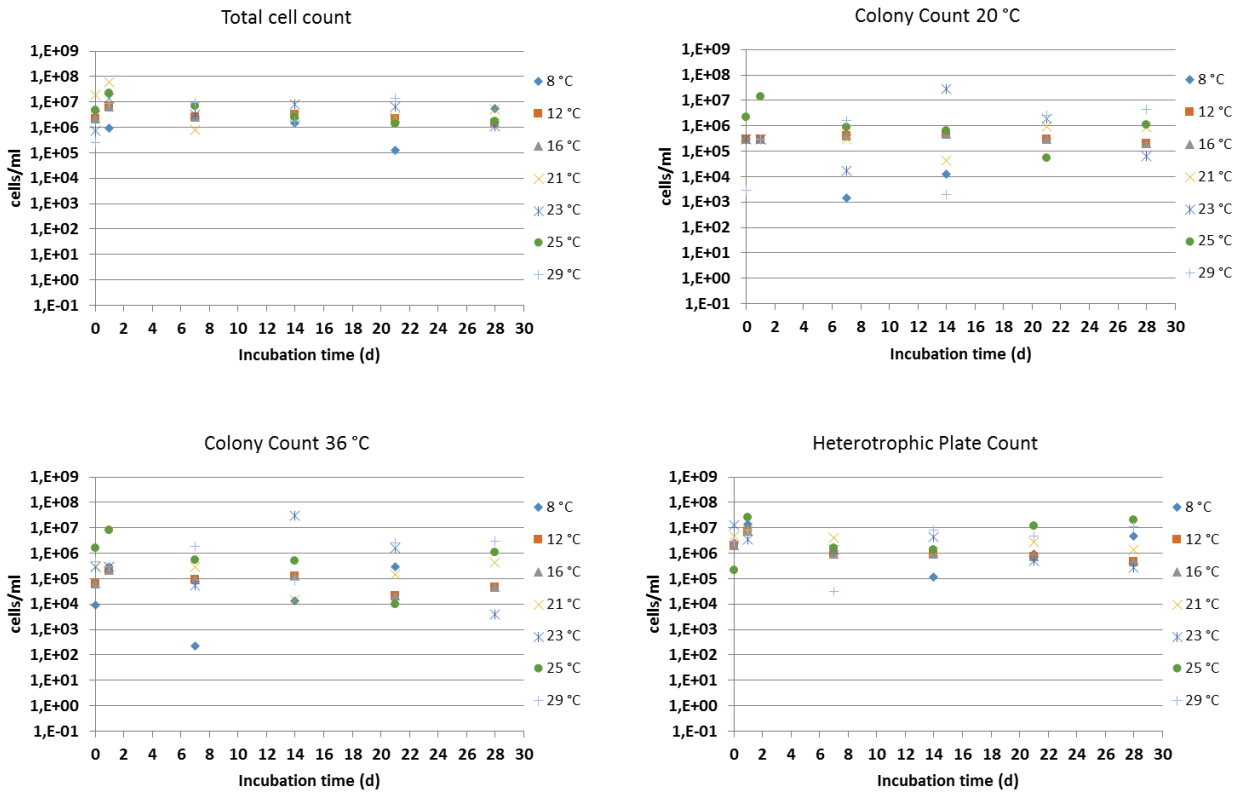


Figure 24: Total cell counts, HPC, and colony counts in the effluents of the annular reactors inoculated with *P.aeruginosa* and *L.pneumophila* under copiotrophic conditions.

4.2.2 Biofilm formation at various temperatures

Total cell counts

Total cell counts of the biofilms grown on PE80 and EPDM were only marginally influenced by temperature. For most of the investigated temperatures, total cell counts already reached maximum cell densities after the 14 d of biofim establishment and varied only slightly during the 28 d of operation. Only on PE80, cell densities showed higher variations under oligotrophic conditions (Figure 25). The total cell count slightly decreased of about 1.5 log units after 21 days of incubation at 21 °C while at 27 °C cell densities increased of about 2 log units during the 28 days after inoculation. The coupon material influenced biofilm formation. Total cell counts on EPDM were about one to two log units higher than on PE80 under oligotrophic conditions. Nutrient addition

significantly enhanced biofilm formation on both materials. While under oligotropic conditions total cell counts on PE80 varied between 3.6 x 10^4 cfu cm^{-2} and 4.8 x 10^7 cfu cm^{-2} and between 2.3 x 10^6 cfu cm^{-2} and 9.9 x10^7 cfu cm^{-2} on EPDM, total cell counts were about 2 log units higher under copiotrophic conditions on both materials.

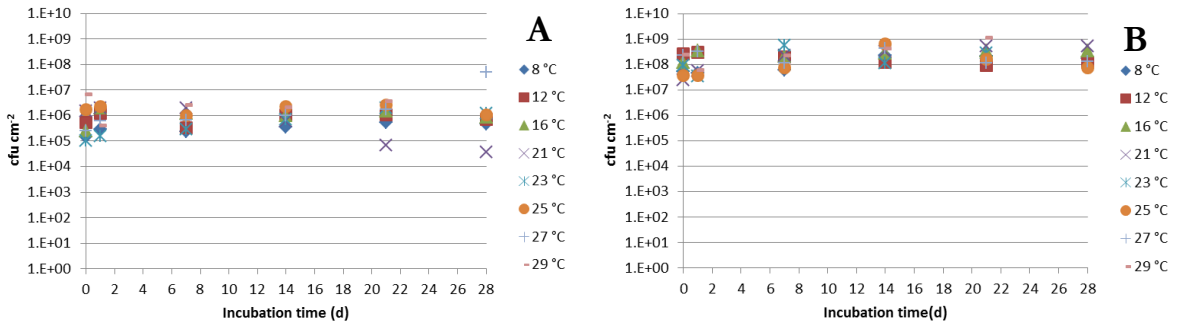


Figure 25: Total cell counts on PE8: (A) oligotrophic conditions, (B) copiotrophic conditions

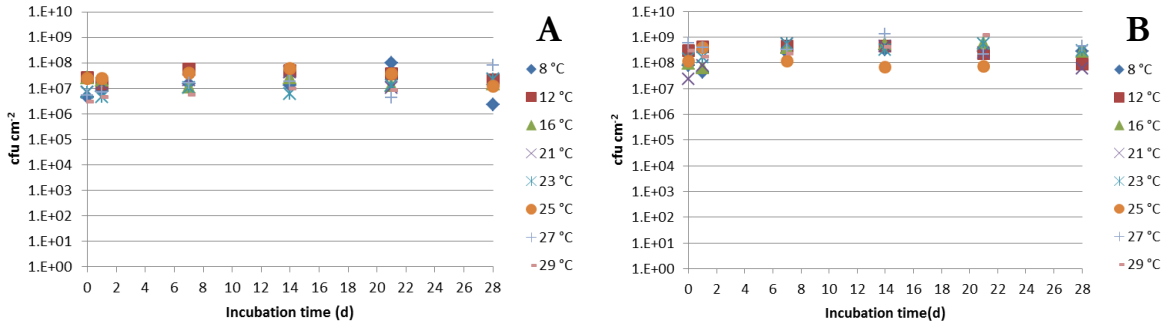


Figure 26: Total cell counts on EPDM: (A) oligotrophic conditions, (B) copiotrophic conditions

HPC

The HPC were also not significantly affected by temperature and were within a range of 10^4 cfu cm^{-2} to 10^6 cfu cm^{-2} on PE80 under oligotrophic conditions. However, at 21 °C colony counts were lowest and varied between 3.4 x10^3 cfu $cm^{-2}$and 8.9 x 10^4 cfu cm^{-2}. Another exception was detected at 27 °C. The HPC significantly increased of about four log units after 28 d of incubation. This trend was not observed for the other temperatures. When nutrients were added, the HPC was at least two log units higher for all temperatures compared to oligotrophic conditions. In contrast to the oligotrophic conditions, colony counts were highest at 21 °C. However, no distinct influence of

temperature was observed on PE80 either under copiotrophic conditions and the availability of nutrients had major influence on biofilm formation.

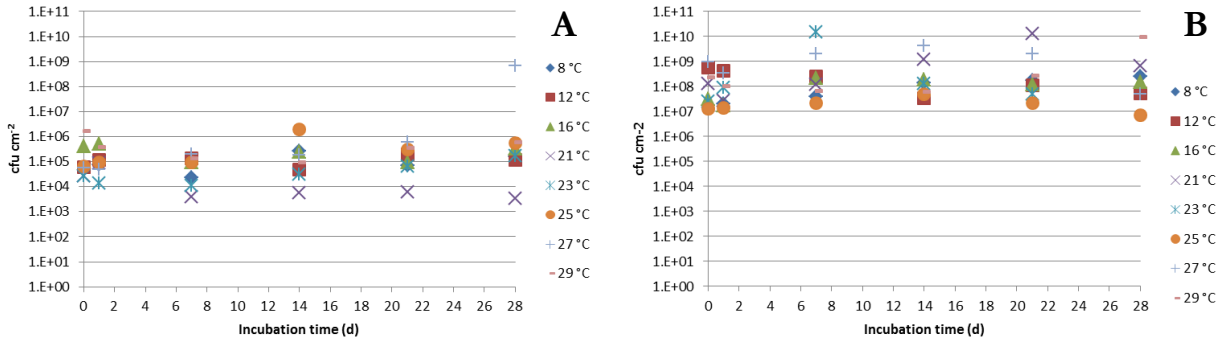


Figure 27: HPC on PE80: (A) oligotrophic conditions, (B) copiotrophic conditions

On EPDM, the HPC varied between 10^5 cfu cm^{-2} and 10^7 cfu cm^{-2} prior to inoculation of the target organisms and between 10^5 cfu cm^{-2} and 10^8 cfu cm^{-2} at the end of the experiment under oligotrophic conditions. At 23 °C an increase of about two log units was observed 24 h after inoculation. Moreover, the HPC was highest at 23 °C during the complete period of the experiment. Under copiotrophic conditions, the increase of the HPC of about two log units after inoculations was also observed at 23 °C. Here, the HPC even increased of about three log units after seven days of incubation. However, a decrease of about 2.5 log units occurred after 14 days of incubation. At 27 °C the HPC increased of about 1.5 log units from T_0 to $T_{14\ days}$ and then decreased of about 2.5 log until the end of the experiment. At 21 °C an increase of about three log units was observed until 21 days of incubation followed by a slight decrease of about one log unit. As already observed for the total cell counts, the material influenced the HPC which was higher on EPDM than on PE80.

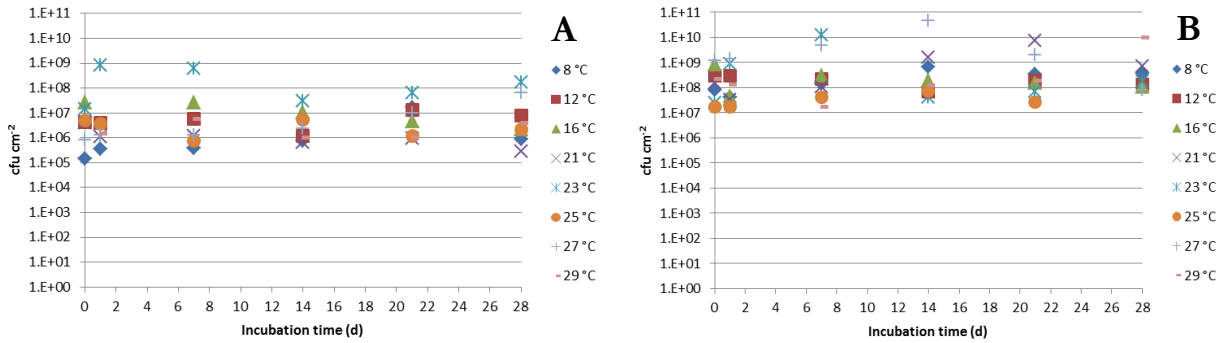


Figure 28: HPC on EPDM: (A) oligotrophic conditions, (B) copiotrophic conditions

Colony Counts

No significant influence of temperature on the colony counts at 22 °C was observed under oligotrophic conditions. Colony counts were highest at 12 °C and lowest at 21 °C. Conspicuous was a significant increase of about three log units of the number of cfu at 25 °C directly after inoculation of the target organisms. Compared to the total cell counts and the HPC, the colony counts at 22 °C showed the highest variation between the different temperatures ranging from 2.5 10^2 cfu cm^{-2} to 1 10^5 cfu cm^{-2} prior to inoculation with the target organisms and 10^1 cfu cm^{-2} to 10^7 cfu cm^{-2} at the end of the experiment. Under copiotrophic conditions, numbers of the cfu were significantly lower at 21 °C compared to the cfu at the other temperatures whereas the variations between the other investigated temperatures were not as high as under oligotrophic conditions. Moreover, an increase in the number of colony forming units of about two log units was observed at 8 °C and 16 °C from T_0 to $T_{14\ days}$.

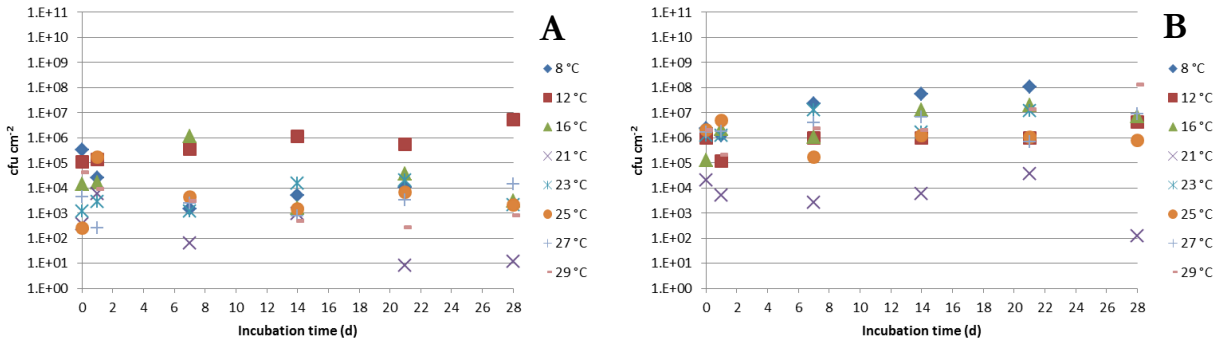


Figure 29: Colony Counts (22°C) on PE80: (A) oligotrophic conditions, (B) copiotrophic conditions

On EPDM, results were similar. The variations between the different temperatures were higher under oligotrophic conditions than under copiotrophic conditions. However, no distinct influence of temperature was observed. As already reported for the total cell counts and the HPC, the addition of nutrients also had a promoting effect on the formation of cfu at 22 °C both, on PE80 and on EPDM.

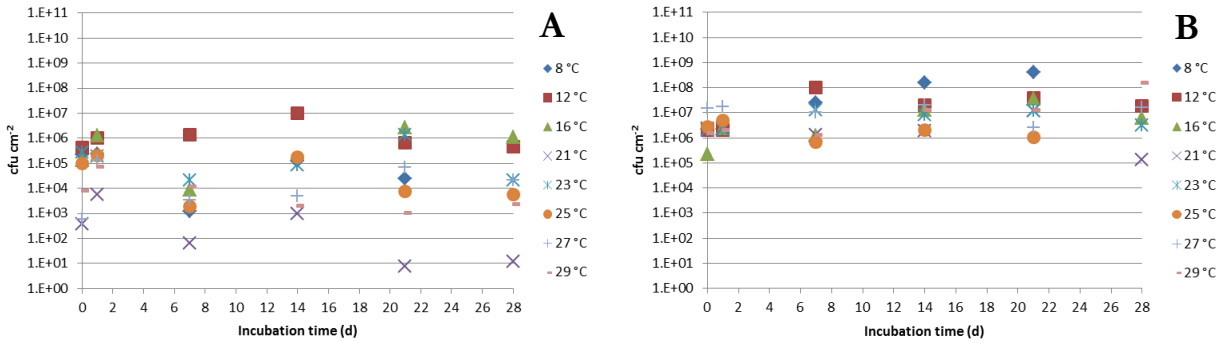


Figure 30: Colony Counts (22°C) on EPDM: (A) oligotrophic conditions, (B) copiotrophic conditions

Colony counts at 36 °C were also ambiguously influenced by temperature on PE80 under oligotrophic conditions. However, a significant decrease of the number of cfu was detected 1 day after inoculation with the target organisms at 8 °C whereas the converse effect occurred at 25 °C. Under copiotrophic conditions, variations between the different temperatures were smaller than under copiotrophic conditions as already detected for the colony counts at 22 °C. No significant effect of temperature on the numbers of the cfu was observed also under copiotrophic conditions.

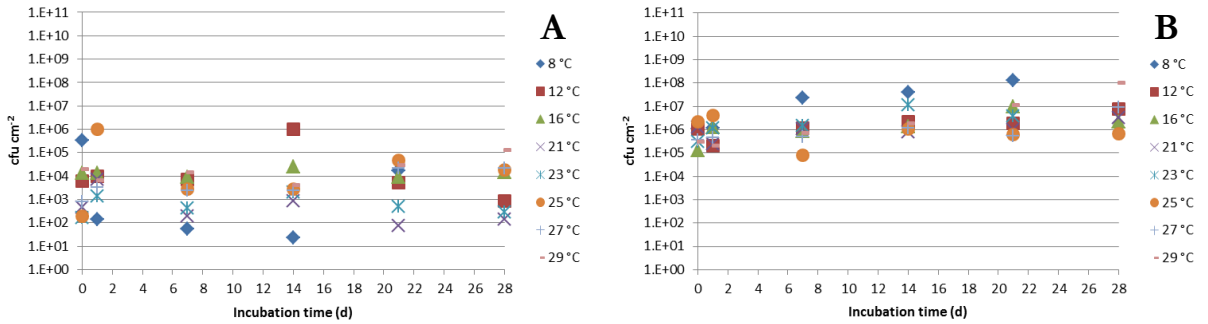


Figure 31: Colony Counts (36 °C) on PE under oligotrophic conditions (A) and under copiotrophic conditions (B)

Results on EPDM were similar to those on PE80. No significant influence of temperature was observed on the colony counts at 36 °C. Variations between the different temperatures slightly decreased when nutrients were added.

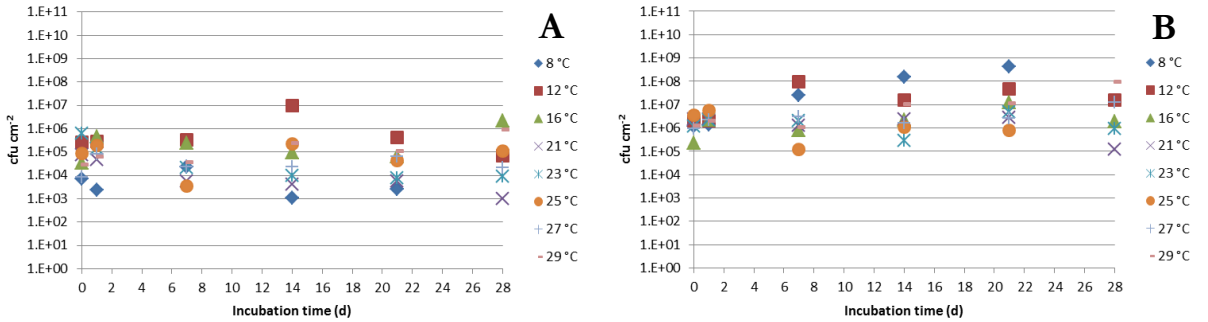


Figure 32: Colony Counts (36 °C) on PE: (A) oligotrophic conditions, (B) copiotrophic conditions

Summary

Temperature only marginally influenced the formation of drinking water biofilms in this study. Total cell counts, HPC and colony counts regarding the German drinking water ordinance were hardly affected by the different temperatures. However, nutrient availability significantly influenced the biofilm formation in the annular reactors. The addition of nutrients always resulted in elevated total cell counts, HPC, and colony counts. Moreover, the material had an effect on the biofilm formation. Total cell counts and HPC were higher on EPDM than on PE80.

4.2.3 Incorporation of hygienically relevant bacteria into drinking water biofilms at various temperatures

To investigate the ability of hygienically relevant bacteria to integrate into drinking water biofilms, 14 day old drinking water biofilms grown on PE80 and EPDM in annular reactors were spiked with either a combination of the environmental bacteria *P. aeruginosa* and *L. pneumophila* or with a combination of the indicator bacteria *E. coli* and *K. pneumoniae*. Prior to inoculation the target organisms were incubated in sterile filtered drinking water from a copper-free pipe at room temperature for 24 h in order to adapt to drinking water conditions within the annular reactors. To evaluate the incorporation of the target organisms into the established biofilms, determination of the bacteria was conducted using cultural and culture-independent methods prior to inoculation and one day after incubation.

E. coli

Prior to inoculation, *E. coli* was not detected in the drinking water biofilms on PE80 under both oligotrophic and copiotrophic conditions. Regardless of temperature and nutrient availability, *E. coli* was capable of integrating into the existing biofilms (Figure 33). Numbers of bacteria determined with cultural methods one day after inoculation varied between 10^1 MPN cm^{-2} and 10^3 MPN cm^{-2} under oligotrophic conditions. MPNs decreased at temperatures above 21 °C. Under copiotrophic conditions this effect did not occur. No *E. coli* was detectable at 25 °C regardless of the nutrient situation. Moreover, incorporation of *E. coli* was only promoted by nutrient addition at temperatures above 21 °C. Total cell counts significantly increased with nutrient addition as already mentioned in section 0. Numbers of FISH-positive *E. coli* were about two to three log units higher than the culturally derived numbers. With nutrient addition, numbers of FISH-positive *E. coli* slightly increased on PE80 (Figure 33).

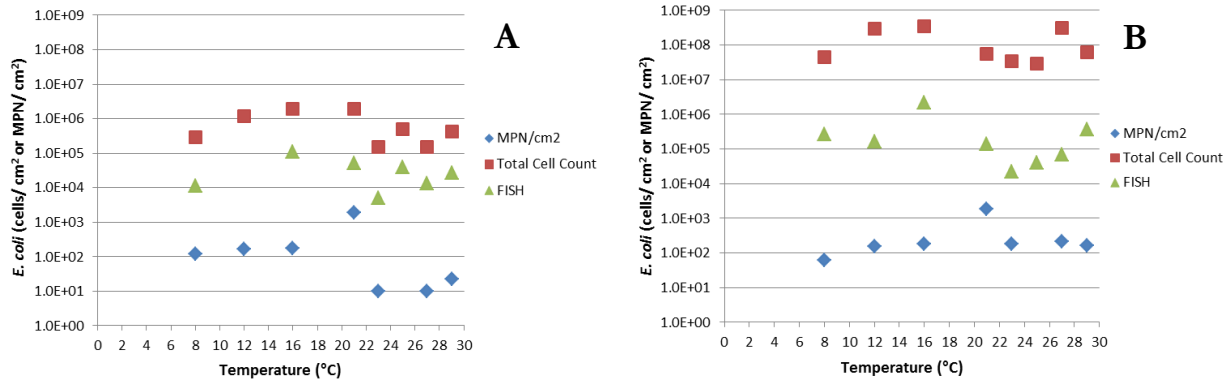


Figure 33: Incorporation of *E. coli* into a 14 days old drinking water biofilm grown on (A) oligotrophic conditions, (B) copiotrophic conditions

As well as on PE80, *E. coli* was not detected in biofilms grown on EPDM prior to inoculation. *E. coli* was able to integrate into the established biofilms on EPDM regardless of temperature and nutrient conditions (Figure 34). On EPDM, numbers of cultivable *E. coli* and FISH-positive *E. coli* were comparable to those detected on PE80 under copiotrophic conditions (Figure 33; Figure 34). Under oligotrophic conditions total cell counts were about one to two log units below the total cell counts when nutrients were added to the system. The significant decrease of cultivable *E. coli* at temperatures above 21 °C was also observed on EPDM under oligotrophic conditions. However, an increase in the number of *E. coli* was detected at 29 °C on EPDM whereas on PE80 concentrations remained almost constant (Figure 33; Figure 34). This effect was not detected on EPDM under copiotrophic conditions. Total cell counts were about one to two log units higher than under oligotrophic conditions when nutrients were added. However, no significant difference between the numbers of FISH-positive *E. coli* under oligotrophic and copiotrophic conditions was observed. As well as in the biofilms grown on PE80, no *E. coli* was detected culturally at 25 °C regardless of the nutrient situation.

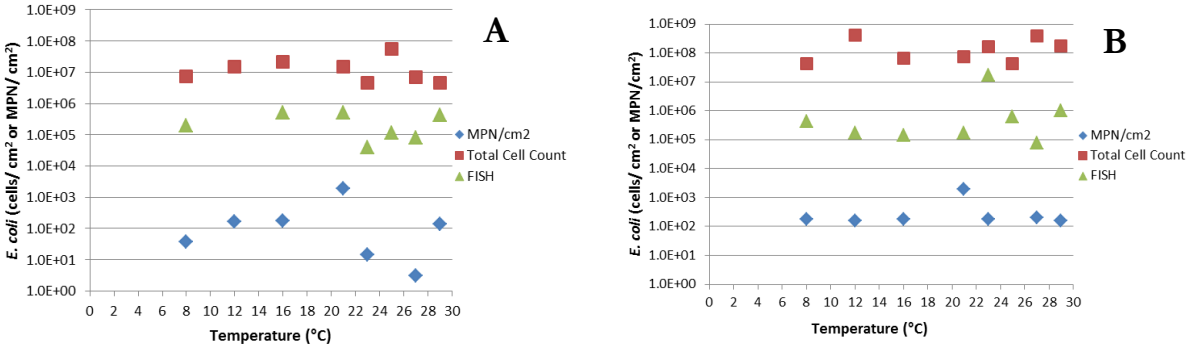


Figure 34: Incorporation of *E. coli* into a 14 days old drinking water biofilm grown on EPDM: (A) oligotrophic conditions, (B) copiotrophic conditions

In Figure 35, the incorporation of *E. coli* into the drinking water biofilms grown on different materials in dependence of the total cell count is depicted. The number of cultural *E. coli* within the drinking water biofilms one day after inoculation increased with increasing total cell counts on PE80 under oligotrophic conditions. When nutrients were added, this correlation was not observed. On EPDM this trend was not as distinct as on PE80. The number of FISH-positive *E. coli* within the established drinking water biofilms grown on PE80 under oligotrophic conditions correlated positively with the total cell counts. Higher total cell counts resulted in higher concentrations of FISH-positive *E. coli*. This trend was more significant on PE80 than on EPDM. Under copiotrophic conditions this association between the total cell counts and the incorporation of *E. coli* was not as distinct as under oligotrophic conditions.

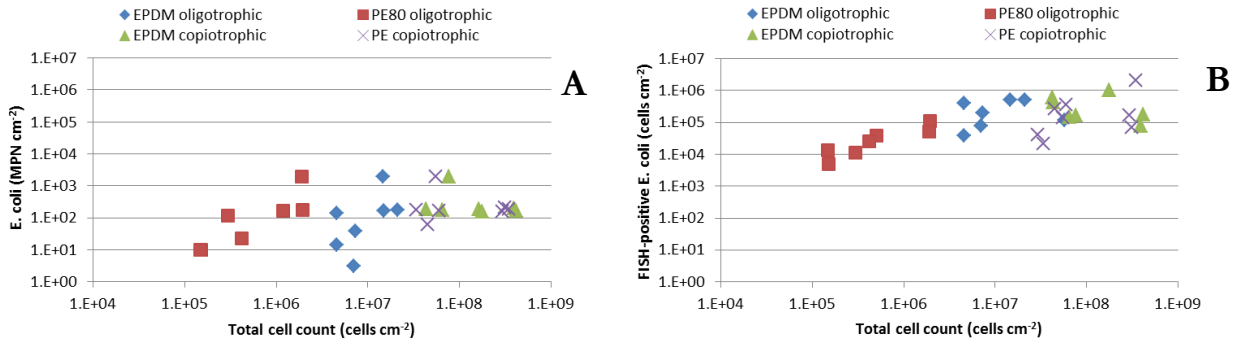


Figure 35: Incorporation of *E. coli* into a 14 days old drinking water biofilm grown on PE80 and EPDM: (A) oligotrophic conditions, (B) copiotrophic conditions

K. pneumoniae

Incorporation efficiency of *K. pneumoniae* was comparable to that of *E. coli* (Figure 36; Figure 37). A decrease of culturable *K. pneumoniae* was detected at temperatures above 21 °C on PE80 under oligotrophic conditions. Other than observed for *E. coli*, numbers of FISH-positive *K. pneumoniae* also decreased at 23 °C and 27 °C. However, at 25 °C and 29 °C this decrease in FISH-positive *K. pneumoniae* did not occur (Figure 36). The same effect was observed under copiotrophic conditions with FISH (Figure 37). Regarding the culturability, this effect was not as distinct as under oligotrophic conditions. Total cell counts were significantly higher under copiotrophic conditions compared to oligotrophic conditions. As also observed for *E. coli*, *K. pneumoniae* was not detected using cultural methods at 25 °C.

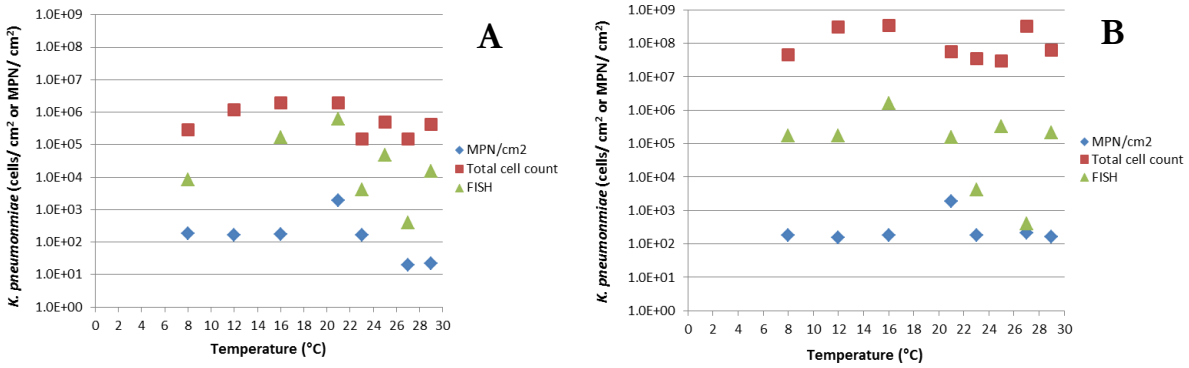


Figure 36: Incorporation of *K. pneumoniae* into a 14 days old drinking water biofilm grown on PE80: (A) oligotrophic conditions, (B) copiotrophic conditions

On EPDM the incorporation efficiency of *K. pneumoniae* was comparable to that on PE80 (Figure 37). There was also a significant decrease of culturability detected at temperatures above 21 °C under oligotrophic conditions. However, this effect was not observed using FISH as well as under copiotrophic conditions. Under copiotrophic conditions, a slight increase of FISH-positive *K. pneumoniae* was detected at temperatures above 21 °C with one exception at 27 °C. Total cell counts remained constant both under oligotrophic and copiotrophic conditions. However, nutrient addition resulted in an increase in the number of total cells of one to two log units.

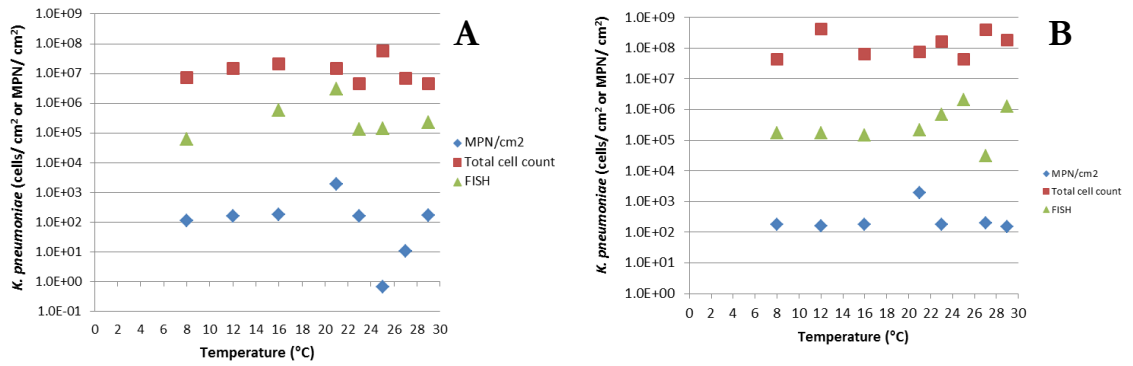


Figure 37: Incorporation of *K. pneumoniae* into a 14 days old drinking water biofilm grown on EPDM one day after incubation. (A) oligotrophic conditions, (B) copiotrophic conditions.

In Figure 38, the incorporation of *K. pneumoniae* into the drinking water biofilms grown on different materials in dependence of the total cell count is depicted. No correlation between the number of cultural *K. pneumoniae* one day after inoculation and the total cell counts was observed regardless of material and nutrient situation. The number of FISH-positive *K. pneumoniae* within the established drinking water biofilms grown on PE80 under oligotrophic conditions correlated positively with the total cell counts. Increasing total cell counts resulted in higher concentrations of FISH-positive *K. pneumoniae*. This trend was more significant on PE80 than on EPDM. Under copiotrophic conditions no influence of the total cell counts on the occurrence of FISH-positive *K. pneumoniae* was observed.

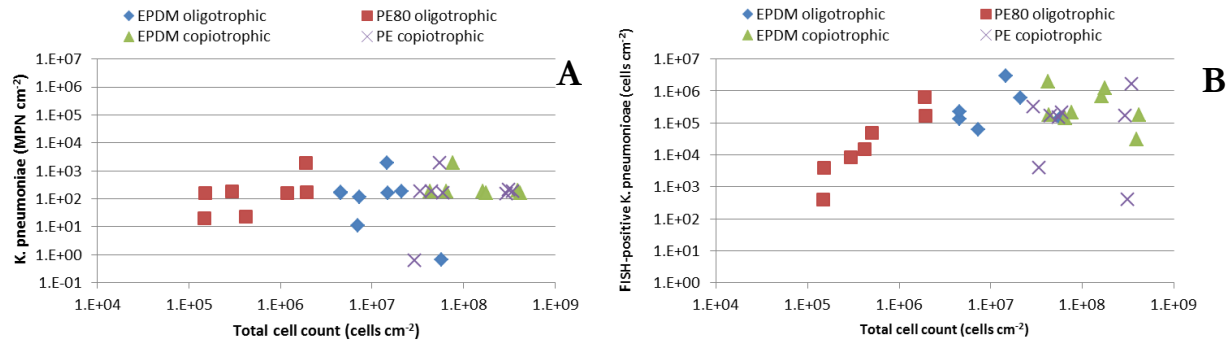


Figure 38: Incorporation of *K. pneumoniae* into a 14 days old drinking water biofilm grown on PE80 and EPDM under oligotrophic conditions and copiotrophic conditions at different temperatures one day after incubation in dependence of the total cell count. (A): culturable *K. pneumoniae*, (B): FISH-positive *K. pneumoniae*

P. aeruginosa

In contrast to *E. coli* and *K. pneumoniae*, *P. aeruginosa* was detected in the drinking water biofilms prior to inoculation using culture-independent FISH (Figure 39). Numbers on PE80 under oligotrophic conditions were within a range of 1.6 x 10^3 cells cm^{-2} and 6.4 x 10^4 cells cm^{-2} while under copiotrophic conditions numbers of FISH-positive *P. aeruginosa* were about one to two log units higher and within a range of 3 x 10^4 cells cm^{-2} to 1.9 x 10^6 cells cm^{-2}. However, at 12 °C and 25 °C *P. aeruginosa* was not detected within the drinking water biofilms (Figure 39). On EPDM, *P. aeruginosa* was also detected in in the drinking water biofilms at t_0. Under oligotrophic conditions numbers of FISH-positive *P. aeruginosa* varied between 6.5 x 10^4 cells cm^{-2} and 6.6 x 10^5 cells cm^2. Nutrient addition only resulted in significant higher numbers at 8 °C while at the other temperatures numbers of FISH-positive *P. aeruginosa* were similar regardless of the nutrient situation. Like it has been observed for biofilms grown on PE80, *P. aeruginosa* was not detected at 12 °C and 25 °C (only under oligotrophic conditions).

No clear temperature dependence was observed for the occurrence of *P. aeruginosa* in the established drinking water biofilms prior to inoculation grown on both PE80 and EPDM regardless of the nutrient situation.

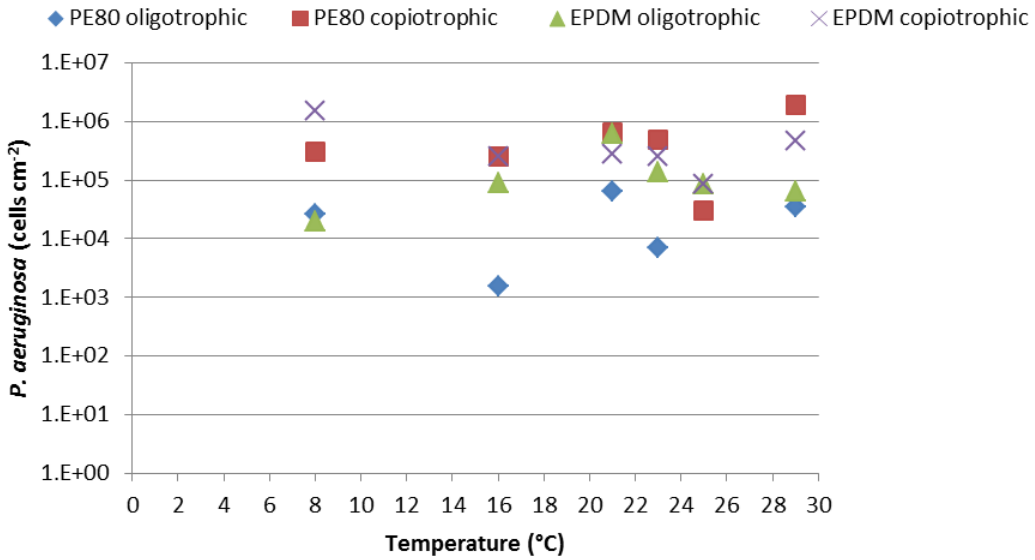


Figure 39: Detection of *P. aeruginosa* in drinking water biofilms prior to inoculation (t_0) using FISH.

Incorporation efficiency of cultural *P. aeruginosa* on PE80 under oligotrophic conditions was influenced by temperature (Figure 40). At 8 °C about 1 x10^1 cfu cm^{-2} were detected whereas at 29 °C the number of colony forming units was about three log units higher. However, the most significant increase in the number of culturable *P. aeruginosa* occurred at 12 °C. Colony counts were about 2.5 log units higher at 12 °C compared to 8 °C. The increase in the numbers of colony forming *P. aeruginosa* at temperatures above 12 °C was not as distinct as it was between 8 °C and 12 °C and within the range of one to 1.5 log units (Figure 40). The number of FISH-positive *P. aeruginosa* at 8 °C was significantly higher than the number of culturable *P. aeruginosa* and the number of FISH-positive *P. aeruginosa* was within a range of 10^4 to 10^5 FISH-positive cells cm^{-2} for all temperatures. Under copiotrophic conditions the effect of temperature on the incorporation of *P. aeruginosa* was not as nonambiguous as under oligotrophic conditions. Colony forming units at 8 °C were about two log units higher when nutrients were added. Between 12 °C and 23 °C numbers of culturable *P. aeruginosa* were similar and within a range of 1.1 x10^4 cfu cm^{-2} and 1.8.x 10^3 cfu cm^{-2}. A significant increase in the number of colony forming units occurred at 25 °C. 1.6 x 10^6 cfu cm^{-2} were detected at this temperature under copiotrophic conditions (Figure 40). However, it has to be noticed, that the number of FISH-positive *P. aeruginosa* at 25 °C was about 1.5 log units below the number of culturable *P. aeruginosa*. Except the decreased number of FISH-positive *P. aeruginosa* at 25 °C, numbers were within a range of 1.2 x 10^5 cfu cm^{-2} and 1.6 x 10^6 cfu cm^{-2}. Total cell counts were not influenced by temperature but increased with the addition of nutrients. Under copiotrophic conditions, the number of the total cells in the drinking water biofilms was about 2 to 2.5 log units higher than under oligotrophic conditions.

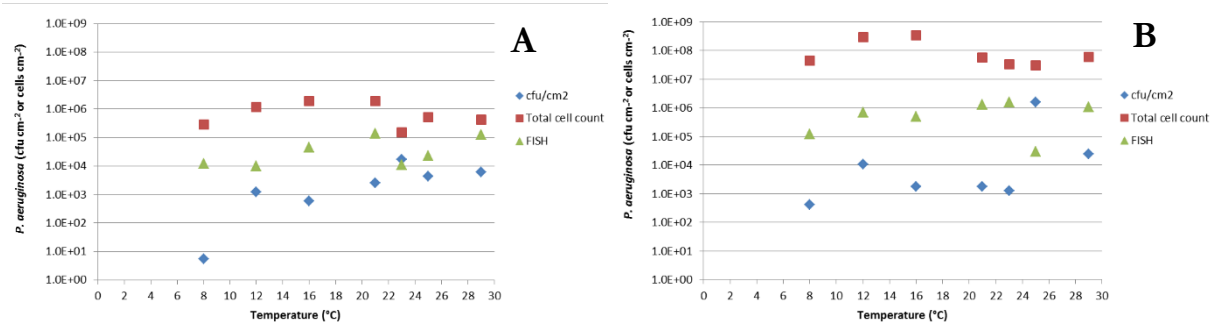


Figure 40: Incorporation of *P. aeruginosa* into a 14 days old drinking water biofilm grown on PE80 one day after incubation. (A) oligotrophic conditions, (B) copiotrophic conditions.

On EPDM, the number of culturable *P. aeruginosa* increased at temperatures above 21 °C under oligotrophic conditions (Figure 41). However, a decrease of colony forming units was detected at 29 °C. This effect was observed under copiotrophic conditions, respectively. The number of FISH-positive *P. aeruginosa* increased with elevating temperatures under oligotrophic conditions. At 8 °C 2.8 x 10^4 cells cm^{-2} were detected whereas at 29 °C the number of target cells was about two log units higher. Under copiotrophic conditions numbers of FISH-positive *P. aeruginosa* increased at 12 °C, remained constant between 12 °C and 21 °C and then slightly increased again. Total cell counts remained constant regardless of temperature and nutrient availability. However, nutrient addition resulted in higher numbers of the total cell counts. On PE80 the promoting effect of nutrient addition on the total cell counts was more distinct (Figure 40).

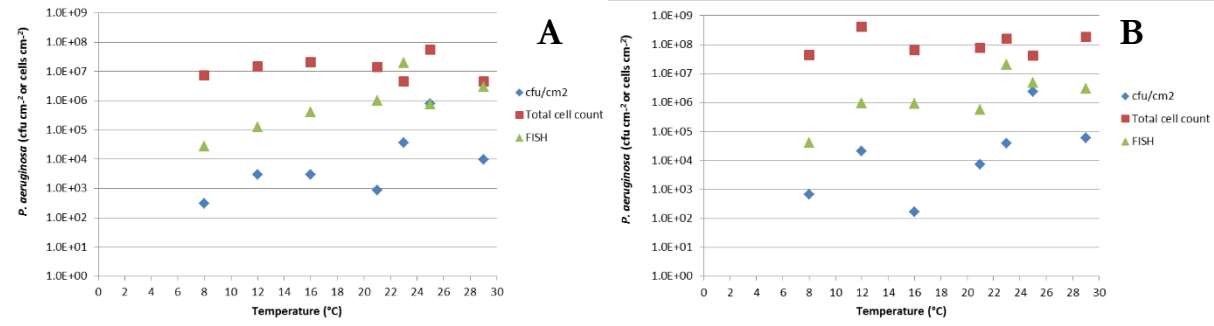


Figure 41: Incorporation of *P. aeruginosa* into a 14 days old drinking water biofilm grown on EPDM one day after incubation. (A) oligotrophic conditions, (B) copiotrophic conditions.

In Figure 42, the incorporation of *P. aeruginosa* into the drinking water biofilms grown on different materials in dependence of the total cell count is depicted. A slightly inverse correlation between the number of cultural *P. aeruginosa* one day after inoculation and the total cell counts was observed on PE80 under oligotrophic conditions. The same trend occurred for the number of FISH-positive *P. aeruginosa* on EPDM under oligotrophic conditions. Nutrient addition resulted in higher total cell counts, but not in higher numbers of culturable and FISH-positive cells.

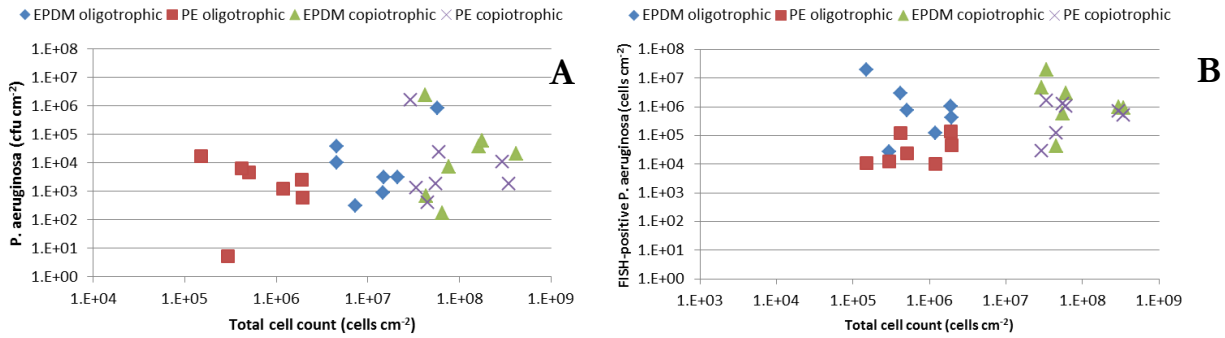


Figure 42: Incorporation of *P. aeruginosa* into a 14 days old drinking water biofilm grown on PE80 and EPDM under oligotrophic conditions and copiotrophic conditions at different temperatures one day after incubation in dependence of the total cell count. (A): culturable *P. aeruginosa*, (B): FISH-positive *P. aeruginosa*

As well as *P. aeruginosa*, *L. pneumophila* was also detected in the drinking water biofilms prior to inoculation using culture-independent FISH (Figure 43). Numbers on PE80 under oligotrophic conditions showed a high variation and were within a range of 1 x 10^3 cells cm^{-2} and 6.4 x10^4 cells cm^{-2} while under copiotrophic conditions numbers of FISH-positive *L. pneumohila* were similar and within a range of 2.4 x 10^4 cells cm^{-2} to 2.6 x 10^5 cells cm^{-2}. However, at 12 °C and 29 °C *L. pneumophila* was not detected within the drinking water biofilms on PE80 under oligotrophic conditions (Figure 43). On EPDM, *L. pneumophila* was also detected in the drinking water biofilms at t_0. Under oligotrophic conditions numbers of FISH-positive *L. pneumophila* were similar to those detected on PE80 under copiotrophic conditions and varied between 3 x 10^4 cells cm^{-2} and 1.6 x 10^5

cells cm^{-2}. Nutrient addition resulted in higher variations between the different temperatures. No *L. pneumophila* was detected at 12 °C and 16 °C.

No distinct temperature dependence was observed for the occurrence of *L. pneumophila* in the established drinking water biofilms grown on both PE80 and EPDM prior to inoculation regardless of the nutrient situation.

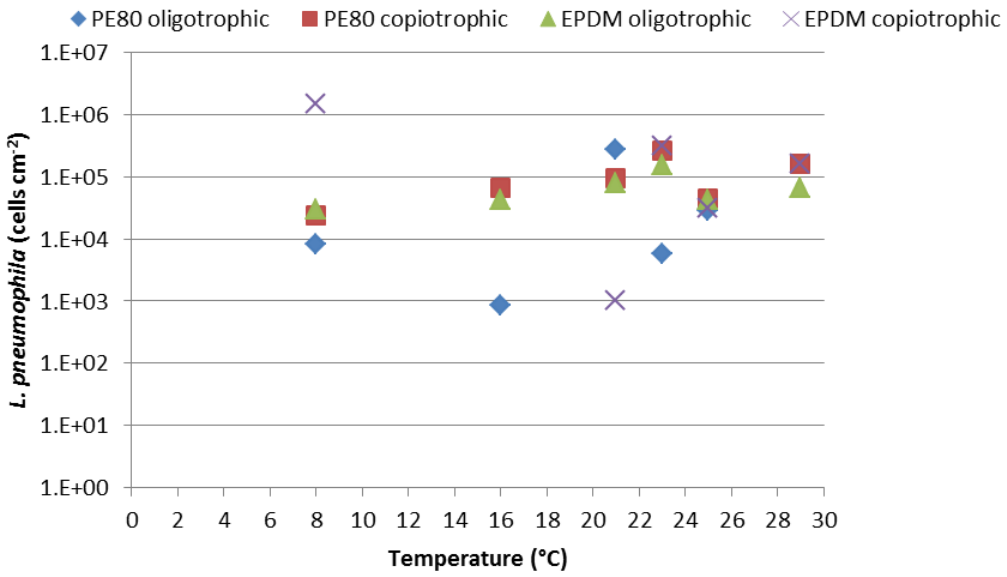


Figure 43: Detection of *L. pneumophila* in drinking water biofilms prior to inoculation (t_0) using FISH.

Incorporation of *L. pneumophila* into biofilms grown on PE80 and EPDM respectively under oligotrophic conditions was hardly effected by temperature (Figure 44; Figure 45). At temperatures above 8 °C the number of culturable *L. pneumophila* increased of about one to two log units and remained constant at the other temperatures with exceptions at 21 °C and 25 °C on both materials. At 21 °C no culturable *L. pneumophila* was detectable. At 25 °C the number of culturable *L. pneumophila* decreased on both materials. There was no distinct influence of temperature detectable the number of FISH-positive *L. pneumophila* on PE80 (Figure 44). On EPDM, numbers of FISH-positive *L. pneumophila* slightly decreased at temperatures above 21 °C under oligotrophic conditions (Figure 45). Under copiotrophic conditions results were similar

both on PE80 and EPDM. The number of culturable *L. pneumophila* increased of about two log units at temperatures above 8 °C on both materials. However, no culturable *L. pneumophila* were detectable at 21 °C and at temperatures ≥ 25 °C. This effect was observed on PE80 as well as on EPDM. No distinct influence of temperature was observed on the number of FISH-positive *L. pneumophila*.

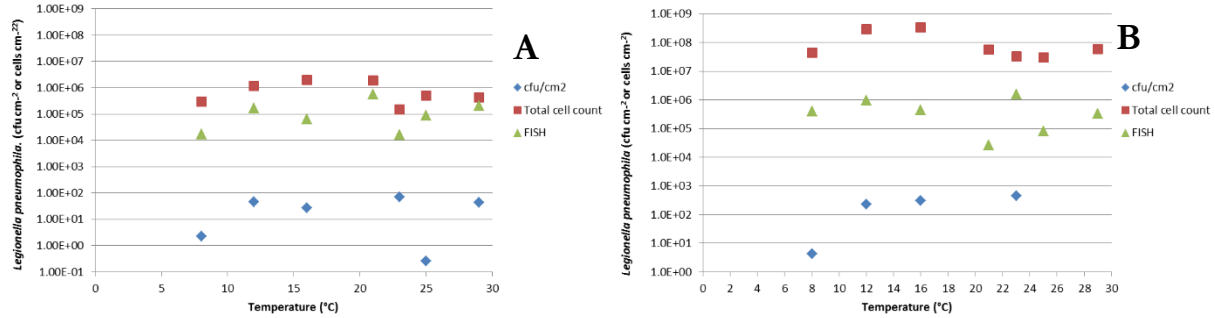


Figure 44: Incorporation of *L. pneumophila* into a 14 days old drinking water biofilm grown on PE80 one day after incubation. (A) oligotrophic conditions, (B) copiotrophic conditions.

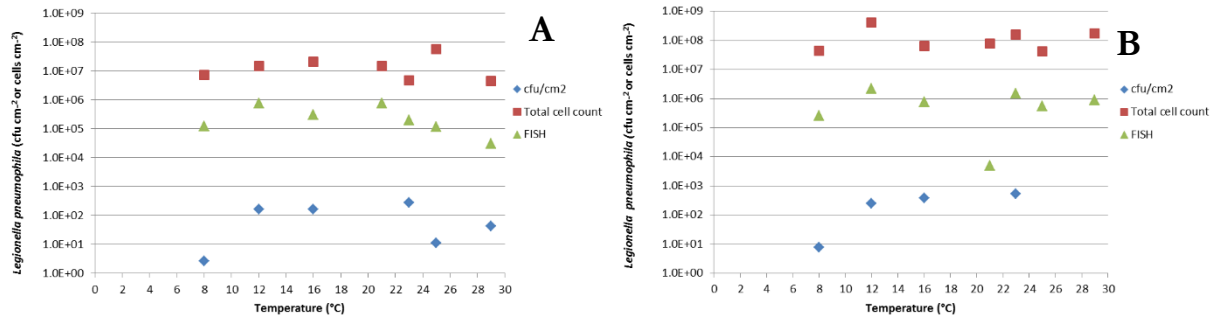


Figure 45: Incorporation of *L. pneumophila* into a 14 days old drinking water biofilm grown on EPDM one day after incubation. (A) oligotrophic conditions, (B) copiotrophic conditions.

In Figure 46, the incorporation of *L. pneumophila* into drinking water biofilms grown on PE80 and EPDM in dependence of the total cell count is depicted. No correlation between increasing total cell counts and the incorporation of cultural *L. pneumophila* was observed. However, an increase in the total cell counts was observed, when nutrients were added. Regarding the incorporation of FISH-positive cells a promoting effect of increasing total cell counts occurred on PE80 under oligotrophic conditions. This trend was not observed under copiotrophic conditions.

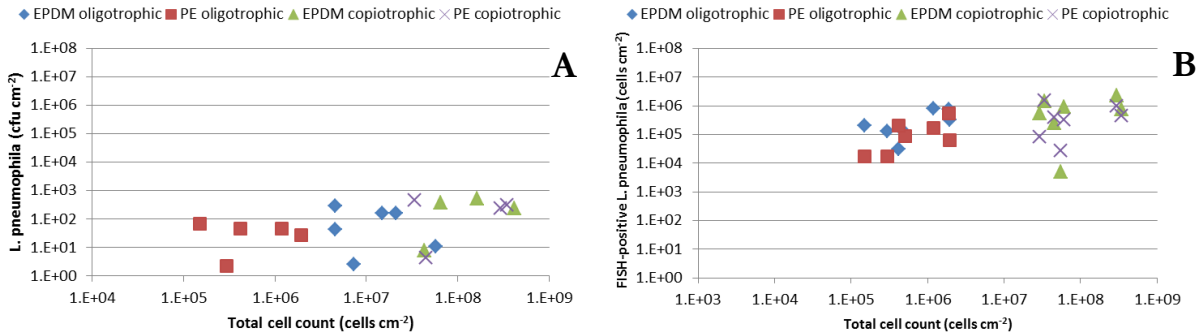


Figure 46: Incorporation of *L. pneumophila* into a 14 days old drinking water biofilm grown on PE80 and EPDM under oligotrophic conditions and copiotrophic conditions at different temperatures one day after incubation in dependence of the total cell count. (A) culturable *L. pneumophila*, (B): FISH-positive *L. pneumophila*.

Summary

No *E. coli* and *K. pneumoniae* were detected culturally and with FISH prior to inoculation. Incorporation of *E. coli* and *K.pneumoniae* was influenced by temperature. At temperatures above 21 °C the number of both culturable *E. coli* and culturable *K. pneumoniae* decreased under oligotrophic conditions. However, this trend was not observed under copiotrophic conditions. Neither *E. coli* nor *K. pneumoniae* were detected culturally at 25 °C whereas they were detectable using culture-independent FISH. Increasing total cell counts enhanced the incorporation of cultural as well as FISH-positive *E. coli* on PE80 under both oligotrophic and copiotrophic conditions. For *K. pneumoniae* this trend was only observed with FISH and on PE80.

No cultural *P. aeruginosa* and *L. pneumophila* were present within the drinking water biofilms prior to inoculation. However, with FISH both organisms could be detected in the established drinking water biofilms. No distinct influence of drinking water temperature and nutrient situation was observed for any of the two pathogens within the established drinking water biofilms with FISH. After inoculation of the drinking water biofilms with the target strains, *P. aeruginosa* and *L. pneumophila* were detected culturally. Incorporation of *P. aeruginosa* was influenced by temperature. On PE80, the number of culturable *P. aeruginosa* increased with elevated temperatures. However,

nutrient addition weakened this effect. On EPDM, the number of culturable as well as FISH-positive *P. aeruginosa* correlated positively with increasing temperature regardless of the nutrient situation. On PE80, the number of culturable *P. aeruginosa* slightly decreased with increasing total cell counts under oligotrophic conditions. The same trend was observed for the number of FISH-positive *P. aeruginosa* on EPDM under copiotrophic conditions. The incorporation of *L. pneumophila* was hardly influenced by temperature. Numbers of culturable *L. pneumophila* increased between 8 °C and 12 °C and remained constant between 12 °C and 29 °C with exceptions at 21 °C and 25 °C. No culturable *L. pneumophila* were detected at 21 °C regardless of nutrient availability and material. At 25 °C the number of culturable *L. pneumophila* decreased on both materials. On EPDM, the number of FISH-positive *L. pneumophila* decreased at temperatures above 21 °C under oligotrophic conditions. On PE80, the number of FISH-positive *L. pneumophila* increased with an increasing number of total cell counts.

4.2.4 Persistence of hygienically relevant bacteria in drinking water biofilms

In order to investigate the ability of hygienically relevant bacteria to persist within drinking water biofilms, 14 day old drinking water biofilms grown on PE80 and EPDM in annular reactors were spiked with *P. aeruginosa*, *L. pneumophila*, *E. coli*, and *K. pneumoniae*. To evaluate their persistence, the target organisms were quantified using cultural methods and culture-independent FISH over a period of 28 d. Additionally, effluents of the annular reactors were analysed for the target organisms in order to evaluate the possible contamination potential biofilms may pose to the drinking water.

Persistence of *E. coli* in drinking water biofilms grown on PE80 was not influenced by temperature. At six of eight tested temperatures, *E. coli* was culturally detectable within the drinking water biofilms only one day after inoculation. At 12 °C cultural *E. coli* was detectable over the complete period of the experiment. At 25 °C no cultural *E. coli* was

detected. With FISH, *E. coli* was detected over 28 d regardless of the temperature. In the effluents, *E. coli* was only detected one day after inoculation with one exception at 12 °C. Here, culturable *E. coli* occurred seven days after spiking. Under copiotrophic conditions, temperature also did not influence the persistence of *E. coli* within the drinking water biofilms. However, a significant increase in the duration of the detection of cultural *E. coli* occurred when nutrient were added. Cultural *E. coli* was at least detected 21 d after spiking of the drinking water biofilms. At five of eight temperatures, *E. coli* was even quantified culturally until the end of the experiment. As it was already the case in the low-nutrient drinking water, no *E. coli* was culturable at 25 °C. In the effluents, the duration of culturability of *E. coli* was also enhanced by nutrient addition. At five of eight temperatures, *E. coli* was culturally detectable over the complete period of the experiment. Only at 25 °C no *E. coli* was detected. At 12 °C cultural *E. coli* occurred seven days after spiking of the drinking water biofilms and at 16 °C *E. coli* was only detectable one day after inoculation.

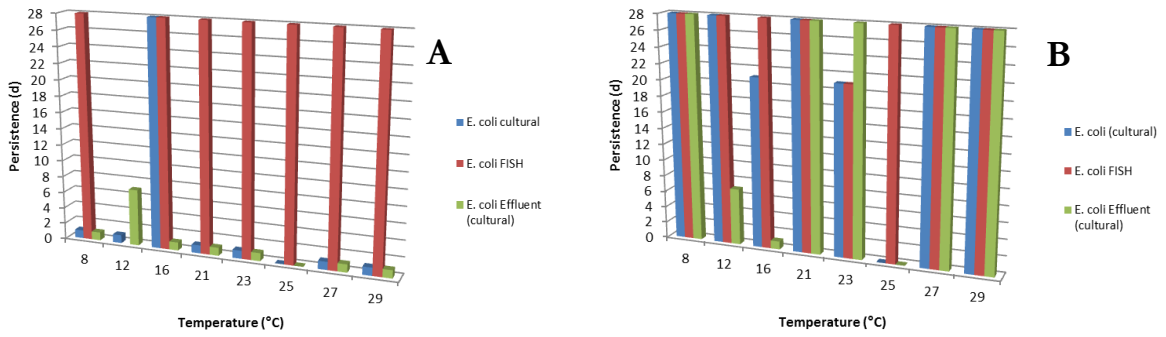


Figure 47: Persistence of *E. coli* in a 14 days old drinking water biofilm grown on PE80. (A) oligotrophic conditions, (B) copiotrophic conditions.

Persistence of *E. coli* in drinking water biofilms grown on EPDM was similar to persistence of *E: coli* in biofilms grown on PE80. At four of eight investigated temperatures, *E. coli* was only detectable using cultural methods one day after spiking of the biofilms under oligotrophic conditions. At 12 °C and 16 °C *E. coli* remained cultural

for seven days and for 14 days at 29 °C, whereas no culturable *E. coli* was detected at 25 °C. With FISH, *E. coli* was detected at least 21 d after inoculation. The effluent for PE80 and EPDM was the same therefore, results are the same as described earlier for PE80. A significant effect of nutrient addition was also observed on EPDM. Under copiotrophic conditions, persistence of *E. coli* was strongly enhanced and *E. coli* was culturally detected over the complete period of the experiment at five of eight temperatures. As already observed on PE80 and on EPDM under oligotrophic conditions, no culturable *E. coli* was detected at 25 °C.

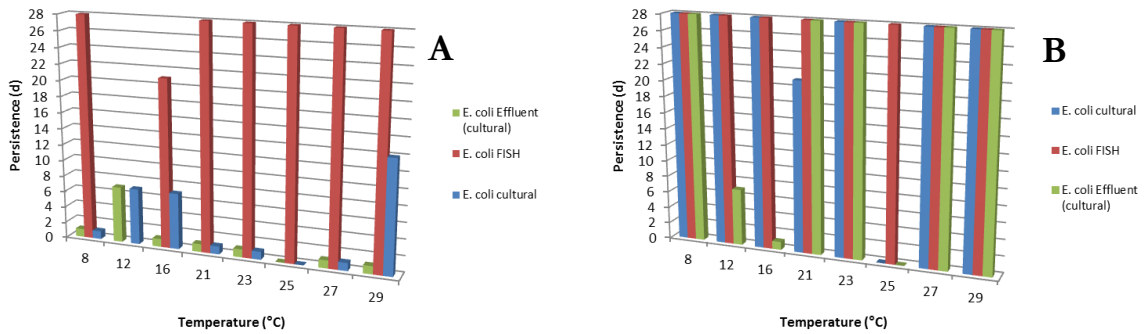


Figure 48: Persistence of *E. coli* in a 14 days old drinking water biofilm grown on EPDM (A) oligotrophic conditions, (B) copiotrophic conditions.

K. pneumoniae remained culturable within drinking water biofilms grown on PE80 under oligotrophic conditions for at least one day. No influence of temperature on the persistence of *K. pneumoniae* was detected (Figure 49). At five of eight investigated temperatures. *K. pneumoiae* was culturally detected one day after inoculation. However, at 16 °C culturable *K. pneumoniae* was detected over the complete period of the experiment whereas at 25 °C no culturable *K. pneumoniae* occurred. At 29 °C, *K. pneumoniae* remained culturable within the drinking water biofilms for 14 days. With FISH, *K. pneumoniae* was detected at least 21 days after spiking of the biofilms. In the effluents, culturable *K. pneumoniae* was detectable within the drinking water biofilms one day after inoculation at six of eight temperatures. At 12 °C, *K. pneumoniae* was detectable

with cultural methods seven days after the experiment started. Conspicuous was the detection of culturable *K. pneumoniae* in the effluent over the complete period of the experiment at 27 °C. Nutrient addition significantly enhanced culturablity of *K. pneumoniae* within the biofilms grown on PE80 (Figure 49). At six of eight temperatures, *K. pneumoniae* was culturally detectable for at least 21 days. Even at 25 °C, *K. pneumoniae* was detected culturally seven days after inoculation. The detection of FISH-positive *K. pneumoniae* was neither influenced by temperature nor by nutrient additions and results were comparable to those for biofilms grown on PE80 under oligotrophic conditions. In the effluents, the duration of cultural detection of *K. pneumoniae* was also significantly enhanced by nutrient addition. Cultural *K. pneumoniae* was detected at least 21 days after inoculation and at five of eight temperatures *K. pneumoniae* was even culturally detected over the complete period of the experiment. Only at 25 °C, *K. pneumoniae* was only detectable one day after the beginning of the experiment.

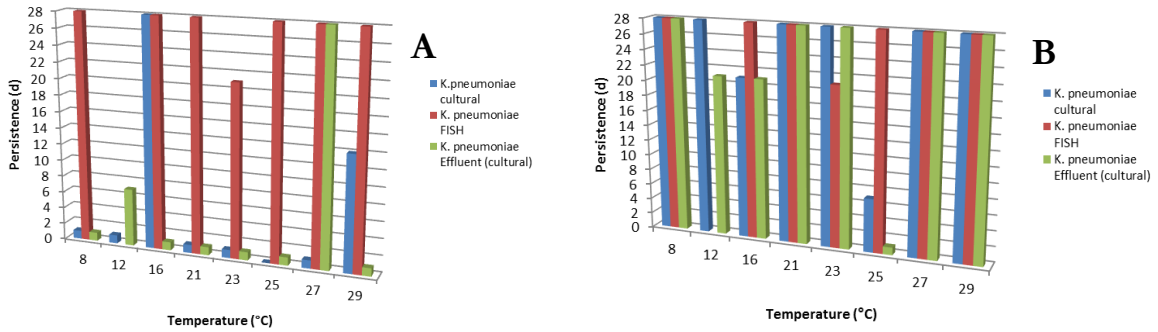


Figure 49: Persistence of *K. pneumoniae* in a 14 days old drinking water biofilm grown on PE80 (A) oligotrophic conditions, (B) copiotrophic conditions.

On EPDM, persistence of *K. pneumoniae* within the drinking water biofilms under oligotrophic conditions was longer than within biofilms grown on PE80 under oligotrophic conditions (Figure 50). At 29 °C, *K. pneumoniae* was culturally detected over the complete period of the experiment, at 8 °C and 16 °C, *K. pneumoniae* remained

culturable for 21 days, and at 23 °C, the duration of cultural detection was 14 days. At 21 °C and 27 °C, *K. pneumoniae* was only detectable one day after inoculation and at 25 °C, no culturable *K. pneumoniae* occurred. With FISH, *K. pneumoniae* was at least detectable for 21 days and no influence of temperature was observed. Results of the duration of culturability of *K. pneumoniae* within the effluents were the same as for PE80 under oligotrophic conditions, respectively. Nutrient addition had a promoting effect on the persistence of *K. pneumoniae* within the drinking water biofilms. *K. pneumoniae* remained culturable over the complete period of the experiment at seven of eight temperatures. However, at 25 °C culturability was only detectable seven days after inoculation. With FISH, *K. pneumoniae* was detected for at least 21 days and no influence of temperature was detected.

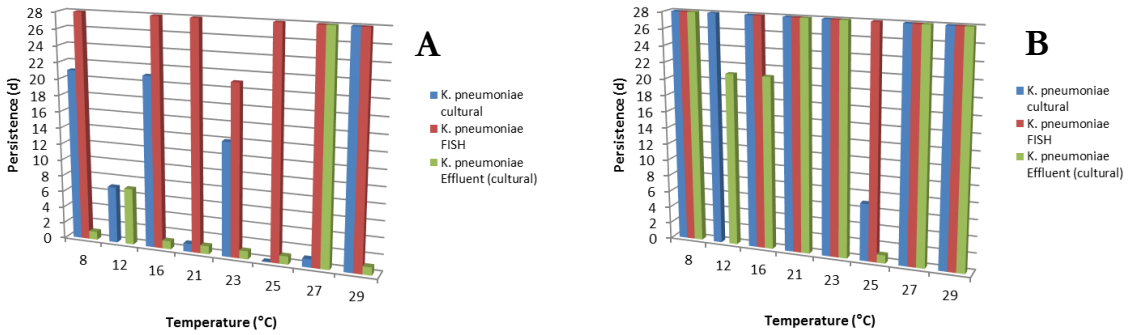


Figure 50: Persistence of *K. pneumoniae* in a 14 days old drinking water biofilm grown on EPDM (A) oligotrophic conditions, (B) copiotrophic conditions.

P.aeruginosa

Persistence of *P. aeruginosa* within drinking water biofilms was neither influenced by temperature nor by nutrient addition regardless of the surface material (Figure 51; Figure 52). On both, PE80 and EPDM *P. aeruginosa* was detected with cultural methods at least 21 d after inoculation (Figure 51; Figure 52). Using FISH, *P. aeruginosa* was detected over the complete period of the experiment on PE80 under both oligotrophic and copiotrophic conditions. The addition of nutrients resulted in a decrease in the

duration of cultural detection of *P. aeruginosa* in the effluents at 12 °C and 21 °C of about 14 d at 12 °C and seven days at 21 °C. On EPDM, nutrient addition only shortened the cultural detection of *P. aeruginosa* at 25 °C. Under oligotrophic conditions, *P. aeruginosa* was detectable in the drinking water biofilm for 28 d using cultural methods and FISH. Under copiotrophic conditions, this time was shortened to 21 d after inoculation. At 12 °C and 21 °C, the cultural detection of *P. aeruginosa* in the effluents was also shortened by nutrient addition. Duration of detection of FISH-positive *P. aeruginosa* within the drinking water biofilm grown on EPDM at 8 °C was shorter than the duration of cultural detection (Figure 52). In the effluents, cultural *P. aeruginosa* was detected over the complete period of the experiment. However, using FISH, *P. aeruginosa* was only detected 21 d after inoculation at 8 °C and 16 °C.

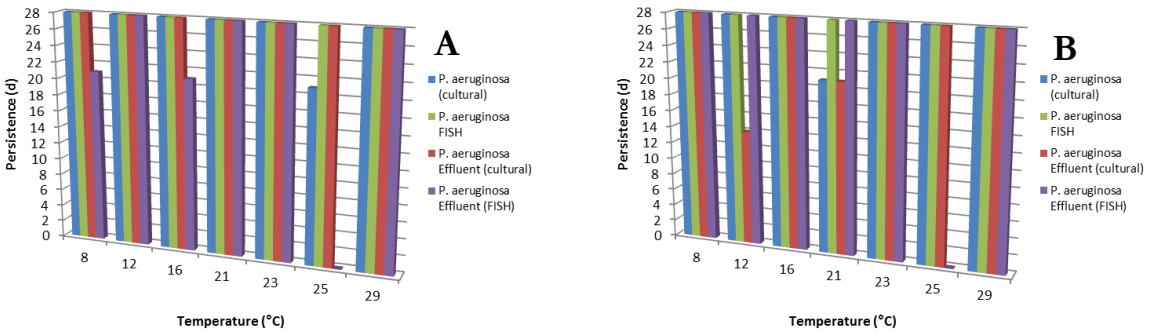


Figure 51: Persistence of *P. aeruginosa* in a 14 days old drinking water biofilm grown on PE80 (A) oligotrophic conditions, (B) copiotrophic conditions.

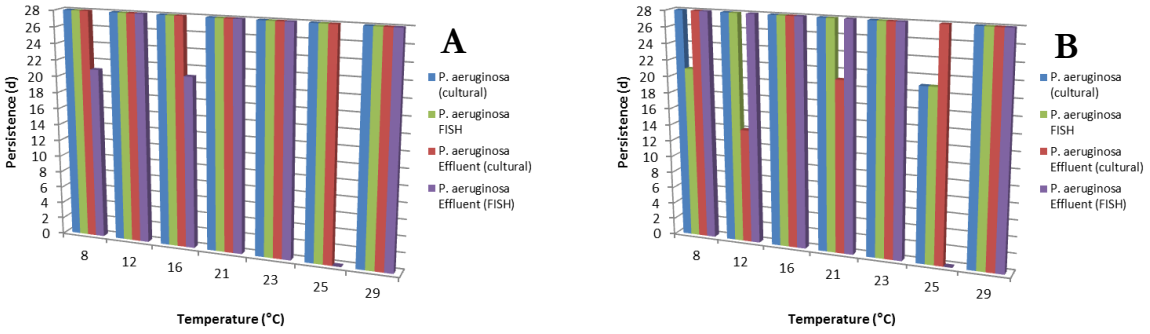


Figure 52: Persistence of *P. aeruginosa* in a 14 days old drinking water biofilm grown on EPDM (A) oligotrophic conditions, (B) copiotrophic conditions.

Different from *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, persistence of culturable *L. pneumophila* within drinking water biofilms grown on PE80 under oligotrophic conditions was influenced by temperature (Figure 53). At temperatures above 21 °C, persistence of cultural *L. pneumophila* was longer than at temperatures below 21 °C (5.3 d in mean below 21 °C and 21 d in mean above 21 °C). However, nutrient addition shortened the persistence of culturalbe *L. pneumophila*. Under copiotrophic conditions the maximum time of cultural detection of *L. pneumophila* was one day with one exception at 12 °C (28 d) whereas under oligotrophic conditions persistence was significantly longer. Using FISH, *L. pneumophila* was detected within the drinking water biofilms over the complete period of the experiment regardless of temperature and nutrient condition. In the effluents, the period of cultural detection of *L. pneumophila* was shortest at 12 °C and 16 ° (one day) and longest at 23 °C and 25 °C (21 d). However, culturability in the drinking water was also negatively influenced by nutrient addition and the duration of cultural detection of *L. pneumophila* was diminished under copiotrophic conditions.

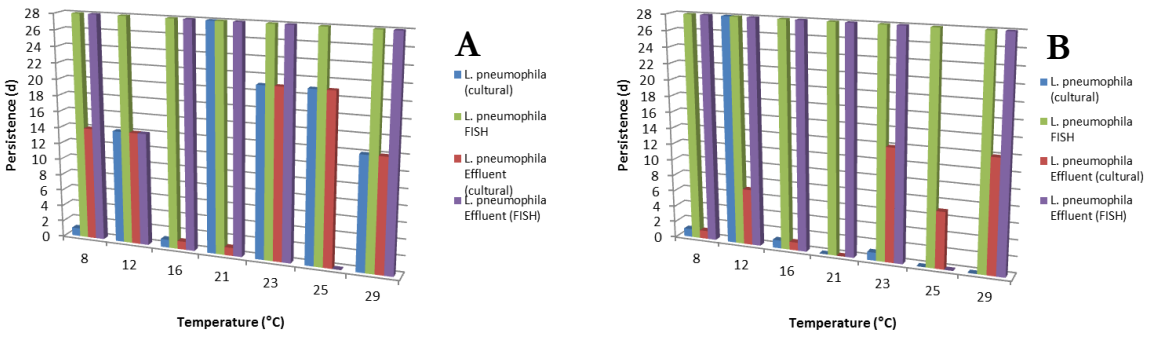


Figure 53: Persistence of *L. pneumophila* in a 14 days old drinking water biofilm grown on PE80 (A) oligotrophic conditions, (B) copiotrophic conditions.

On EPDM, the influence of temperature on the persistence of cultural *L. pneumophila* under oligotrophic conditions was not as distinct as on PE80 (Figure 54). The maximum

time of detection of culturable *L. pneumophila* was 21 d at 12 °C, 21 °C, and 23 °C. No cultural *L. pneumophila* were detected at 29 °C. However, using FISH, *L. pneumophila* was detectable within the drinking water biofilms over the complete period of the experiment regardless of the temperature. Nutrient addition significantly decreased the duration of cultural detection of *L. pneumophila* while the time of detection of FISH-positive *L. pneumophila* was not affected (Figure 54).

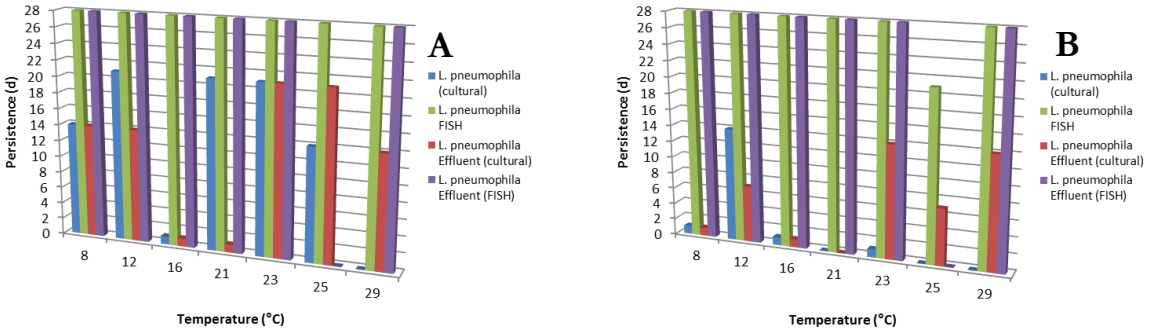


Figure 54: Persistence of *L. pneumophila* in a 14 days old drinking water biofilm grown on EPDM (A) oligotrophic conditions, (B) copiotrophic conditions.

Summary

For *E.coli*, *K. pneumoniae*, and *P. aeruginosa* no influence of drinking water temperature on the persistence wihin the drinking water biofilms was observed using cultural methods as well as cultureindependent FISH. However, for *E.coli* and *K. pneumoniae* an increase in the duration of dectection was detected when nutrients were added. This effect was not observed for *P. aeruginosa*.

Persistence of *L. pneumophila* was influenced by drinking water temperature. At temperatures above 21 °C, persistence of cultural *L. pneumophila* in mean was 15 days longer than at temperatures below 21 °C (5.3 d in mean below 21 °C and 21 d in mean above 21 °C). Nutrient addition significantly shortened the persistence of cultural *L. pneumophila*. Using FISH, *L. pneumophila* was detected within the drinking water biofilms over the complete period of the experiment regardless of temperature and nutrient condition.

Pulsed Field Gel Electrophoresis

To investigate if the positive results regarding the target organisms in the laboratory experiments could be referred to spiking of the drinking water biofilms with the target organisms or to a contamination with other strains, isolates of *P. aeruginosa* were exemplary analyzed using pulsed field gel electrophoresis (PFGE). As a reference DNA of the strain *P. aeruginosa* AdS used for spiking of the biofilms grown in the annular reactors was applied to the gel.

In the PFGE, DNA fragments are exposed to electric fields of periodically altering directions in which migrating molecules have to reorient continuously. Smaller molecules are able to reorient more quickly than bigger molecules. Thus, DNA molecules migrate through the agarose gel in a size-dependent manner (Schwartz and Cantor, 1984; Gardiner 1991; Herschleb et al., 2007). Figure 55 shows the band patterns of different isolates of *P. aeruginosa* isolated from the drinking water biofilms in the laboratory experiment generated using PFGE. It is unambiguous that all strains identified in the biofilms are identical as they express the same band patterns. Therefore, a contamination of the annular reactors resulting in positive results regarding the target organisms is unlikely.

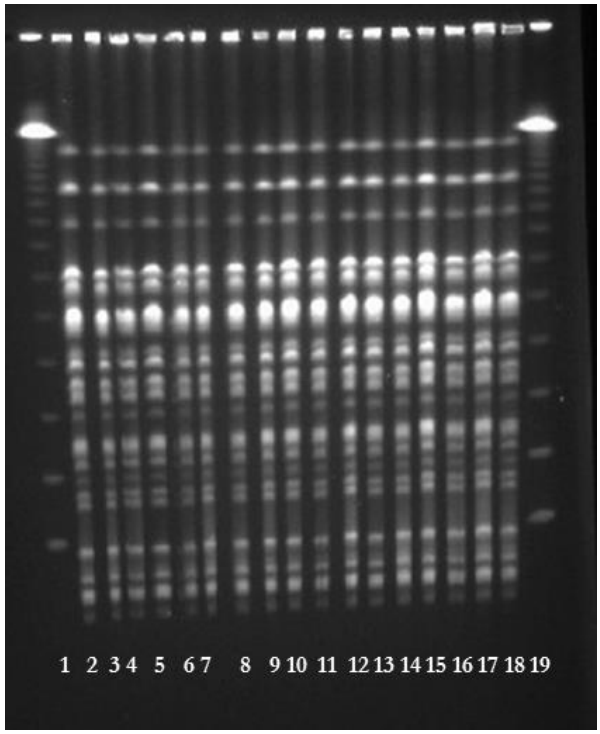


Figure 55: Comparison of the band patterns (PFGE) of *P. aeruginosa*-isolates from teh annular reactors with the reference strain *P. aeruginosa* AdS. lanes 1 und 19: Marker; lanes 2, 6, 9, 12 ,17: isolates from drinking water, lanes 3, 4, 5, 7, 8, 10, 11, 13, 14, 15, 16: isolates from drinking water biofilms; lane 18: reference strain *P. aeruginosa* AdS.

4.2.5 Denaturing Gradient Gel Electrophoresis

To investigate the influence of drinking water temperature on biofilm diversity, biofilms established and grown in the annular reactors at different temperatures were analyzed by means of denaturing gradient gel electrophoresis (DGGE). In the field experiments biofilms grown on EPDM were seasonally sampled and biofilm diversity was evaluated. Taken into account each climatope individually, biofilms were most similar between autumn and winter (84%) which were also the season with the lowest difference in temperature (2,9°C). A comparison of biofilms established during summer and winter resulted in 76 % similarity. For summer and autumn biofilms showed lowest similarity with 66% although the difference in temperature was higher between summer and winter. However, it has to be mentioned that for all seasonal comparisons similarities of the biofilm commiunitites were on a comparable level. A comparison of the similarity of the biofilms between all climatops showed a mean similarity of 46 % for all seasons. That means, similarities of the biofilm populations at the same location were higher than

between the different climatopes. Moreover, seasonal effects did not have significant impact on the comparability of the biofilms.

Table 18: Mean temperature differences of drinking water on the day of sampling and similarities of biofilm populations between all climatopes

	Δ T(°C)	**Similarity of biofilm population (%)**
Summer/Winter	10,5	46
Summer/Autumn	9,4	46
Autumn/Winter	3,1	46

Table 19: Mean temperature differences of drinking water on the day of sampling and similarities of biofilm populations for the individual climatopes

	Δ T(°C)	**Similarity of biofilm population (%)**
Summer/Winter	10,7	76
Summer/Autumn	7,9	66
Autumn/Winter	2,9	84

In addition to the drinking water biofilms exposed in the drinking water distribution network, drinking water biofilms established and grown in annular reactors at various temperatures (8°C to 29 °C) in the laboratory were analyzed to elucidate a possible influence of water temperature on the biofilm population. Therefore, band patterns of biofilms established on EPDM in oligotrophic as well as in copiotrophic drinking water at different water temperatures (8, 16, 21, 23, 29 °C) and for different exposure times (two weeks of biofilm formation, two weeks of biofilm formation+one day after inoculation, two weeks of biofilm formation+four weeks after inoculation) were compared. Drinking water temperature influenced the biofilm population in so far, that biofim populations were most similar within the individual reactors when temperature was the same (Table 20). That means biofilm populations within the biofilms established in the same reactor and grown for four weeks were more similar at the same temperature

than biofilms in the same reactor grown at different temperatures. However, there was no increase in biofilm diversity with increasing Δ T. For biofilms established under copiotrophic conditions, similarities were lower than for biofilms grown under oligotrophic conditions.

Table 20: Similarities of the drinking water biofilms grown at different temperatures in annular reactors and sampled at different time points under oligotrophic and copiotrophic conditions (Similarities were calculated using the Sørensen Index).

EPDM oligotrophic	S1 8°C	S2 8°C	S6 8°C	S1 16°C	S2 16 °C	S6 16 °C	S1 21 °C	S2 21 °C	S6 21 °C	S1 23 °C	S2 23 °C	S6 23 °C	S1 29°C	S2 29 °C
S1 8°C														
S2 8°C	86													
S6 8°C	50	61												
S1 16°C	67	70	62											
S2 16 °C	58	61	54	100										
S6 16 °C	62	64	57	64	64									
S1 21 °C	20	21	19	38	38	29								
S2 21 °C	32	33	30	55	55	40	67							
S6 21 °C	42	44	40	46	46	41	63	81						
S1 23 °C	35	36	32	40	40	21	26	31	29					
S2 23 °C	45	48	42	33	33	15	20	26	24	96				
S6 23 °C	64	67	58	50	50	38	47	58	48	61	55			
S1 29°C	37	38	35	35	35	32	43	64	49	31	21	47		
S2 29 °C	26	27	25	30	30	41	39	51	37	31	21	42	93	
S6 29 °C	39	40	37	42	42	44	45	49	47	32	22	44	81	65

EPDM copiotrophic	S1 8°C	S2 8°C	S6 8°C	S1 16°C	S2 16 °C	S6 16 °C	S1 21 °C	S2 21 °C	S6 21 °C	S1 23 °C	S2 23 °C	S6 23 °C	S1 29°C	S2 29 °C
S1 8°C														
S2 8°C	78													
S6 8°C	80	73												
S1 16°C	36	24	59											
S2 16 °C	32	27	58	89										
S6 16 °C	39	29	53	48	60									
S1 21 °C	22	30	17	42	46	44								
S2 21 °C	30	33	38	48	38	44	43							
S6 21 °C	31	34	45	35	45	38	52	30						
S1 23 °C	32	36	33	30	17	33	40	38	26					
S2 23 °C	17	30	45	32	27	36	30	33	28	73				
S6 23 °C	25	29	61	31	35	28	41	24	40	55	45			
S1 29°C	27	41	39	46	39	38	51	47	37	39	41	29		
S2 29 °C	21	32	22	37	30	27	42	33	34	30	32	22	72	
S6 29 °C	31	26	56	43	48	39	50	59	38	48	52	33	43	43

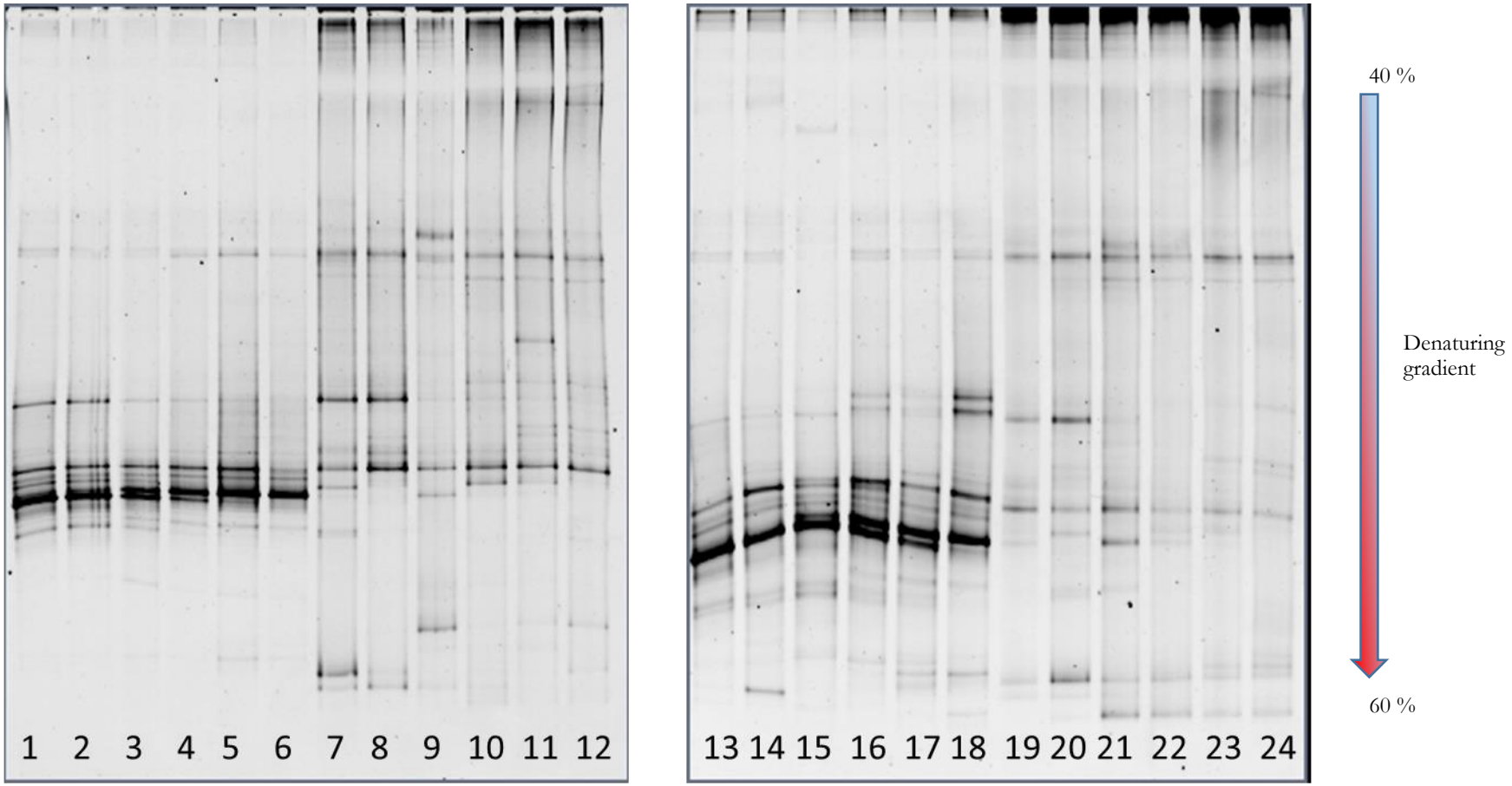


Figure 56: Example of a DGGE band patterns of drinking water biofilms (grown at 16 °C): Lanes 1-6: Reactor 1 (*P. aeruginosa/L. pneumophila* oligotrophic conditions) lane one: prior to inoculation with target organism, lane two: one day after inoculation, lane three: one week after inoculation, lane four: two weeks after inoculation, lane five: three weeks after inoculation, lane six: four weeks after inoculation; lanes 7-12: Reactor 2 (*E.coli/K.pneumoniae* oligotrophic conditions; respectively), lanes 13-18: Reactor 3 (*P. aeruginosa/L. pneumophila*, copiotrophic conditions, respectively), lanes 19-24: Reactor 4 (*E.coli/K.pneumoniae*, copiotrophic conditions, respectively)

5 Discussion

To investigate the influence of temperature on the survival of hygienically relevant bacteria in drinking water and drinking water biofilms, biofilm reactors containing different materials (EPDM, PE80/100, stainless steel) were inserted into a German drinking water distribution system as a bypass in a field study. Samples were taken in different seasons (autumn, winter, summer) in order to encompass a possible influence of variations in drinking water temperature on the occurrence of hygienically relevant bacteria. Total cell counts, HPC, and colony counts according to the German drinking water ordinance were conducted to monitor drinking water quality and biofilm formation within the biofilm reactors. Moreover, the presence of hygienically relevant bacteria was investigated using conventional cultural methods as well as the culture-independent FISH-method.

Additionally, laboratory experiments were performed to investigate the influence of temperature on the survival of hygienically relevant bacteria in drinking water and drinking water biofilms. Therefore, biofilms were grown on coupons of elastomeric (EPDM) and plastic (polyethylene PE80) material in modified RotoTorque annular reactors at different temperatures (8 °C- 29 °C). Continuous flow-through of the reactors was performed with UV-disinfected drinking water from a surface water treatment plant in Germany with low assimilable organic carbon (AOC: 6 µg/L acetate-C-equivalent), and, for nutrient rich conditions, water supplemented with tryptic soy broth (AOC: 150 µg/L acetate-C-equivalent). After 14 d of biofilm establishment, the reactors were inoculated with a combination of the coliform indicator bacteria *E. coli*

and *K. pneumoniae*, and separately with a combination of the environmental pathogens *P. aeruginosa* and *L. pneumophila* and operated for 28 d. Samples were taken prior to inoculation, one day after inoculation and then weekly over a period of four weeks. In addition, samples of the influents and effluents of the reactors were monitored to encompass possible contamination potential of the biofilms regarding the drinking water.

5.1 Drinking water quality and biofilm formation

5.1.1 Drinking water quality

In the field experiments, elevated drinking water temperatures did not result in an impaired biological stability of the drinking water analyzed in the field experiments independent of the season. For the evaluation of the biological stability of the tested drinking water, total cell counts, colony counts, and the HPC were taken into account in this study. Total cell counts as well as HPC and colony counts were not affected by increasing water temperatures. The colony counts according to the German drinking water ordinance did not exceed the threshold of 100 cfu mL^{-1}, but remained within the single or low double-digit range regardless of the season. Results were comparable for the influents of the annular reactors used for biofilm establishment in the laboratory experiments. Neither of the analyzed parameter was significantly influenced by temperature. However, all mentioned parameters showed higher concentrations in the effluents of the annular reactors compared to the influents (depending on the parameter, numbers were up to five log units higher). Moreover, the nutrient situation had a major influence on the total cell count, colony counts, and HPC. Copiotrophic conditions resulted in higher concentrations for all parameters. A reason for the stable drinking water quality in the field experiments and in the influents of the annular reactors in the laboratory experiment might be the low AOC content of the applied drinking water after treatment (6 µg/L acetate-C-equivalent). In addition, the molar C:N:P (AOC:NO_3-

N:PO_4-P) ratio (100:45.000:380) showed a significant C-limitation. For bacterial growth a molar C:N:P ratio of 100:10:1 is suggested to be optimal. This and the AOC below 10 µg/L acetate-C-equivalent indicated a biological stable drinking water which did not promote bacterial growth. Volk and LeChevallier (2000) investigated AOC levels in 95 effluents of water treatment plants and reported L^{-1}. They average AOC concentrations in plant effluents of about 100 µg suggested that removal of biodegradable organic matter during water treatment might be an effective method to maintain the biological quality of water without using excessive amounts of disinfectants. The reduction of AOC as a useful method to control bacterial regrowth was also demonstrated at Osaka Water Works (Watanabe 2014). Moreover, Van der Kooij (1989) suggested an AOC value of 10 µg L^- for water to be considered biologically stable. Vital et al. (2010) investigated the influence of AOC on growth of various pathogens. They found that in samples of one drinking water treatment plant, increasing AOC contents were associated with increasing numbers of *P. aeruginosa* and *E.coli*, while in another treatment plant growth of the two organisms was promoted although the concentration of AOC decreased. They suggested that not only the concentration of AOC, but also the composition is crucial for growth. Despite water temperatures which were temporarily close to optimal growth temperatures, the drinking water investigated in this study remained biologically stable which might be due to the low AOC content. In the drinking water supplied with nutrients, water temperature also did not significantly influence the drinking water quality. It is possible that in this case the effect of the increased nutrient availability covered the effect of increasing water temperatures. However, Kerneïs et al. 1995 earlier described the necessity to associate thresholds for nutrients prohibiting bacterial growth in a drinking water distribution system with elevated drinking water temperatures as the biodegradation of BDOC is temperature-dependent. He suggested, that at higher water temperatures, the minimum BDOC concentration, which is required to control bacterial growth in a distribution network is lower than at low water temperatures as biodegradation of organic compounds is less in

winter compared to summer. Moreover, Szewzyk et al. (2000) stated that organic compounds are not the main nutrition source for bacterial growth but other nutrients such as phosphate and nitrate have to be taken into consideration as growth-promoting compounds. They reported, that in recent years studies in Nordic European countries did not reveal an association between microbial growth and the concentration of organic compounds in drinking water. However, addition of phosphate into the drinking water immediately supported bacterial growth. As described by the studies mentioned before, bacterial growth also was promoted by the addition of nutrients in the present study. To compile the result of the present study and the studies mentioned before it can be said, that it is inevitable to consider nutrient availability as a limiting factor for the biological stability of water systems and its microbiological quality, but that the effect of temperature on the impact of nutrient availability should not be neglected.

5.1.2 Formation of drinking water biofilms within the biofilm reactors

Biofilms are ubiquitous and can be found on all surfaces in drinking water distribution systems. As described in various studies, the majority of all bacteria present in a drinking water distribution system is attached to surfaces (O'Toole et al., 2000; Donlan, 2002; Flemming and Wingender 2010, Wingender and Flemming 2011). For that reason, not only the water phase was analyzed in the field experiments, but also the biofilms grown in the biofilm reactors. Biofilms were established at least for six months on various materials (EPDM, PE80/100, stainless steel) within the biofilm reactors, which were inserted into the drinking water distribution network as a bypass. In addition, biofilms were established under laboratory conditions at various temperatures to investigate the influence of temperature on the survival of hygienically relevant bacteria in drinking water biofilms. For a general characterization of the drinking water biofilms, total cell counts, HPC and colony counts according to the German drinking water ordinance were determined.

In the field experiments as well as in the laboratory, total cell counts of the biofilms were only marginally influenced by drinking water temperature which ranged from 6 °C to 21 °C at the different days of sampling in the field experiments. The formation of drinking water biofilms was hardly affected by temperature and only marginal variations were detected. Total cell counts, HPC and colony counts regarding the German drinking water ordinance were similar at all investigated temperatures (8 °C – 29 °C) during the laboratory investigations. Both, in the laboratory and the field experiments, biofilm formation was influenced by the applied materials rather than temperature. Cell densities were highest on EPDM (10^7 to 10^8 cells cm^{-2}) and about two to three log units lower on PE80/100 and stainless steel. Already 30 years ago the influence of pipe materials on biofilm formation in drinkig water systems has been investigated (Colbourne 1985; Schoenen 1989). Enhanced biofilm formation on elastomeric or plastic plumbing materials has been reported in various studies (Moritz et al., 2010, Bressler et al., 2009, Kilb et al., 2003). In their study, Moritz et al. (2010) found total cell counts of 14 d old drinking water biofilms to be 50 to 260 times higher when grown on EPDM compared to PE or copper. However, this effect was not observed after 43 days of biofilm formation. The influence of pipe mterials on early stage of biofilm development was also described by Prest et al. (2016). Lehtola et al. (2004) also reported enahnced biofilm formation on plastic plumbing materials. In their study they investigated biofilm development in a pilot drinking water distribution system with copper and plastic pipes. Moreover, they found that the plastic PE material they used in their study released a significant amount of Phosphorus into the drinking water over a period of two to three weeks. It was suggested that in drinking water with Phosphorus being the limitting factor the released Phosphorus may promote biofilm formation. The role of phopshate in the regulation of bacterial grwoth was also reported by Szewzyk et al. (2000). Moreover, the findings of Lehtola et al. (2004) were supported by a study of Silhan et al. (2006). When investigating the influence of temperature and pipe material on biofilm formation they reported enhanced biofilm formation on plastic materials as well. 32 d

were necessary to establish a steady state biofilm on plastic material, whereas on other materials cell densities did not reach a steady state during the experiment. Temperature also affected the formation of drinking water biofilms as the development of biofilm occurred much more faster at 35 °C compared to 15 °C. The influence of temperature on the formation of drinking water biofilms was also described in an earlier study by Hallam et al. (2001). In their study they investigated the growth of biofilms in water distribution systems. They reported a higher biofilm quantity (pg ATP cm^{-2}) in summer and fall (14 °C and 15 °C) compared to winter (5 °C). Despite these findings, no distinct effect of temperature on biofilm formation was observed. However, it is possible, that the effect of temperature was disguised by the more significant effect of the applied materials. Higher nutrient availability enhanced biofilm formation in the laboratory experiments. Elevated total cell counts, HPC, and colony counts were observed under copiotrophic conditions. This is coherent with the results of Fang et al. (2009). They suggested that the addition of phosphorous promoted the growth of biofilms. Jang et al. (2012) also investigated the influence of phosphorous on biofilm formation. Corroborating the results of Fang et al. (2009), they also observed a significant promoting influence of phosphate on growth of biofilms within annular reactors. Pang and Liu (2006) reported that carbon removal due to biological filtration resulted in biofilms of reduced thickness and biovolume. Allan et al (2002) observed a diminished biofilm biomass when *Citrobacter sp.* was grown under glucose, phosphorous or nitrogen limitation. They also showed that not only carbon is required for biofilm formation, but that it is crucial which source of carbon is available. Biofilm biomass produced by Citrobacter under glucose limitation was 90 % lower than under lactose limitation.

5.2 Influence of temperature on hygienically relevant bacteria in drinking water and drinking water biofilms

5.2.1 Occurrence of hygienically relevant bacteria in drinking water

With cultural methods, coliform bacteria (without *E. coli*), enterococci, and aeromonads were detected in the drinking water from the field experiments. Within the drinking water biofilms, *E. coli*, coliform bacteria, enterococci, and *L. pneumophila* were detected culturally. Using FISH, drinking water and drinking water biofilms were analyzed according to the presence of non-culturable *E. coli*, *Legionella* spp., *L. pneumophila*, and *P. aeruginosa*. In all analyzed samples at least one of the target organisms was detected with FISH. In the drinking water, no association of the occurrence of hygienically relevant bacteria with the drinking water temperature was observed. With FISH, the trend of decreasing numbers of *L. pneumophila* occured with elevated temperatures in the different seasons (frequency of detection (%) from autumn to summer: 75; 100; 12.5). In the laboratory experiments, none of the target organisms were culturally detectable in the influents of the annular reactors. However, in the effluents, all of the target organisms were at last detectable one day after inoculation. *P. aeruginosa* was detectable in the effluents almost over the entire time of the experiment at all temperatures. Rather than temperature, the nutrient situation influenced the concentration of the target organisms in the effluents of the reactors. In the reactors, which were perfused with copitrophic drinking water, concentrations of the target organisms in the effluent water was higher than under oligotrophic conditions.

Van der Wielen and van der Kooij (2013) investigated the growth of fungi, nontuberculous mycobacteria, and opportunistic pathogens in unchlorinated drinking water of eight drinking water treatment plants in the Netherlands. Using selective quantitative PCR methods, they detected amongst others, *L. pneumophila* as well as *P. aeruginosa* in the drinking water. However, these pathogens were not detected in the

treated water samples prior to distribution indicating that proliferation of the organisms occurred during distribution. Comparable to the present study, no *L. pneumophila* could be cultivated from the water samples in the study of van der Wielen and van der Kooij (2013) although the culture-independent method revealed the presence of non-culturable *L. pneumophila* in the drinking water. A possible reason for the absence of culturable *L. pneumophila* in this study might be the presence of the organisms in the VBNC-state. The ability of *L. pneumophila* to undergo a transition from a culturable into a non-culturable state was demonstrated in various studies (Moritz et al., 2010; Ducret et al. 2014; Gião et al. 2014). In their study, Van der Wielen and van der Kooij (2013) did not observe a seasonal influence on the occurrence of *L. pneumophila* and *P. aeruginosa* in most of the analyzed drinking waters. Only in two treatment plants, seasonal effects were observed. Here, the occurrence of *L. pneumophila* was higher in summer than in winter. This is contradictory to the results obtained in the present study as the number of FISH-positive *L. pneumophila* in the drinking water decreased in the warmer seasons. In another study, two distribution systems in the Netherlands were analyzed for the occurrence of opportunistic pathogens in September 2009 and February 2010 (van der Wielen et al. 2014). The *mip* gene of *L. pneumophila* was only detected in the water samples of one of the tested distribution systems and the number of copies in February was higher than in September. However, cultivation of *L. pneumophila* from the same samples was not possible. In the same study, *P. aeruginosa* was only detectable in February but not in September using qPCR. In an earlier study, van der Wielen and van der Kooij (2013) did not observe a clear seasonal influence on the occurrence of *P. aeruginosa* in drinking water. Karagiannis et al (2009) investigated the influence of weather on the abundance of Legionnaire´s disease (LD) in the Netherlands. They reported the highest incidence of LD in summer when the weather was warm and wet. Eber et al. (2011) reported a seasonal and temperature-associated increase in bloodstream infections (BSIs) accounted to gram-negative bacteria such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. However, in his review, Richet (2012) reported conflicting results of two independent

studies regarding the seasonality of BSIs accounted to *Klebsiella* spp. The described observations and contradictory results suggest that not only the temperature, but also other factors like water composition, biocoenosis, pipe materials or treatment processes contribute the occurrence of opportunistic pathogens in drinking water. The biofilm reactors in the field study were open systems and the annular reactors in the laboratory experiments were perfused with drinking water instead of sterile deionized water. The complexity of the biological, chemical, and physical interactions occurring in such intricate systems should not be underestimated when evaluating the obtained results. It is strongly recommended to include such parameters when investigating the occurrence of hygienically relevant organisms in drinking water (Van der Wielen and van der Kooij 2013; van der Wielen et al. 2014).

5.2.2 Occurrence of hygienically relevant bacteria in drinking water biofilms

The potential of drinking water biofilms to serve as a possible habitat for pathogens was described in various studies (Szewzyk et al., 2000, Flemming et al., 2002, Kilb et al. 2003). .In addition to the general parameters total cell counts, HPC, and colony counts according to the German drinking water ordinance, the presence of hygienically relevant bacteria in drinking water and drinking water biofilms was investigated in the field experiments as well as in the laboratory experiments. In this study, hygienically relevant bacteria were detected within the investigated drinking water biofilms over the entire period of the experiment. Within the drinking water biofilms, 18 % of the culturally analyzed samples from the field experiments were positive for hygienically relevant bacteria. Many studies reported an association between elevated water temperatures and increasing numbers of bacteria (Volk and Joret, 1994, LeChevallier et al., 1996, Liu et al., 2013, Prest et al., 2016). However, in this study only the occurrence of the coliform bacteria (except *E. coli*) correlated with the drinking water temperature when cultural methods were used for quantification. Slightly increasing detection frequencies were

determined with elevated temperatures. This is consistent with the results of LeChevallier et al. (1996) who observed an enhanced occurrence of coliform bacteria at temperatures above 15 °C when investigating the regrowth of coliform bacteria in drinking water in 31 water systems in North America. With FISH, an association of the occurrence of *E. coli* and drinking water temperature as well as the surface material was observed in this study. The occurrence of FISH-positive *E. coli* increased with elevated temperatures (25 % to 63 %) in biofilms grown on PE80/100. In an earlier study, Silhan et al. (2006) reported a significant influence of temperature and pipe material on the survival of *E. coli* in drinking water distribution pipes. In their study, pipes of different materials (galvanized steel, copper, PE and cross-linked PE (PEX)) were filled with drinking water and inoculated with *E. coli*. Survival of *E. coli* in the water phase was longer at 15 °C than at 35 °C. In contrast to the present study and many other studies, which proved *E. coli* to be able to incorporate into biofilms (Mezule et al. 2012; Juhna et al. 2007a; Juhna et al. 2007b; LeChevallier et al. 1987), no *E. coli* was detected within any of the biofilms analyzed in the study of Silhan et al. (2006). A reason for this might be the competition with the indigenous microflora in these biofilms. Banning et al. (2003) also suggested a too competitive indigenous microflora as a reason for a reduced persistence of *E. coli* in ground-water derived biofilms.

In the laboratory, incorporation (presence of the target organisms in the biofilms one day after inoculation) and persistence (maximal time of detection of the target organisms during the experiment) were monitored using cultural methods and culture-independent FISH. Quantification was conducted prior to inoculation, as well as 1, 7, 14, 21, and 28 days subsequently. All target organisms integrated into the established drinking water biofilms. The ability of pathogenic bacteria to integrate into drinking water biofilms was demonstrated in several studies (Taylor et al. 2013; Stewart et al. 2012; Moritz et al. 2010; Bressler et al. 2009; Lethola et al. 2007; Kilb et al. 2003). This study also proved that hygienically relevant bacteria are capable of incorporating into already existing drinking water biofilms. Moreover, they are able to persist within these biofilms. Depending on

the strain, incorporation as well as persistence of the target organisms were influenced by temperature, nutrient availability, and material. In this study, the material only slightly influenced incorporation and persistence of all four target organisms. However, the influence of plumbing materials on the incorporation of pathogens into biofilms was described in several studies. Rogers et al. (1994b) stated that the promoting effect of certain plumbing materials on biofilm growth is evident. They investigated the influence of different materials on TOC when incubated in water and reported elevated TOC-concentrations in the water after incubation of ethylene propylene, polyethylene and latex. However, in their opinion leaching of nutrients was not the major reason for the effect on growth of biofilms but rather the surfaces of the plumbing materials. Crevices and hollows on the surface make it easier for organisms to adhere to the surfaces and successively produce biofilms. Hambsch et al. (2014) investigated the growth of *P. aeruginosa* on different materials (polyethylene, polypropylene, polyvinylchloride, rubber material (EPDM)). They reported growth of *P. aeruginosa* on each of the tested materials, which is coherent with the results of the present study. However, in absence of previously established drinking water biofilms EPDM was reported to be most promoting for growth of *P. aeruginosa*. When exposed to surface material covered with an established biofilm, incorporation efficiency decreased especially on EPDM. The promoting effect of elastomeric materials on the growth of *L. pneumophila* was demonstrated earlier by numerous studies (Benölken et al. (2010), Colbourne and Ashworth (1986), Schönen (1986)) and Colbourne (1985) reported a supporting effect of ethylene propylene polymer on the growth of *P. aeruginosa* already 30 years ago.

In the laboratory experiments, no *E. coli* and *K. pneumoniae* were detected culturally and with FISH prior to inoculation. Incorporation of *E. coli* and *K. pneumoniae* was influenced by temperature. At temperatures above 21 °C the number of both, culturable *E. coli* and culturable *K. pneumoniae* decreased under oligotrophic conditions, which is consistent with the promoted survival of *E.coli* at 15 °C compared to 35 °C observed in the study of Silhan et al. (2006). However, this trend was not observed under copiotrophic

conditions, indicating that nutrient availability affects survival of *E. coli* to a bigger extent than temperature. Vital et al. (2012) investigated the influence of nutrient availability and temperature on the competition of *E. coli* with autochthonous bacterial communities in drinking water. They observed enhanced ability of *E. coli* to compete with other drinking water bacteria at higher AOC contents. Juhna et al. (2007) investigated the influence of phosphorus on the survival of *E. coli* in drinking water. They observed that the addition of phosphorus corresponded with enhanced cultivability of *E.coli* in drinking water biofilms. This has to be taken into account when evaluating the effects of plumbing material used in this study since EPDM and PE are known to release phosphorus. Van Nevel et al. (2013) described the effect of nutrient availability on *Pseudomonas putida* which is closely related to *P. aeruginosa*. They stated, that the addition of acetate as a form of an easily degradable carbon source did not result in the expected enhanced growth or survival of *P. putida*. However, combined with phosphate and nitrogen, growth and survival of the pathogen was promoted. Szabo et al. (2006) also mentioned the importance of nutrients for biofilm associated *K. pneumoniae*. They reported that not only shear forces resulted in detachment of the bacteria, but also competition for nutrients with other organisms in the biofilm. The influence of nutrients on the culturability of bacteria was demonstrated in the study of Silhan et al. (2006). Cells were grown in nutrient rich medium prior to inoculation and entering the VBNC-state might have been a response to the quick change in nutrient availability when exposed to nutrient-poor drinking water without any possibility of adaptation. This assumption is strengthened by the results of a study of Camper et al. (1996). In their study, they investigated the influence of growth conditions and substratum composition on the persistence of coliforms in biofilms grown under oligotrophic conditions. They found that coliforms with the lowest growth rate showed the longest persistence in the investigated biofilms. In the study of Silhan et al (2006), the bacteria were not able to adapt to drinking water conditions prior to inoculation, but were directly transferred

from a nutrient rich medium to nutrient poor drinking water possibly resulting in the transition from a cultural to the VBNC-state.

Neither *E. coli* nor *K. pneumoniae* were detected culturally at 25 °C whereas they were detectable using culture-independent FISH. Increasing total cell counts enhanced the incorporation of cultural as well as FISH-positive *E. coli* on PE80 under both, oligotrophic and copiotrophic conditions indicating that the present microflora had synergistic effects on the incorporation of *E.coli* in the investigated drinking water biofilms. For *K. pneumoniae* this trend was only observed with FISH and on PE80. Cultural detection of *P. aeruginosa* and *L. pneumophila* was only possible after inoculation. However, with FISH, both organisms were detected in the established drinking water biofilms. No influence of temperature and nutrient situation was observed for any of the two pathogens within the established drinking water biofilms prior to inoculation. However, incorporation of *P. aeruginosa* was influenced by temperature. On PE80, the number of culturable *P. aeruginosa* increased with elevated temperatures. Nutrient addition weakened this effect. On EPDM, the number of culturable as well as FISH-positive *P. aeruginosa* correlated positively with increasing temperature regardless of the nutrient situation. On PE80, the number of culturable *P. aeruginosa* slightly decreased with increasing total cell counts under oligotrophic conditions. The same trend was observed for the number of FISH-positive *P. aeruginosa* on EPDM under copiotrophic conditions. The incorporation of *L. pneumophila* was hardly influenced by temperature. Numbers of culturable *L. pneumophila* increased between 8 °C and 12 °C and remained constant between 12 °C and 29 °C with exceptions at 21 °C and 25 °C. No culturable *L. pneumophila* were detected at 21 °C regardless of nutrient availability and material. At 25 °C the number of culturable *L. pneumophila* decreased on both materials. On EPDM, the number of FISH-positive *L. pneumophila* decreased at temperatures above 21 °C under oligotrophic conditions. On PE80, the number of FISH-positive *L. pneumophila* increased with an increasing number of total cell counts. In all experiments, numbers of

the target organisms were at least one log unit higher with FISH compared to cultural methods. These results are in accordance with Moritz et al. (2010). In their study, numbers of culturable *P. aeruginosa* were significantly below the numbers obtained with FISH. In the same study, similar results were observed for *L. pneumophila*. Various studies described the discrepancy between culturally derived concentrations of *L. pneumophila* in drinking water biofilms and FISH-positive results (Långmark et al. 2005; Lehtola et al.,2007; Gião et al. 2009, Gião et al. 2015). Li et al. (2014) mentioned this possible underestimation of viable cells in their review on the importance of VBNC-state of pathogens. The ability of the target organisms to enter the VBNC-state, which was demonstrated in various studies (Aurass et al. 2011; Arana et al. 2010; Bjergbæk and Roslev, 2005; Garcia-Armisen and Servais, 2004; Villarino et al. 2000), might be a possible explanation for the higher cell numbers with FISH in this study. In the present study, the transition from the diluted broth into the drinking water might have induced the VBNC-state. However, it cannot be excluded, that rRNA of dead or lysed cells was targeted by the FISH oligonucleotide probes applied in this study leading to false positive results. The possible detection of rRNA of dead or injured cells was described earlier (Cenciarini et al. 2008, Rochelle et al. 2011). Moreover, Prescott and Fricker (1999) assumed that rRNA is detectable within cells long after cell death and therefore suggested FISH to be inappropriate to distinguish between viable and dead cells. Also, the physiological state of the possibly of the starved target organisms can influence the efficiency of hybridization (Douterelo et al. 2014). Nevertheless, the FISH-method was chosen in this study to gain information about the viability of the target organisms in the investigated drinking water biofilms because many studies proved this method to be appropriate for the detection of VBNC-cells. For *P. aeruginosa* a negative correlation between the drinking water temperature and the detection frequency of FISH-positive cells was observed in this study. This is similar to the results described by Arana et al. (2010). In their study, they investigated the effect of temperature and nutrient

deprivation on the survival of *E. coli* and *Pseudomonas fluorescens*. They reported an inverse correlation of the survival of *P. fluorescens* at higher temperatures. Culturability significantly decreased at 25 °C and 37 °C compared to 5 °C and 15 °C. Moreover, the fraction of VBNC cells was clearly dominant at 5 °C and 15 °C, but decreased significantly at 25 °C and 37 °C, whereas the fraction of nonviable cells significantly increased at the higher temperatures. Contradictory results were obtained in a study performed by Hambsch et al. (2014). In their investigations regarding the survival of *P. aeruginosa* in drinking water systems, they reported a positive correlation between drinking water temperature and the occurrence of *P. aeruginosa*. At 10 °C, growth of *P. aeruginosa* in pure cultures was significantly lower than at 15 °C or 20 °C. The addition of nutrients only showed a minor effect on growth of *P. aeruginosa*. However, the exposition to non-sterile drinking water present in the drinking water resulted in decreasing numbers of *P. aeruginosa* possibly related to competition with the mixed bioscoenosis. The influence of autochthonous microorganisms in biofilms was demonstrated by Gião et al. (2011). They investigated the interaction of *L. pneumophila* with other species present in drinking water biofilms. *Sphingomonas* sp. as well as *Acidovorax* sp. were shown to have antagonistic influence on the culturability of *L. pneumophila*. This corroborates the results of an earlier study performed by Toze et al. (1990). They demonstrated the inhibiting impact of heterotrophic bacteria present in chlorinated drinking water on growth of *L. pneumophila*.

As a resume of the various studies described above and the present study, it clearly has to be mentioned, that very complex interactions took place in the investigated microenvironments and that for each system various important parameters such as temperature, nutrient availability, autochthonous microorganisms, etc. has to be taken into consideration for the evaluation of pathogen-associated health risks.

5.3 Influence of temperature on population diversity in drinking water biofilms

The influence of temperature on the population diversity of drinking water biofilms grown on EPDM in the field as well as in the laboratory experiments was analyzed by DGGE. Therefore, 16S rDNA fragments of the isolated DNA from the drinking water biofilm samples was separated in acrylamidic gels in order to encompass possible impacts of temperature on population dynamics. In the filed experiments, the mean similarity of the biofilm populations for the individual climatopes was in the range of 66 % to 84 % depending on the seasons, which were compared (

Table 16). Biofilm populations showed the highest similarity when both, the difference in temperature and the difference in exposure time were lowest. However, similarities of the biofilm populations were only 8 % less when the difference in temperature between the seasons was highest (10.7 °C). Biofilm populations were less similar between summer and autumn when Δ T was 7.9 °C (18 % less similarity). Biofilms established in the annular reactors in the laboratory were investigated, respectively. Band patterns of biofilms grown in the annular reactors for six weeks under oligotrophic as well as copiotrophic conditions at different temperatures were compared after defined exposition times to elucidate the influence of temperature on population diversity in drinking water biofilms. It was observed that biofilm populations showed the highest similarity within the same reactor at the same temperature. Moreover, similarities were higher at copiotrophic conditions than at oligotrophic conditions. However, when biofilm populations were analyzed at different temperatures, increasing Δ T did not correlate with increasing biofilm diversity. I.e. for example, that there was no higher similarity observed between the biofilm populations when biofilms were compared at 8 °C and 12 °C or at 8 °C and 23 C.

The effect of temperature on biofilm populations in drinking water was investigated in

several studies. Ling et al (2016) investigated the diverstity of drinking water biofilms in an urban drinking water distribution system over 2 years. They collected 213 biofilm samples and 20 samples from tap water. Among other parameters they determined the correlation between water temperature and varieties in biofilm communities and observed a seasonal variation in biofilm communitites. They hypthesized that in the investigated DWDS water temperature was the major effect influencing bacterial composition of the biofilms and other engineering factors had a lower impact on biofilm diverstity. As a possible reason they assumed that biofilms in DWDS are more resistant to perturbations and disinfection, because they establish and persist for a long time and are protected by their EPS. Pinto et al (2014) investigated bacterial community dynamics in drinking water of a drinking water distribution system in Ann Arbor. They mentioned that localized effects (e.g. pipe age, pipe material, water demand, etc.) also have to be taken into consideration when bacterial community of a drining water distribution systems is investigated. However, they noticed, that temporal effects have a much greater impact on bacterial communities than the spatial effects. Analysis of similarity (ANOSIM) and permutational analyses of variance (PERMANOVA) also revealed, that temporal effects (month and season) were the major factors influencing the bacterial community dynamics and not locations of the drinking water system. Moreover, Pinto et al (2014) observed a cyclical pattern of the bacterial community within the investigated drinking water system.They detected a shift in the bacterial community from summer to winter and back to summer. Bacterial communites in the summer showed no association of the operational taxonomic units (OUT) with each other, but only with the two dominant OTUs and were characterized by very low network densitiy. In winter, there were less OTUs, but those were well connected and showed a significantly higher network densitiy. Possible reasons for this might be found in variations in water quality (nutrient availability) and water temperature. Lührig et al (2015) analyzed the bacterial community of drinking water biofilms grown in a drinking water distribution system in Southern Sweden by means of next generation sequencing

of the 16S r RNA genes. Sampling was done in March, April, and June. Biofilms of two parallel installed water meters as well as additional drinking water biofilms were investigated. The results of their study showed, that in the investigated drinking water distribution system, differences in the bacterial communites were mainly caused by ecological factors and by the age of the biofilm. In the study of Zlatanović et al (2017) differences in biofilm populations within the same drinking water distribution system were explained by different local conditions and consumption behavior. Among the pipe material, Douterolo et al (2016) mentioned the source water to be considered as an important factor for bacterial dynamics in drinking water systems. Since drinking water treatment plants are not capapble of removing all microorganisms from the source water, bacteria from the source water may enter the drinking water distribution system. Different qualities of the source water regarding temperature and nutrients can then favour different bacterial communities. Changes in temperature and nutrient availability in the source water are more distinct for surface water as a source for drinking water than for ground water. In the present study the drinking water was finished river water. Similarities of the drinking water biofilm communities in the drinking water distribution system were highest within the same climatopes when Δ T was lowest indicating that the microclimate of the individual climatopes and drinking water temperature were the main factors influencing the investigated biofilm. However, it has to be mentinoned that the similarities did not decline significantly for higher Δ T. Moreover, the influence was more apparent under oligotrophic conditions rather than when nutrients were added. An explanatrion for that might be a correlation between nutrient availability and bactierial diversity.

The different findings of the mentioned studies above reveal how complex the interactions in drinking water distribution systems are and that one parameter such as water temperature cannot be seen as the only influencing factor.

5.4 Conclusion

This study provides information about the cabability of hygienically relevant bacteria to incorporate into drinking water biofilms and persist whithin these and the impact of drinking water temperature on the survival of these bacteria within the drinking water as well as in the drinking water biofilms to elucidate possible impacts of global warming induced climate change on drinking water quality. .

It was demonstrated that hygienically relevant bacteria were able to incorporate and persist within drinking water biofilms at various water temperatures under defined laboratory conditions. All the test strains were proved to incorporate into the 14 days old drinking water biofilms independent of the water temperature. Persistence was strain- dependent and influenced by nutrient availability rather than water temperature. In addition to the ability of the target organisms to remain cultivable, the transiton from the culturable to the VBNC state was investigated by means of FISH.

To gain information about the impact on water temperature on hygienically relevant bacteria in drinking water and drinking water biofilms, investigations were also performed in a drinking water distribution system. Therefore, biofilm reactors were installed into a drinking water system as a bypass. In order to observe possible influences of climatic conditons, eight different climatopes representing different microclimates were choosen for the field study. Over an experimental period of 10 months biofilms were established and sampled in three sampling campaigns (Nov. `10, March `11, Sep. `11). It was demonstrated that within the different climatopes, water temperature only had a marginal impact on the water quality and the target organisms. Rather than the water temperature, the pipe material influenced water quality. The effect of different pipe materials on hygienically relevant bacteria and biofilm formation was described in various studies (Rogers et al. 1994, Moritz et al. 2010, , Roeder et al. 2010, Michalowski 2012) and results were coherent with the findings of the present study. In the laboratory

as well as in the field experiments bacterial concentrations were highest on EPDM, followed by PE and stainless steel.

A low impact of temperature on coliform bacteria present in the drinking water biofilms was observed in the field experiments (increase from 4 % in winter to 17 % in summer). However, for the evaluation of the results it has to be taken into account that numbers of the target organisms were partially very low (single digit range). In the drinking water hygienically relevant bacteria were only present in three of the 24 samples and absent in the repetition samples. Numbers varied from one to two cfu/ 100 ml and no influence of water temperature was observed. However, using FISH, the target organisms were detected in all samples which were taken. This indicates, that in a drinking water distribution system hygienically relevant bacteria may be present in the VBNC state and a transition into a cultivable state might be possible under favourable conditions. The ability of hygienically relevant bacteria to enter the VBNC state and resuscitate under favourable conditions has been demonstrated in numerous studies (Oliver 2010; Pinto et al. 2011, Mezule et al. 2012, Li et al. 2014).

In the present study, the influence of water temperature on the detection frequency of the target strains by means of FISH was strain dependent in the field experiments. The detection frequency of FISH-positive *E. coli* increased with elevated temperature and on EPDM (EPDM> PE80/100 > stainless steel). The detection frequency of *P. aeruginosa* decreased with rising temperatures whereas the detection frequency of *Legionella* spp. was independent of season and material. The detection frequency of *L. pneumophila* was highest in winter.

In the laboratory experiments, neither *E. coli* nor *K. pneumoniae* were detected culturally and with FISH prior to inoculation. However, incorporation of *E. coli* and *K.pneumoniae* was influenced by temperature and nutrient availability. For *P. aeruginosa* and *L. pneumophila* the impact of drinking water temperature on the incorporation also did not follow a distinct pattern. Neither of both strains was culturally detectable within the drinking water biofilms prior to inoculation, but with FISH both organisms could be

detected in the established drinking water biofilms. Incorporation of *P. aeruginosa* was influenced by temperature as well as pipe material and nutrient situation. The incorporation of *L. pneumophila* was only marginaly influenced by temperature. Persistence of the target strains within the drinking water biofilms was only influenced by temperature for *L. pneumophila*. Nutrient addition significantly shortened the persistence of cultural *L. pneumophila*. For *E.coli*, *K. pneumoniae*, and *P. aeruginosa* no influence of drinking water temperature on the persistence wihin the drinking water biofilms was observed using cultural methods as well as culture-independent FISH. However, for *E.coli* and *K. pneumoniae* an increase in the duration of dectection was detected when nutrients were added. This effect was not observed for *P. aeruginosa*.

The present study provided information about the influence of drinking water temperature on hygienically relevant bacteria. It was demonstrated that the influence of drinking water temperature on the survival of hygienically relevant bacteria in drinking water biofilms was strain-dependent and can only be taken into consideration as one factor amongst others affecting incorporation and persistence of the target strains. Nutrient availability and pipe material often had a major impact on the pathogenic organisms. Drinking water distribution systems are very complex systems and the analysis of pathogens and their behavior within these systems is very demanding. The local condtions may change within a distribution system and different microclimates mayrepresent divers environmental conditions hygieneically relevant microorganisms have to adapt to. The local microflora can be as important as the water demand, pipe age, and pipe material (Pinto et al. 2014, Zlatanović et al. 2017). Research in the field of the influence of global warming induced climate change on microbial drinking water quality is very complex and therefore has to be thoroughly planned and the focus of the study has to be choosen very precisely to gain valuable information in this very complex field of study.

6 Literature

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Appendix

Publikationsliste

2015	"Influence of water temperature on hygienically relevant bacteria in drinking water biofilms" **J. Wagner** , S. Grobe , G. Schaule , J. Wingender. Poster auf der IWA specialized conference "Biofilms in drinking water systems", Arosa
2014	„Sicherung der Trinkwasserqualität bei der Wasserverteilung bei veränderten Bodentemperaturen" Grobe, S., **Wagner, J**., Wingender, J. 2014 dynaklim Publikation No. 52
2014	„Einfluss der Wassertemperatur auf das Überleben hygienisch relevanter Bakterien in Trinkwasserbiofilmen." Grobe, S., **Wagner, J**., Schaule, G., Wingender, J. Postervortrag und Beitrag im Tagungsband der Wasser 2014 - Jahrestagung der Wasserchemischen Gesellschaft, Haltern am See
2014	"Influence of water temperature on hygienically relevant bacteria in drinking water biofilms" **Wagner, J**., Grobe, S., Schaule, G., Wingender, J. Poster auf der Biofilms VI Konferenz, Wien.
2013	"Influence of temperature on incorporation and persistence of culturable and non-culturable *Escherichia coli* and *Klebsiella*

	pneumoniae in drinking water biofilms" S.Grobe, **J. Wagner**, G. Schaule, J. Wingender Poster auf der Konferenz "How dead is dead III - Life Cycles "06. – 07. Juni, Berlin
2012	„Mikrobiologische Trinkwasserqualität - Welche Folgen hat der Klimawandel?" Grobe, S., **Wagner, J**., Schaule, G., Flemming, H.-C., Wingender, J. Poster und Beitrag im Tagungsband der Wasser 2012 - Jahrestagung der Wasserchemischen Gesellschaft, Neu-Ulm
2009	Genes involved in copper resistance influence survival of *Pseudomonas aeruginosa* on copper surfaces Elguindi, **J.; Wagner**, J.; Rensing, C. JOURNAL OF APPLIED MICROBIOLOGY Volume: 106 Issue: 5 Pages: 1448-1455

Erklärung

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

„ Influence of temperature on the survival of hygienically relevant bacteria in drinking water and drinking water biofilms"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Dissertation in dieser oder ähnlicher Form bei keinem anderen Promotionsverfahren eingereicht wurde.

Essen, im Mai 2020 (Janine Wagner)

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.