Technische Universität Berlin

Investigation of mature biofilm populations in the distribution of drinking water with attention to bacteria of hygienic relevance

vorgelegt von
Diplom-Biologin Bianca Conradi
aus Greetsiel

von der Fakultät III-Prozesswissenschaften
der Technischen Universität Berlin

zur Erlangung des akademischen Grades
Doktorin der Naturwissenschaften
-Dr. rer. nat.-

genehmigte Dissertation

Promotionsausschuss

Vorsitzender: Prof. Dr. rer. nat. Wolfgang Rotard, TU Berlin

Berichter: 1. Prof. Dr. rer. nat. Ulrich Szewzyk, TU Berlin

Berichterin: 2. Prof. Dr. rer. nat. Isolde Röske, TU Dresden

Tag der wissenschaftlichen Aussprache: 31.03.2011

Berlin 2011

D83

meiner Großmutter E. Hübel

1 Introduction

Nowadays, despite of the good drinking water quality in industrialized countries, diseases related to drinking water have been reported. For example the “Centers for Disease Control and Prevention” in the U. S. reported that in 1999 and 2000 twenty outbreaks of waterborne disease could be associated with pathogens (Centers for Disease Control and Prevention 2002). In addition, the World Health Organization described an outbreak of *E. coli* O157 in Walkerton, Ontario, Canada in 2000 that resulted in the death of five people and more than two dozen hospitalized people (Leclerc et al. 2002; WHO 2000). This remains of the importance of high microbiological quality of drinking water.

Despite the author is aware that in addition to the microbial item the chemical safety of drinking water is the second point of major concern (Anonymous 2008; Heberer 2002), this study focussed on the impact of bacteria on quality of drinking water. Regarding the subject of microbiological drinking water quality the reviews of Szewzyk et al. 2000, Leclerc et al. 2002 and the WHO guidelines for drinking water quality of 2008 give a comprehensive overview of pathogens belonging to bacteria, viruses, protozoa, and Helminths with concern in drinking water (Anonymous 2008; Leclerc et al. 2002; Szewzyk et al. 2000). Szewzyk et al. 2000 divided bacterial pathogens in two groups, those with fecal origin and those that originated in water or soil. The first group comprises bacteria like *Campylobacter* species, enterohemorrhagic *Escherichia coli*, *Salmonella* species, *Vibrio cholerae*, *Yersinia enterocolitica*, or *Helicobacter pylori*. The second group of pathogens inhabit water or soil and are transported from these habitats into drinking water. These bacteria are able to grow if parameters are getting appropriate and include representatives of *Legionella* species, *Pseudomonas aeruginosa*, *Aeromonas* species, *Acinetobacter* species, and environmental Mycobacteria. (Szewzyk et al. 2000) Furthermore, *Mycobacterium avium*, *Legionella pneumophila* and *Legionella* species as well as *Campylobacter* species and *P. aeruginosa* have been shown to survive in drinking water biofilms (Buswell et al. 1998; Lehtola et al. 2007b; Moritz et al. 2010; Rogers et al. 1994).

In oligotrophic environments as drinking water with a low nutrient content of the free water phase the formation of biofilms at solid-liquid interfaces has been described as

typical (Fletcher and Marshall 1982). Moreover, it has been found for example by Van der Wende et al. and Block et al. that biofilms in drinking water systems play an important role in contamination of the water phase (Block et al. 1993; van der Wende and Characklis 1990). The rough calculation of Flemming that approximately 95 % of the biomass in distribution systems is found in the biofilms that are not routinely examined and only 5 % in the water phase reflect the importance of the biofilms (Flemming 2003).

In the last decades, a lot of work has been done in different natural and artificial drinking water systems. These previous investigations on drinking water biofilms differed in four main aspects: The drinking water resource, systems in which the biofilms were exposed, the substratum for development of microbial biofilms, the time surfaces were exposed, and the methods applied to investigate the biofilm. For an overview a selection of drinking water studies will be described.

The systems used for development or exposition of biofilms can be divided in three categories. The smallest systems are devices of a portable dimension which allow a simple exchange of biofilm coupons and can be installed easily in different locations. Pedersen et al. used a rectangular box (polycarbonate and aluminium) with an o-ring sealed lid, a test pile and two diffusors inside. They investigated biofilm development on hydrophilic stainless steel and hydrophobic PVC surfaces for 4 to 5.6 months (Pedersen 1990). Donlan et al. exposed test cylinders of cast iron in a device incorporated in water mains for up to 3.8 months (Donlan et al. 1994). Furthermore, Hallam et al. fitted 45 cm pipe sections of MDPE, PVC, or cement between two PVC end plates and exposed them for 21 days (Hallam et al. 2001). Niquette and co-workers constructed a PVC cylinder for the exposition of coupons. They investigated different materials that are used to different extent in distribution systems (PVC, PE, cemented steel, asbestos-cement, cemented cast iron, tarred steel and grey iron) and exposed them for 2 to 8 months. (Niquette et al. 2000) The materials PVC and cement were also investigated by Camper et al.. In addition, ductile iron and an epoxy material were exposed in an annular reactor for 3 to 8 months. (Camper et al. 2003) Zacheus et al. constructed a combined system of a basin-like device with coupons and pipe sections. They exposed the coupons of PVC and stainless steel from one week to 4.4 months and the PVC and PE pipe sections up to about 5.4 months. (Zacheus et al.

2000) A cylinder-shaped stainless steal device named “Robbin`s device” or modifications of this device were used by several investigators. Kalmbach et al. exposed the materials PE, PVC and as a non supporting material glass in this system. Incubation was done 14 to 70 days. (Kalmbach et al. 1997b; Kalmbach 1998) An incubation time of 8 to 15 days for drinking water biofilms is found in Schwartz et al. who investigated steel and copper additionally to HDPE and PVC (Schwartz et al. 1997; Schwartz et al. 1998). A modified Robbin`s device was also used by Kerr et al.. They exposed cast iron, MDPE, and PVC up to 10 months. (Kerr et al. 1999)

Others used pipe systems of a greater dimension in which operating conditions were expected to be more similar to the distribution system. Percival et al. 1998 studied different grades of stainless steal that were exposed to drinking water for one and two years. The stainless steel coupons were sorted horizontal to the flow direction in the middle of eight centimetre stainless steel pipe sections. (Percival et al. 1998a; Percival et al. 1998b) Martiny et al. also used a pipe system and investigated biofilms on coupons installed in test plug modules from one day up to approximately three years (Martiny et al. 2003). In a pilot-scale system with removable pipe sections of PVC and iron pipes Norton et al. exposed the sections one to eight weeks before scraping and washing the biofilms from the interior of PVC and iron pipes (Norton and LeChevallier 2000). One month biofilms of a pilot plant with six pipe loops in which removable coupons of PVC and cement were exposed have been investigated by Block et al. (Block et al. 1993). Frias et al. investigated exposed PE surfaces up to 66 days in a pilot system of 200 m length with a diameter of 1.5 cm (Frias et al. 2001). Lethola et al. used a pilot scale system of PE and copper pipes for exposure of biofilms for approximately five months (Lehtola et al. 2006). Deines et al. investigated up to 11 days old biofilms on coupons developed in a pilot pipe loop system constructed from actual distribution system PE pipes (Deines et al. 2010).

The last category describes pipe sections directly taken out of the drinking water distribution system. An early study was done by Olson and Ridgeway in 1981 who investigated approximately 40 years old biofilms developed on cement lined iron and a galvanized iron pipe removed from the distribution system (Olson et al. 1981). Hallam et al. removed HDPE pipes of 18 months and 10 years out of the distribution system (Hallam et al. 2001). Coupon samples of 8 to 90 years old cast iron, cement lined

cast iron, ductile iron, and asbestos cement pipes were sampled by LeChevallier et al.. They also investigated deposits obtained from mechanical cleaning (pigging) or scraping of pipe surfaces with a sterile spatula. (LeChevallier et al. 1987)

Traditionally, for the description of drinking water quality cultivation techniques have been used and have been transferred to biofilm investigations. They described the heterotrophic bacteria or focussed on indicator or pathogenic bacteria. Cultivation has been used in several of the above described systems and further studies for investigations of drinking water biofilms (Block et al. 1993; Carter et al. 2000; Donlan et al. 1994; Dutkiewicz and Fallowfield 1998; Percival et al. 1998b). Since scientists in microbial ecology got aware of the limitation that cultivation only detects a small percentage of the total bacterial population, they looked for methods to circumvent this bias for phylogenetic identification and quantification of bacteria (Amann and Kühl 1998; Ward et al. 1990). One favourite method for determination of total bacterial cell counts is staining of double stranded DNA by fluorescent dyes like DAPI or acridine orange and subsequent epi-fluorescent microscopy (Block et al. 1993; Schwartz et al. 1998; Zacheus et al. 2000). For phylogenetic identification Kalmbach et al. and Schwartz et al. used total cell counts in combination with rRNA targeted oligonucleotide probes of different phylogenetic levels (Kalmbach 1998; Kalmbach et al. 2000; Schwartz et al. 1998). The CARD(catalyzed reported deposition)-FISH method with the advantage of enhanced signal intensity was successfully applied by Deines et al. in 3 to 11 days old biofilms (Deines et al. 2010). With further development of molecular techniques in microbial ecology scientists investigated genomic DNA in drinking water biofilms. Schwartz et al. used the polymerase chain reaction in combination with southern blot hybridization for identification of facultative pathogens in a drinking water biofilm (Schwartz et al. 1998). More recently Martiny and co-workers identified bacterial DNA after extraction of total genomic DNA of drinking water biofilms grown on stainless steel by cloning with subsequent sequencing (Martiny et al. 2003). In addition, for comparison of population profiles of different biofilm samples on a rougher phylogenetic level they used a fingerprinting technique. Therefore, after DNA extraction and PCR Martiny et al. used 16S rDNA targeted terminal restriction fragment length polymorphism (T-RFLP) and were able to detect correlations between young (1 to 94 days) and old (571 to 1093 days) biofilms and the population profiles (Martiny et al. 2003). Röder et al. used the fingerprinting technique DGGE (denatur-

ing gradient gel electrophoresis) in disinfection experiments. Drinking water biofilms were grown 10 to 38 months on bacterial growth supporting silicone rubber tubes before disinfection. (Roeder et al. 2010) This technique was also used by Deines in 3 to 11 days old biofilms (Deines et al. 2010). Schmeisser et al. 2003 also analyzed the bacterial population on a rubber-coated valve taken out of the distribution system by a cloning sequencing approach but also did the next step from phyologenetic analysis to the description of the metabolic potential by a metagenome analysis (Schmeisser et al. 2003).

In addition to the above described selection of investigations furthermore work has been done and provided a lot of invaluable insights about biofilms in drinking water. In general different factors influencing the microbiological water quality direct or indirect have been of interest including e. g. water source, concentration and kind of disinfectant, temperature, hydraulic conditions, assimilable organic carbon (AOC), age of biofilms, material used for biofilm development and survival potential of selected bacteria with focus on pathogen potential. Nevertheless, the findings of the investigation of the last decades left unanswered questions:

- Is the bacterial composition of old biofilms comparable to the primarily described young biofilms?
- Are biofilms of the drinking water distribution system a reservoir for opportunistic pathogenic, pathogenic or indicator bacteria and therefore a risk to human health?

In the present study a biofilm reactor was constructed out of PE pipe sections with a dimension that is routinely used in the Berlin distribution system. In each pipe section, coupons of glass, copper, PE, stainless steel, and PVC were installed on the inner surface in horizontal direction to the flow. The system was supplied directly by a drinking water distribution system pipe. In this reactor system materials were exposed 6 to 24 months and determined as “middle aged biofilms”. Furthermore, pipe samples were taken from the actual distribution system in the Ruhrgebiet and Berlin. The investigation focussed mainly on pipe materials of relevance in Germany like cast iron, grey cast iron, and cement lined cast iron as well as PE and PVC. These pipe samples have been exposed for 8 to 99 years in the distribution system and are therefore named as “old biofilms” in this study. For investigation of the mature

biofilms of these systems it was the aim to get a comprehensive insight by use of cultivation in combination with molecular techniques.

This work was part of the cooperation project with focus on “Detection of growth and the contamination risk of biofilms in distribution of drinking water” (Flemming 2003). The dimension of the project and the need of specific methods and experience resulted in the organization in a network of specialized scientists and drinking water suppliers in Germany. The relevant organisms and project partners are summarized in the subsequent table.

Investigated microorganisms	**Project partner**
Pseudomonas sp., *Legionella* sp., *E. coli*, coliform bacteria	H.-C. Flemming, J. Wingender Universität Duisburg
Mycobacteria	R. Schluze-Röbbecke, B. Ilg Universität Düsseldorf
Aeromonas sp.	R.H.W. Schubert Universität Frankfurt
Campylobacter sp. and *Yersinia* sp.	I. Feuerpfeil, A. Hummel Federal Environmental Agency Germany, Bad Elster
Helicobacter pylori	M. Exner, A. Rechenburg Universität Bonn
Cryptosporidium and *Giardia*	M. Exner, C. Koch Universität Bonn
Viruses	K. Botzenhart Universität Tübingen
Amoeba	R. Michel, R. Hoffmann Ernst-Rodenwald-Institute Koblenz
Fungi	E. Göttlich, H.-C. Flemming IWW, Mülheim a. d. Ruhr

2 Material and Methods

2.1 Reactor systems at different locations

2.1.1 Reactor system Berlin (Germany)

2.1.1.1 Setup and function

The Berlin reactor system was designed in cooperation with the Berliner Wasserbetriebe (BWB, the local water supplier in Berlin) and built up in a technical supply station of the Berliner Wasserbertriebe in Berlin Lichterfelde. The drinking water influx in the system was supplied via a 25 m (diameter: 8 cm) ductile cast pipe from the main pipe near the supply station. After passage through the system the water was discarded. Construction and function had to be as equivalent as possible to the real drinking water distribution system. Only material routinely used in drinking water distribution systems was utilized. In PE pipe sections with an inner diameter of 10 cm and a height of each segment of 15.5 cm (hole column of 5 segments: 77.5 cm), the coupons (consisting of PE-HD: polyethylene high density, PVC: polyvinylchloride, Cu: copper, V2A-steel, and glass) were fit to the inner surface of the segments in flow direction (figure 1). PE, PVC, Cu and V2A-steel are materials which can be found in the dinking water distribution system and in house installations. The coupons were treated by the laboratory of the BWB with the purifier Extron (Merck, Germany) and rinsed with sterile destilled water, incubated for some minutes in isopropanol (70 %) and air dried before being inserted in the segments. Each of the five reactors arranged in series included one hundred coupons of the different materials that were allocated to the project partners. The valves at the inlet and outlet of each reactor allowed sampling of one reactor without the need of draining all reactors. Each reactor was equipped with a fire resistant sampling valve at the outlet for taking bulk water samples. The median water flow was 5 m³/h (range 0.5 to 45 m³/h) to day 134 and 0.4 m³/h (range 0.05 to 3 m³/h) thereafter. During the first operation phase up to day 110 flushing operations with enhanced flow rate (range 9 to 48 m³/h, lasting 2 to 19 h) were performed 3 to 5 days every week. Furthermore, stagnation operations of 4 to 6.5 h and from day 235 stagnation times of 16.5 to 19.5 h were done. The bulk water phase was regularly investigated for heterotrophic plate counts (GDWR 1990, chapter 2.3.2) and *P. aeruginosa* (DIN EN 12780, chapter 2.3.4). Coupons were exposed to drinking water for 6, 12, 18, and 24 months.

Fig. 1: Overview of the reactor system in Berlin (left). Two PE pipe sections with copper coupons inside (right).

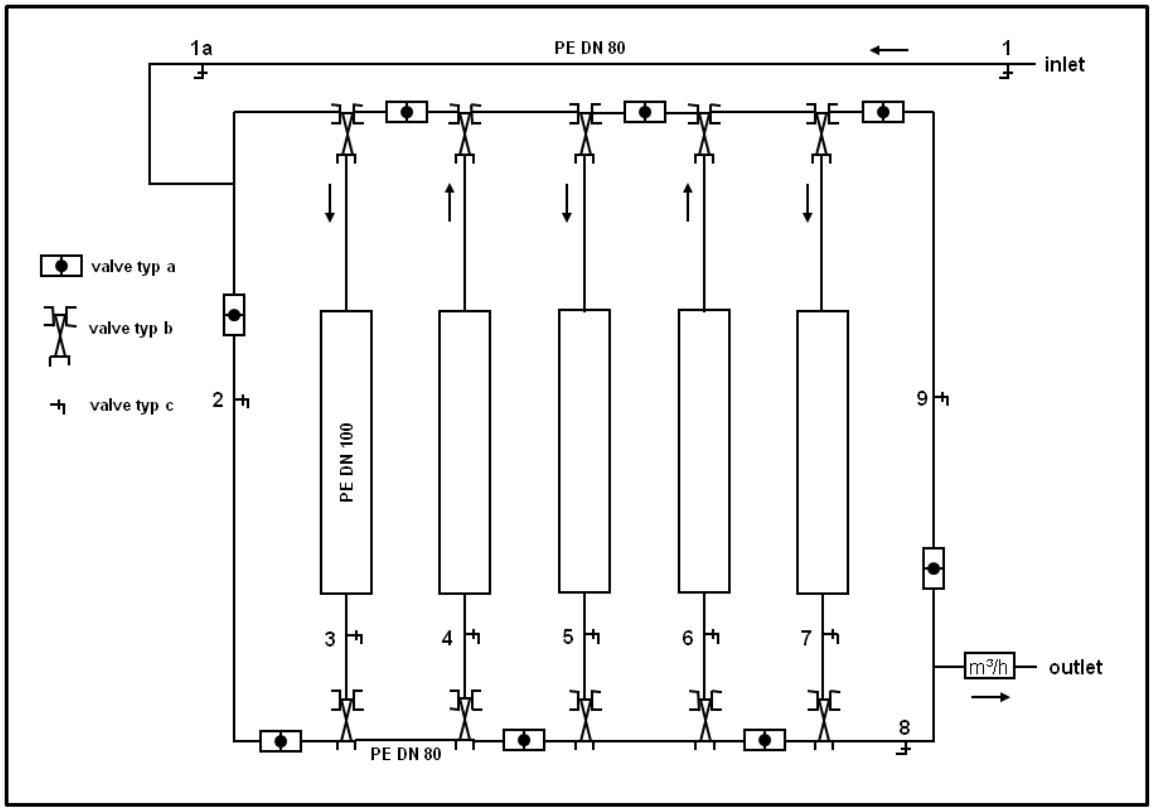


Fig. 2: Layout of the Berlin reactor system with five reactors arranged in series. typ a) valve for regulation of flow direction typ b) valve used during coupon sampling typ c) fire resistant valve used for water sampling

2.1.1.2 Sampling of material coupons

To sample the coupons water flow in the reactor system was stopped but only the sampled reactor was drained. After one reactor with coupons has been taken out an equivalent sterile reactor without coupons was inserted and the water flow was started again. The openings of the sampled reactor were covered with sterile plastic bags to avoid contamination and drying and the reactor was transported to the laboratory. In the laboratory the coupons were released of the segments and inserted into a humid chamber (50 mL centrifugal tubes with some millilitres of drinking water on cellulose paper were autoclaved before use) to avoid drying of the deposits and stored at approx. 8°C until further preparations at the sampling day. To detach the deposits from coupons the drinking water exposed surface (17.25 cm^2) was scraped with a metallic scraper. Afterwards the scraper and the surface were rinsed with a defined volume of sterile drinking water in a sterile petri dish to avoid loss of bacteria. At the end the biofilm suspension was filled in 1.5 mL reaction tubes (Eppendorf, Germany) for further applications.

2.1.2 Reactor systems Duisburg (Germany)

The two reactor systems installed by the Duisburger project partner (working group of Prof. H.-C. Flemming) had basically the same setup and function as the Berlin reactor system. One reactor was located at the outlet of the treatment plant Dorsten-Holsterhausen the other within the distribution system in Gladbeck, approx.. 15 km from the treatment plant Dorsten-Holsterhausen. The treatment plant supplied non-disinfected water form a groundwater source. The material coupons (PE, PVC, Cu, V4A-steel) with a surface area of 16.5 cm^2 were fit to the inner surface of the reactors (PE, height: 760 mm, inner diameter: 100 mm) and the 5 reactors were arranged in series as in the Berlin reactor system. Coupons and reactors were cleaned with methanol and sterile destilled water before installation and the whole system was disinfected with H_2O_2 for 24 h (400 mg/L) before operation. In Dorsten-Holsterhausen flow rate was regulated to 4 m^3/h (velocity: 0.12 m/s). In Gladbeck a flow rate of 2 m^3/h (velocity: 0.06 m/s) was regulated from 6 a.m. to 18 p.m. and during night the

flow was reduced to 0.5 m³/h (velocity: 0.016 m/s). (Flemming 2003; Wingender and Flemming 2004)

2.1.3 Reactor system Lundtofte (Denmark)

Operation and sampling of the pilot plants

In cooperation with Adam C. Martiny of the University of Denmark a third reactor system was investigated. Biofilm and bulk water samples were taken from the distribution system located in Lundtofte, Denmark (Boe-Hansen et al. 2002) and in Berlin, Germany. In the two reactor systems neither disinfectant agents nor ozone or UV irradiation was used. Both systems were supplied with groundwater. Physico-chemical and microbiological parameters of both systems are shown in table 1. All analyses were performed according to standard methods. The material coupons were placed parallel to flow direction to avoid turbulence in the water flow and were exposed to drinking water for more than 6 months. In the German reactor system the materials PVC, PE, and steel were included in the comparative investigations, whereas in the Danish system only steel coupons were positioned.

Bulk water from the inlet (Lundtofte) and the outlet (Berlin) was sampled in 100 mL aliquots and stored on ice until further analysis (max. 2 h). Material coupons of the Danish system were scraped off with a wetted sterile cotton tip and transferred to a test tube containing 10 mL autoclaved, 0.22 μm filtered drinking water. The tube was vigorously shaken for 1 min. German coupons were treated as described in chapter 2.1.1.2. The Danish biofilm and bulk water samples were plated on standard R2A medium (Reasoner and Geldreich 1985), whereas samples from Germany were plated on modified R2A medium (starch replaced by Tween 80, see chapter 2.3.1). All isolated strains from Berlin were tested positive for the ability to grow on standard R2A medium. Plates were incubated for 7 (Berlin) respectively 10 days (Lundtofte) at room temperature (approx. 23°C) and transferred twice on the cultivation medium. One hundred colonies were isolated from each sample, 400 in total. To minimize possible biases introduced by selection, colonies were picked systematically from one side of the plate towards the other (Lundtofte), while colonies in Berlin were picked according to colony morphology.

Tab. 1: Physico-chemical and microbiological parameters of bulk water and biofilm in the pilot plants of Lundtofte (Denmark) and Berlin (Germany).

Parameters[a)]	Lundthofte	Berlin
Biofilm		
Exposure time of coupons [days]	319	197
Plate count[b)], steel [cfu/cm²]	7.7×10^5	1.0×10^5
Plate count[b)], PE [cfu/cm²]	-	1.3×10^5
Plate count[b)], PVC [cfu/cm²]	-	1.4×10^5
Bulk water		
pH	7.6	7.6
Iron [mg/l]	0.04	0.02
Manganese [mg/l]	<0.005	<0.02
Hardness [dH]	17.8	10.9
Calcium [mg/l]	93	95
Ammonia [mg/l]	0.1	0.052
Nitrite [mg/l]	0.03	<0.03
Nitrate [mg/l]	2.5	3.6
Phosphate [mg/l]	<0.02	0.092
Chloride [mg/l]	109	55
Sulfate [mg/l]	11	78
NVOC [mg/l]	2.3	3.6
AOC [µg/l]	6.1	n.d.
Temperature [°C]	12.3	16.3
Flow rate [m³/h]	0.5	1 - 10
Plate count [cfu/mL]	680	265

a)determined by standard methods of the drinking water suppliers b)modified R2A

2.2 Pipe sections from the drinking water distribution system

In Berlin the pipes were sampled in cooperation with the Berliner Wasserbetriebe. After excavation of the selected pipe section the pipe`s outer surface was cleansed with a brush and sterile Milli-Q water, treated with 10 % H_2O_2 for 5-10 min and rinsed again with sterile Milli-Q water. The water supply was turned off and a first cut was done in the pipe to slowly drain the water from inside the pipe. The pipes were cut with different tools according to the pipe material. PVC pipes were cut with a professional hacksaw and the metallic pipes were cut with a professional pipe cutter which avoided any heating of the cutting site. Only the first Berlin test sample BWB I was cut with a circular saw. Cutting tools were cleaned with H_2O_2 and Milli-Q water before application as described above. Water was drained form the excavation to prevent contamination by the re-entering water. After draining of the pipe the second cut was carried out to completely excise the pipe section. Water which drained from the main was pumped away before it could re-enter the pipe and cause contamination. The openings of the pipe section were covered with sterile plastic bags to avoid contamination and drying of the interior. Subsequent the pipe was directly transported to the

laboratory (20 to 40 min) for further treatment. At warm days the pipes outer surface was cooled with cool packs.

2.2.1 Free water samples of the distribution system

Before each pipe sampling water samples were collected at a standpipe which was placed downstream the sampling site. The standpipe was flushed for about 20 min prior to sample collection. Temperature and pH were determined on site. Water samples for microbiological analysis were taken in sterile glass bottles after fire sterilization and short flush of the tap to cool it down.

2.2.2 Treatment of pipe deposits on the inner surface

As described in chapter 3.1.1 there was a great difference in the macroscopic shape of the inner surface. The PVC and cement pipes with less voluminous coatings and the metallic pipes with voluminous irregular coatings. According to these differences appropriate standard treatment protocols were developed with the working group of the Universität Duisburg to ensure equal treatment of the pipe samples in both laboratories. The first 4 to 5 cm at each opening were discarded to minimize contamination of the samples.

2.2.2.1 Treatment of PVC pipes

One opening of the pipe was closed with two layers of sterile plastic bags and fixed strongly with tape (not in contact with the inside). One hundred gram sterile glass pearls (diameter 0.5 cm) and 150 mL of sterile Milli-Q water were filled in. After the second opening was closed with sterile plastic bags as described above the PVC pipe with the glass pearls was shaken and rotated by hand for 10 min. The water phase was decanted, fresh 100 mL sterile Milli-Q water filled in and shaken and rotated again for 10 min. The procedure was repeated until the inner surface was clean by optical inspection. Finally glass pearls were washed two times with 50 mL Milli-Q water. The biofilm suspensions were pooled, homogenized by shaking and subdivided into sterile glass bottles for the participating laboratories.

2.2.2.2 Treatment of metallic pipes

For the metallic pipes a scraping method was used. One opening of the pipe was put in a sterile bowl and the deposits were scraped from the other opening with different kinds of metal scrapers. This was done from both sides of the pipe and in the end the pipe was rinsed with sterile drinking water. If necessary, fragments were crushed with a metal stick. Finally the biofilm suspension was homogenized by shaking and subdivided as described before.

2.3 Analysis of bacterial populations by cultivation techniques

2.3.1 Aerobic cultivation on modified R2A medium

Cultivation of the biofilm suspension was performed on modified R2A medium (Tween 80 instead of soluble starch 0.1 % v/v) as described by Kalmbach (Kalmbach et al. 1999; Reasoner and Geldreich 1985). After scraping and homogenization (chapter 2.1, 2.2) the samples were serially diluted in sterile Berlin drinking water (autoclaved 20 min at 121°C and filtered, 0.2 µm pore size Supor-200 membrane WAT200539, Waters Corporation, Ann Arbor, Michigan, USA) and R2A medium inoculated with 100 µL by the spread-plate method in three to four replicates at 20±2°C in the dark. Colony forming units (CFU) were determined after 7 and 14 days. For further investigations two to three colonies of each morpho-type were selected and transferred to a fresh R2A plate (Tween 80 modified). This was repeated until a pure culture was obtained. Special focus was laid on *Aquabacterium*-like morphologies as described by Kalmbach (Kalmbach et al. 1999). Because they often grow as small plain colonies plates were scanned by a binocular loupe (Zeiss, magnification 8 - 10 times).

2.3.2 Heterotrophic plate counts according to DIN EN ISO 6222

To get an insight in the alteration of the heterotrophic bacteria in the Berlin reactor system, bulk water samples were cultivated as described in the German Drinking

Water Regulations 1990 (GDWR 1990)[1] according to the standard method DIN EN ISO 6222. One mL sample volume was incubated 44 ±4 h at 20°C ±2°C and 36°C ±1°C on a nutrient rich, peptone and meat extract containing medium (DEV) with the pour plate method. The guideline value is 100 CFU/mL. Regular investigations were done by the laboratory of the Berliner Wasserbetriebe (BWB).

2.3.3 *E. coli* and coliform bacteria according to DIN 38 411 K 6

As it is described in the standard method (DIN EN 38 411 K 6 1991), bacteria of the species *E. coli* are inhabitants of the gut of human and endotherm animals. The detection of *E. coli* in water is assessed as indication of a fecal contamination. Coliform bacteria may have a fecal source but are also able to multiply in sewage and surface water. Primary incubation of the water sample was done in 1 % (w/v) lactose bouillon at 36 ±1°C for 24 ±4 h, if negative up to 44 ±4 h. If gas and acid production is noticed subcultures are prepared on Endo-agar or McConkey agar for 24 ±4 h to do further physiological test for discrimination of *E. coli* and coliform bacteria. Investigated sample volume was 100 mL. The limit value for *E. coli* and coliform bacteria is non in 100 mL. Regular investigations were done by the laboratory of the BWB.

2.3.4 Aerobic cultivation of *P. aeruginosa* according to DIN EN 12780

The standard method DIN EN 12780[2] (DIN EN 12780 2002) detects *P. aeruginosa* after membrane filtration (0.45 µm) and incubation for 44±4 h at 36±2°C as colony forming units on a selective cetrimide-containing medium. Typically blue-green colonies (pyocyanin-production) are accepted as *P. aeruginosa* without further characterization. Additionally, colonies not featuring the typical colour on the selective medium have to be further characterized. If they show fluorescence under UV light on the selective medium and are able to produce ammonia out of acetamide they are also ac-

[1]GDWR 1990 – German drinking water regulation 1990 corresponds in German to Trinkwasserverordnung – TrinkwV vom 5. Dez. 1990 (Bundesgesetzblatt I S. 2600) established law from january the first 1991.

[2] In the beginning of the investigations the method was only described as draft standard. Because the approach of this study is identical with the standard method 2002 this version is cited here.

cepted as *P. aeruginosa*. If they are red-brown on the selective medium they have to be oxidase positive, show fluorescence on King`s B medium under UV irradiation, and have to be able to produce ammonia out of acetamide to be identified as *P. aeruginosa*.

2.4 Investigation of the bacterial population by culture independent methods

2.4.1 Total cell counts (TCC) determined by DAPI staining

2.4.1.1 Staining of biofilm suspensions

Biofilm suspensions were obtained from coupons of the reactors or pipe samples (chapter 2.1, 2.2). Usually, 1 mL of biofilm suspension was mixed with 100 µL of DAPI stock solution (conc. 100 µg/mL, Sigma, D-9542) in 1.5 mL reaction tubes (Eppendorf, Germany). If the counts were above or below the limit of 40-100 counts per ocular grid sample volume and staining solution were proportionally reduced. The suspension was incubated for 5 min in the dark. During incubation, the vacuum filtration element (Sartorius, Germany) was prepared. It was flushed with 70 % ethanol and a cellulose support membrane (0.45 µm pore size, Sartorius, Germany) was placed under the polycarbonate membrane (0.2 µm pore size, 25 mm diameter, GTBP 02500, Millipore, Germany). 10 mL of sterile drinking water was filled in the filtration element and the stained biofilm suspension was transferred. After filtration the filter was air dried and mounted with the anti fading reagent Citifluor AF87 (www.citifluor.co.uk, London, UK) on a microscopic slide.

2.4.1.2 Staining on filter membrane

For this procedure the biofilm sample was filtered on the 0.2 µm polycarbonate membrane as described above and 10 to 15 µL of DAPI stock solution (10 µg/mL) were directly dispersed on the filter and incubated for 20 min in the dark. After air drying the filter was mounted as described above. If necessary, to be in the range of 40 to 100 counts per ocular grid, biofilm suspension was diluted with autoclaved and sterile filtered drinking water (0.2 µm pore size).

2.4.1.3 Staining on coupons

Biofilm associated cells on the material coupons were stained with 50 to 100 µL of DAPI solution (10 µg/mL) for 20 min in the dark. Afterwards the coupons were rinsed with fresh Milli-Q water, air dried and mounted with anti fading reagent as described above.

2.4.1.4 Microscopic examination

Immediately after staining fluorescent signals were counted with an Axioplan 2 (Carl Zeiss, Germany) equipped with a HBO 100 lamp and the Zeiss filter no. 1 for DAPI (excitation 365, dichroic mirror 395 nm, suppression 397 nm). A minimum of 10 randomly chosen microscopic fields and 1000 cells were analysed. Results were documented with the camera Color View 12 (Soft Imaging System GmbH, Berlin, Germany) or a Kodak EES 1600 color reversal film.

2.4.2 Fluorescence *in situ* hybridization (FISH)

2.4.2.1 Fixation of biofilm coupons, suspensions, and pure cultures

Biofilm on coupons

Fixation and washing of the coupons was done in 50 mL centrifugal tubes which were filled with sterile 3.7 % formaldehyde and 1 x PBS (Sambrook and Russel 2001) solution respectively. After the coupons have been taken out of the reactor and transported in the laboratory they were first fixed with formaldehyde at 4°C for two hours and then washed twice in 1 x PBS. At the end the coupons were air dried and stored at room temperature in the dark. (Kalmbach 1998)

Biofilm suspension

Formaldehyde fixation:

Seven hundred µL of the biofilm suspension were filled in a 1.5 mL reaction tube (Eppendorf, Germany) and centrifuged at 13,000 rpm for 5 min. The supernatant was discarded. After addition of 700 µL 3.7 % sterile formaldehyde solution (0.2 µm pore

size filtered, GTBP, Millipore, Germany) the pellet was resuspended by Vortex mixing or with a manual micro-homogenisator. The resuspended pellet was incubated for 2 h at 4°C in the dark. Afterwards the pellet was washed twice with sterile 1 x PBS (Sambrook and Russel 2001) and centrifuged as described above. Finally the pellet was resuspended in 50 to 500 µL PBS (1x)/ethanol (96 %) mixture (1:2) and stored at -20°C.

Ethanol fixation:

After centrifugation as described above the biofilm suspension was resuspended in 50 to 500 µL PBS (1x)/ethanol (96 %) mixture (1:2), incubated for 2 h at 4°C in the dark and stored at -20°C.

Bacterial pure cultures

Formaldehyde fixation:

Bacterial pure cultures were incubated in the appropriate medium to the exponential phase. Afterwards 700 to 1500 µL were centrifuged for 5 min at 13,000 rpm to get a 3 to 4 mm diameter pellet. The supernatant was discarded, 700 µL 3.7 % sterile formaldehyde added, the pellet resuspended, and incubated for 2 h at 4°C. After centrifugation (13,000 rpm, 5 min) the pellet was washed two times with 1 x PBS and finally resuspended in PBS (1x)/ethanol (96 %) mixture (1:2) solution and stored at -20°C.

Ethanol fixation:

Bacterial cultures were incubated and pelleted as described for the formaldehyde fixation and resuspended in an appropriate volume (depending on the suspension density, 50 to 500 µL) of PBS (1x)/ethanol (96 %) mixture (1:2), incubated at 4°C for 2 h and stored at -20°C.

2.4.2.2 Hybridization procedure

Oligonucleotide probes were diluted in sterile Milli-Q water to a working solution of 50 ng/µL and stored at -20°C. To obtain a concentration of 5 ng/µL in the hybridization solution the working solution was diluted 1:10 with hybridization buffer containing 0.9 M NaCl, 20 mM Tris/HCl (pH 7.2), 0.03 % SDS, and an appropriate volume of formamide (molecular biology grade, Merck, Germany). The hybridization was per-

formed for 1.5 to 4h in a humid chamber at 46°C. To remove unbound oligonucleotides the samples were washed 18 minutes in the pre-warmed washing buffer consisting of 20 mM Tris/HCl, 0.01 % SDS, and an appropriate volume of NaCl corresponding to the used formamide stringency. Finally, the hybridized object was rinsed with Milli-Q water, air dried and mounted with antifading reagent Citifluor AF2 (Citifluor Ltd., London, UK). (Manz et al. 1998; Manz et al. 1993)

Counterstaining was done by overlaying the hybridized area with a 1 µg/mL (bacterial cultures) or 10 µg/mL (biofilm suspensions and coupons) DAPI (Sigma, D-9542) solution for 15 to 20 min. After gently rinsing off the staining with Milli-Q water and air drying the fields were mounted with antifading reagent.

Fluorescent signals were counted with an Axioplan 2 (Carl Zeiss, Germany) equipped with an HBO 100 lamp and the Zeiss filter no. 1 for DAPI (excitation 365, dichroic mirror 395 nm, suppression 397 nm), no. 9 oregon green (excitation 450-490, dichromatic mirror 510 nm, suppression 520 nm) and HQ light filter 41007 (excitation 535-550 nm, dichroic mirror 565 nm, suppression 610-675 nm, AF Analysentechnik, Tübingen, Germany) for detection of Cy3 labelled probes. A minimum of ten randomly chosen microscopic fields of the ocular grid and 1000 cells were analysed. Results were documented with the camera Color View 12 (Soft Imaging System GmbH, Berlin, Germany) or a Kodak EES 1600 color reversal film.

Bacterial pure cultures

About 10 µL of fixed bacterial pure culture was placed on the cavities of a teflon-coated microscopic slide (Marienfeld, Bad Mergentheim, Germany) and dried at 46°C in the hybridization oven. The slides were dehydrated with ethanol (50, 80 and 96 %, 3 min each), 10 µL hybridization solution added to each cavity and incubated, washed and mounted with antifading agent as described above.

2.4.2.3 Development of a new oligonucleotide probe

A new probe for *P. aeruginosa* was developed with the ARB software package and the probe design and match tool (Strunk et al. 1999). Specificity was checked by comparative sequence database analysis and hybridization with target and non-target bacteria. For the probes optimization of hybridization stringency was performed as described by Manz et al. (Manz et al. 1998). Hybridization stringency was adjusted by addition of varying formamide concentrations to the hybridization buffer and sodium chloride to the washing buffer. Hybridization was prepared as described above. (chapter 2.4.2.2)

2.4.2.4 Oligonucleotide probes used in this study

Table 2 shows the oligonucleotids used in this study with their specificity and stringency. Oligonucleotids were labeled with the indocarbocyanine dye Cy3 or oregon green by the company Metabion (Martinsried, Germany).

Tab. 2: Oligonucleotide probes used in this study.

Common name	**Probe Squence 5` - 3`**	**Target-organisms**	**FA[a)] %**	**Reference**
EUB 338	GCT GCC TCC CGT AGG AGT	domain Bacteria	20	(Amann et al. 1990)
non-EUB 338	ACT CCT ACG GGA GGC AGC	negative control	20	“
PsearB	TCT CGG CCT TGA AAC CCC	*P. aeruginosa*	40	(Hogardt et al. 2000)
PsearE	CCC ACC CGA GGT GCT GG	*P. aeruginosa*	50	present study
Ps	GCT GGC CTA GCC TTC	most true pseudomonads	35-50	(Schleifer et al. 1992)

a)FA: formamide

2.4.3 Extraction of total DNA from biofilm suspensions

2.4.3.1 Simple preparations of DNA from formaldehyde fixed and non-fixed biofilm suspensions

To test the influence of formaldehyde fixation on extraction and amplification procedure, both fixed sample material and untreated samples were tested:

a) Formaldehyde fixed sample of M IV (chapter 2.4.2.1). The fixed biofilm suspension was washed with 1 x PBS and resuspended in 1 x PBS.
b) Untreated biofilm suspension of BWB III which has been stored in a closed glass bottle in the fridge for some weeks. Because the influence of the chemical compounds was the aim of this approach, changes caused by fridge storage were assessed to be low.

As positive control the DNA of the isolate DK 79 (drinking water isolate of the reactor system in Denmark) or *E. coli* (DSM 5695) was added to the PCR mixture. DNA concentration of both controls was optimized to give a well-defined band on a 1.7 % agarose gel after universal PCR (chapter 2.4.3.6, 0.5 µL of AE diluted alkaline lysis, both). Addition of control DNA to biofilm suspensions after DNA preparation allowed assessment of inhibition and/or adsorption. Amplification of control DNA without biofilm suspension was the confirmation that the approach configuration was suitable in general. The following different approaches were made:

2.4.3.1.1 M IV (formaldehyde fixed) and BWB III (non-fixed) serial diluted

The formaldehyde fixed and the non-fixed biofilm suspensions M IV and BWB III were applied serial diluted (to 10^{-6}) to the PCR test series a) and b).

a) addition of DK 79 DNA
b) without addition of DK 79 DNA

2.4.3.1.2 Alkaline lysis of formaldehyde fixed M IV

Two hundred µL of the biofilm suspension were centrifuged at 13,000 rpm for 5 min (Biofuge 13, Heraeus Instruments, Newtown, CT, USA) and the supernatant was discarded. The general lysis procedure is described in chapter 2.4.4.1. Because of the higher pellet volume the volumina of the solutions were adjusted.

The pellet (75 µL) was resuspended in 100 µL lysis solution and heated at 95°C (Thermomixer, 5436, Eppendorf, Germany) for 30 min. Every 5 min the suspension was shaken by hand and vented if necessary. Finally the lysed suspension was diluted with 900 µL AE buffer (chapter 2.4.4). As described above two PCR test series were accomplished.

a) addition of DK 79 DNA
b) without addition of DK 79 DNA

2.4.3.1.3 Alkaline lysis, enhanced sample volume, and ethanol precipitation

Alkaline lysis was prepared with 2 mL biofilm suspension of formaldehyde fixed M IV and addition of 250 µL lysis solution as described above. After lysis the suspension was transferred to a 50 mL centrifugal tube and 2.5 mL ammonium acetate (end-conc. 2 M, NH_4Ac, Merck, Germany) and 225 µL dextran blue (conc. 1 mg/mL, Fluka, Germany) were added, and cooled for 10 min on ice. After centrifugation (15 min, 12,000 rpm, 15°C, Sorvall RC-5B refrigerated Superspeed Centrifuge DuPont, Wilmington, DE, USA) the double volume of ice cold ethanol (96 %, p. a. Fluka, Germany) was added. The suspension was vortexed and centrifugated again (15 min, 12,000 rpm, 15°C). The supernatant was discarded and the pellet washed with 70 % ethanol and transferred to a 1.5 mL centrifugal tube before centrifugation. After drying at 70°C for 3 min the pellet was resuspended in 30 µL AE buffer (Qiagen, Germany). Afterwards universal PCR was prepared as described in chapter 2.4.3.6) with different dilutions of the DNA suspension and:

a) addition of DK 79 DNA
b) without addition of DK 79 DNA

2.4.3.1.4 Alkaline lysis, enhanced sample volume, and isopropanol precipitation

Five caps of formaldehyde fixed biofilm suspension of M IV were washed with 1 x PBS (in 50 mL centrifugal tube) which resulted in a pellet of 5 mL biofilm suspension. This pellet was resuspended in 5 mL alkaline lysis solution. The lysis was prepared at 95°C in a waterbath for 30 min. Every 5 min the tube was shaken by hand. After lysis, 500 µL 3 M sodium acetate solution and 250 µL dextran blue (conc. 1 mg/mL, Fluka,

Germany) were added and carefully mixed. After centrifugation (6 min, 12,000 rpm, 15°C, Sorvall RC-5B Refrigerated Superspeed Centrifuge DuPont, Wilmington, DE, USA) the supernatant was transferred to a clean centrifugal tube, 5 mL isopropanol (99.9 %, Fluka, Germany) added, and mixed by hand. Once again the solution was centrifugated (6 min, 12,000 rpm, 15°C) and the supernatant discarded. The pellet was washed in 0.8 mL 70 % ethanol (10 min, 15,000 rpm, 15°C) and transferred to a 1.5 mL centrifugal tube before centrifugation. Finally the pellet was dryed at 70°C to remove remaining ethanol and resuspended in 30 µL AE buffer (Qiagen, Germany). PCR was prepared as described in chapter 2.4.3.6 and again the two test series accomplished.

a) addition of DK 79 DNA
b) without addition of DK 79 DNA

The isopropanol precipitation was repeated with the following modifications: *E. coli* (DSM 5695) was used parallel to the biofilm suspension. Furthermore isopropanol precipitation was extended to 15 min.

2.4.3.1.5 Addition of bovine serum albumin (BSA)

PCR preparations were done as described in chapter 2.4.3.6. Bovine serum albumin (BSA, acetylated, Promega, Germany), biofilm suspension (BWB III) or control DNA (*E. coli* DSM 5695) was added to PCR preparations as follows:

a) untreated sample of BWB III serial diluted to 10^{-6} + *E. coli* DSM 5695 + 0.1 µg/µL BSA (conc. in 25 µL PCR volume)
b) untreated sample of BWB III serial diluted to 10^{-6} + 0.1 µg/µL BSA (conc. in 25 µL PCR volume)
c) *E. coli* DSM 5695 + 0.1 µL/µL BSA (conc. in 25 µL PCR volume)
d) *E. coli* DSM 5695

After this first approach the concentration dependent effect of BSA was tested:

a) serial dilution of untreated BWB III sample up to 10^{-6} + 0.1 µg/µL BSA
b) serial dilution of untreated BWB III sample up to 10^{-6} + 0.3 µg/µL BSA
c) serial dilution of untreated BWB III sample up to 10^{-6} + 0.6 µg/µL BSA

2.4.3.2 FastDNA Spin Sample Kit for soil

After sampling total nucleic acids were directly extracted from the biofilm suspensions or frozen at –20°C until extraction procedure. DNA was prepared with the method provided by Bio 101 (1070 Joshua, Vista, CA 92083, USA) as FastDNA Spin Sample Kit for soil according to the manufacturer`s instructions. The extraction method includes a mechanical-chemical lysis with ceramic and silica particles, a protein precipitation and several washing and purification steps. Two hundred fifty µL of the biofilm suspension of different samples were added to the Multimix tube and the DNA was finally eluted with 50 µL DES (kit supplied pure water).

2.4.3.2.1 Extraction of DNA from biofilm suspensions

The extraction of total DNA according to the standard protocol did not succeed for various samples (pipes and material coupons of the reactor, data not shown). To concentrate DNA the DNA extracts of different biofilm suspensions of pipe samples were pooled as described in the following table:

Tab. 3: Pooled biofilm suspensions of pipe samples before DNA extraction.

Biofilm suspension	Tubes pooled	Resulting µL
BWB V	4 tubes	150
BWB VII	2 tubes	80
BWB VII	3 tubes	120
M X	3 tubes	130

To precipitate the extracted DNA with isopropanol chemicals were added as follows:

Tab. 4: Additon of chemicals before isopropanol precipitation.

Biofilm suspension	Resulting µL	Dextran blue (conc. 1 mg/mL) µL	Sodium acetate 3M µL	PCR H_2O	Isopropanol µL
BWB V	150	10	20	20	200
BWB VII	80	5	10	5	100
BWB VII	120	7,5	15	7,5	150
M X	130	10	20	40	200

After the addition of isopropanol (99.9 %) the suspension was mixed by hand and precipitated 15 min at room temperature. The centrifugation was done for 15 min at 15.000 rpm and 15°C and the supernatant discarded. The blue pellet (dextran blue) was dried in the Thermomixer at 70°C for 3 min and finally diluted in 30 µL AE buffer (Qiagen, Germany). The solution was stored at -20°C until PCR preparation.

To test whether the concentration of DNA in the biofilm DNA preparations was to high to amplify it in the PCR, dilutions of the DNA preparations of BWB VI, M VII and RB I PE were made (1:10, 1:100, 1:1000) and different volumina appointed in the PCR.

2.4.3.2.2 Evaluation of extraction efficiency

Defined amounts of DNA of *Aquabacterium citratiphilum* DSM 11900 were appointed to the FastSpin extraction to assess the extraction efficiency. At first *A. citratiphilum* was cultivated in liquid modified R2A medium at room temperature and harvested in the exponential phase. The bacterial cells were lysed under alkaline conditions as described before and diluted in AE buffer (1:10). The concentration of the DNA was determined with the PicoGreen dsDNA Quantitation kit (chapter 2.4.3.6.2). Subsequent the defined DNA amounts were applied to the FastDNA Spin Kit method and concentration of DNA was again measured at the end of the procedure to determine the loss of DNA. To further assess the loss of DNA by the mechanical-chemical lysis the extraction was repeated without the kit recommended lysis.

2.4.3.3 CTAB (hexadecyltrimethylammonium bromide) extraction

The CTAB extration is described more detailed than the other DNA preparations because the classical method allows for more biases than an optimized kit method. The general extraction method was described by Ogram in 1998 and was modified as described in the following chapters (Ogram 1998).

2.4.3.3.1 General extraction procedure

Solutions and technical equipment

- 0.12 M sodium phosphate buffer: 0.11 M Na_2HPO_4 (Sigma, Germany), 0.01 M NaH_2PO_4 (Sigma, pH 8.0)
- alkaline lysis solution: 0.25 % SDS (sodium dodecylsufate, Sigma), 50 mM NaOH (Merck, Germany), sterilised by a 0.2 µm Minisart-filter (Satorius, Germany), Qiagen AE-buffer (Qiagen, Germany)
- 5 M NaCl (Merck, Germany)
- 10 % (w/v) CTAB (cetyltrimethylammonium bromide, Sigma) in 0.7 M NaCl (Merck)
- Chloroform-isoamylalkohol (24:1): chloroform (Merck), isoamylalcohol (Merck)
- 13 % (w/v) PEG-(8000) (polyethylene glycol, Sigma) in 0.7 M NaCl
- dextran blue (1 mg/mL, Fluka)
- 99.8 % ethanol (Roth, Germany)
- 70 % ethanol (99.8 % ethanol diluted with sterile Milli-Q water)
- 1 x TE (Tris-EDTA): 10 mM Tris-Cl (tris(hydroxymethly)- aminomethane hydrochloride (Riedel-de-Haen, Germany), 1 mM EDTA (Sigma), pH 8
- 6 M NH_4Ac (ammonium acetate, Merck): sterilised by a 0.2 µm Minisart-filter (Satorius)
- chloroform-isoamyl alcohol (24:1) (both Merck)
- Sterile glas pipettes and pipettboy (Pipetus-Standard, Hirschmann, Eberstadt, Germany)
- Biofuge 13 (Heraeus Instruments, Newtown, CT, USA) for 1.5 mL reaction tubes

- Sorvall RC-5B Refrigerated Superspeed Centrifuge (DuPont, Wilmington, DE, USA) for 50 mL centrifugal tubes
- Thermomixer Eppendorf 5436

Preparation of biofilm suspenions

1. The sample (1 to 5 g) was prepared into sterile 50 mL centrifugal tubes (Kisker, Steinfurt, Germany), 10 mL sodium phosphate buffer added, vortexed for 1 min and allowed to settle for 10 min with occasional mixing.
2. Suspension was centrifugated at 6,500 rpm for 10 min and the supernatant discarded. Step 1 and 2 were repeated.
3. 16 mL alkaline lysis solution were added (0.25 % SDS, 50 mM NaOH), mixed by hand, incubated in a waterbath for 20 min at 95°C and shaken with hand every 5 min.
4. Suspension again centrifugated at 6,500 rpm for 10 min and supernatant transfered in a clean centrifugal tube. The transferred volume was listed and the pellet discarded.
5. 2.7 mL 5 M NaCl (final conc. 0.7 M NaCl) and 2.1 mL of 10 % CTAB (final conc. 1 % CTAB) were added to the supernatant, mixed by hand and incubated at 65°C in the waterbath.
6. An equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed briefly to achieve emulsion.
7. Suspension was centrifugated at 4,200 rpm for 5 min and the upper aqueous phase transfered to a clean centrifugal tube, the lower phase was discarded.
8. An equal volume of 13 % polyethylene glycol (PEG 8000) in 0.7 M NaCl was added, mixed by hand and keeped on ice for 10 min.
9. Suspension was again centrifugated at 8,500 rpm for 15 min and the supernatant discarded. 500 µL of 70 % ethanol and 30 µL dextran blue solution (1 mg/mL, Fluka) were added, mix and centrifugated for a short time (max. 2,000 rpm, to collect liquid at the bottom). The liquid was transfered to a 1.5 mL reaction tube and centrifugated for 15 min at 12,000 rpm. The supernatant was discarted and the pellet dried at 70°C in the Thermomixer for 3 min. Finally, nucleic acids were resuspended in 750 µL TE.

10. 390 µL 6 M NH_4Ac (final conc. 2 M) were added and the suspension keep on ice for 10 min.
11. Again a centrifugation step was done at 12,000 rpm for 15 min, the supernatant transfered in a clean reaction tube, the transferred volume listed, and the supernatant discarded.
12. Afterwards 2 volumes (listed in step 11) of 99.8 % ethanol were added and centrifugated for 15 min at 8,500 rpm and the supernatant discarded. The pellet was washed with 500 µL 70 % ethanol and shortly centrifugated (max. 2,000 rpm) to collect liquid at the bottom and the liquid was transfered into a clean reaction tube. The suspension was centrifugated at 12,000 rpm for 15 min, the supernatant discarded and, the pellet dryed at 70°C for about 3 min. Finally the DNA pellet was resuspended in 100 µL Qiagen AE buffer and store at -20°C until further applications.

2.4.3.3.2 Variations of the protocol

Some of the above described protocol steps were varied as follows:

Step 5: If the protocol was prepared without the lysis steps, the missing liquid volume was replaced by 10 mL or 16 mL double autoclaved Milli-Q water, when 10 mL water was added the volumes of NaCl and CTAB were adapted.

Step 10: In the case of leaving out the PEG precipitation the added volume of 6 M ammonium acetate was adapted to achieve a final conc. of 1.5 to 2 M NH_4Ac.

Step 12: In the validation process of the method after addition of ethanol in step 12 the solution was frozen over night at -20°C and the next day the procedure was continued with centrifugation.

2.4.3.4 DNA extraction with QIAamp DNA Mini Kit

In this kit the DNA is lysed, bound to a silica-gel membrane, two times washed with different buffers and eluted with buffer AE. The extraction was in principle performed as described in the manual (02/2003, QIAamp DNA Mini Kit, Qiagen, Germany) except the modifications described in the following.

Two different lysis procedures were tested:

a) Kit recommended lysozyme lysis in protocol D (lysozyme 20 mg/mL, from chicken egg white, Sigma, Germany; 20 mM Tris HCl (Riedel de Haen, Germany) pH 8.0; 2 mM EDTA (Sigma); 1.2 % Triton (100 %,Fluka), Qiagen proteinase K
b) Alkaline lysis as described in chapter 2.4.4.1.

The following samples were tested:

a) *E. coli* DSM 5695 liquid culture in nutrient agar (DSMZ media, incubated for 7 days) as control of the extraction method was extracted in parallel. The culture was centrifuged at 12,000 rpm for 15 min (Sorvall, RC-5B) and the supernatant discarded.
b) Formaldehyde fixed biofilm suspension of BWB III. For the larger sample volume the volume of the added solutions was adapted. Six caps (approx. 4.2 mL) of formaldeyd fixed samples were pooled, alkaline lysed (3 mL alkaline lysis solution, 30 min at 95°C, shaken by hand every 5 min) and diluted in 27 mL AE buffer. The mixture was centrifuged again (12,000 rpm, 6 min) to separate the sediment from the DNA in the liquid phase, transferred to a clean tube and an adapted volume of 15 mL 96 % ethanol was added (step 4 Qiagen protocol) before the solution was applied to the spin column in 600 µL portions.

2.4.3.5 Extraction and purification by Qiagen Genomic-tips 20

The extraction was accomplished as described in the manufacturer`s manual (08/2001, Qiagen, Germany) except some variations that will be described below. The manufacturer describes that the procedure is based on an optimized buffer systems for careful lysis of cells followed by binding of genomic DNA to anion-exchange resin under appropriate low salt and pH conditions. Impurities are removed by a medium-salt wash and the genomic DNA is eluted in a high-salt buffer and concentrated and desalted by isopropanol precipitation.

The extraction procedure was divided in two parts:

a) sample preparation and lysis protocol for bacteria

1. The lysozyme solution (from chicken egg white, Sigma; stock solution: 100 mg/mL) was prepared as described in the manual and protease K stock solution used from Qiagen.
2. The bacterial culture was pelleted at 12,000 rpm (Sorvall, RC-5B) instead of 5,000 g because in the environmental sample very small bacteria were expected which might not pellet at low g forces.
3. As a control a 24 to 48 h *E. coli* DSM 5695 liquid culture in nutrient agar (DSMZ media) was used. 800 µL of the culture were centrifuged.
4. As an alternative lysis procedure the alkaline lysis as described in chapter 2.4.4.1 was used. To the pellet of 1 mL biofilm suspension 1 mL lysis solution was added, but the pellet was not washed with 1 x PBS. 30 µL lysis solution were added and after lysis diluted with 270 µL AE (Qiagen, Germany). To avoid a negative influence of SDS included in the lysis solution, in some approaches the anionic detergent was precipitated by 1 M potassium acetate for 15 min in the fridge. This was prepared directly after lysis without dilution with AE buffer.

b) Genomic-tips 20 protocol for isolation of genomic DNA from bacteria

This part of the protocol was to a large extent performed as described in the manual.

1. Genomic tips 20/G were used that were recommended for up to 4.5 x 10^9 cells in the sample extracted.
2. Eluted DNA was collected in sterile 15 mL polypropylen centrifugal tubes (Kisker, Germany).
3. The isopropanol precipitated DNA was centrifuged at 4°C and 5,000 rpm for 15 min to concentrate the DNA at the bottom and the supernatant removed before the DNA pellet was washed with 70 % ethanol. Isopropanol and 70 % ethanol were filtered (0.2 µm pore size single use syringe filter, Satorius, Germany).
4. The final drying of the DNA pellet, after washing with 70 % ethanol, was done at 46°C in a hybridization oven for up to 30 min.
5. The DNA pellet was finally resuspended in 100 or 300 µL AE buffer.

2.4.3.6 Verification of the extraction success

The extraction success was evaluated by two methods, polymerase chain reaction with universal bacterial primers and/or measurement of double-stranded DNA with the sensitive fluorescent nucleic acid stain PicoGreen.

2.4.3.6.1 Control PCR

The above extracted bacterial DNA was amplified by PCR in 25 µL reaction mixtures as described in table 5. To test extracted DNA a volume PCR-H_2O was replaced by a volume of the DNA extract, analogous controls were prepared. The Taq polymerase kit contained the appropriate buffer and $MgCl_2$ (5 U/µL Taq no. 1647687, Roche Diagnostics GmbH, Germany). The universal 16S rDNA primers 616 forward (5`-AGA GTT TGA TYM TGG CTC AG-3`) and 1525 reverse (5`-AAG GAG GTG WTC CAR CC-3`) were used (Lane 1991). Reaction mixtures were incubated in a gradient thermal cycler (Whatman, T-Gradient Thermoblock, Biometra, Germany) with one of the three following cycling conditions.

1. Initial denaturation one cycle at 96°C for 2 min followed by **35 cycles** of 94°C for 30 sec, **60°C** for 2 min, 72°C for 3 min, and a final extension cycle at 72°C for 15 min. **program 1**
2. Initial denaturation one cycle at 96°C for 2 min followed by **35 cycles** of 94°C for 30 sec, **57°C** for 2 min, 72°C for 3 min, and a final extension cycle at 72°C for 15 min. **program 2**
3. Initial denaturation one cycle at 96°C for 2 min followed by **40 cycles** of 94°C for 30 sec, **52°C** for 2 min, 72°C for 3 min, and a final extension cycle at 72°C for 15 min. **program 3**

Tab. 5: Composition of PCR reaction mixture per 25 µL total volume. To test extracted DNA the appropriate PCR-H_2O was replaced by the sample volume.

Solution	**Conc. stock solution**	**Volume in 25 µL total PCR reaction mixture**
Buffer	10 x	2.5
$MgCl_2$	25 mM	1.5
dNTP	10 mM (2.5 mM each base)	0.5
Primer 616 F	10 pmol/µL	0.25
Primer 1525 R	10 pmol/µL	0.25
Taq	1 U/µL	1.9
PCR-H_2O		18,1
total volume		25

PCR products were visualized on a 1.7 % or 1 % agarose gel (SeaKem LE agarose, FMC BioProductss, Rockland, Maine, USA) casted in a biozym chamber (Biozym midi chamber, Biozym Scientific GmbH, Hess. Oldendorf, Germany). The investigated DNA solution was mixed with the loading buffer on a parafilm M strip. As loading buffer a Ficoll based buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 15 % Ficoll Type 400, (Sambrook and Russel 2001) or the buffer offered with the ladder (GeneRuler 100bp DNA Ladder Plus, MBI Fermentas, Leon-Rot, Germany) was used. The running conditions are shown in table 6.

Tab. 6: Running conditions of agarose gels.

Agarose% w/v	TAE[a)] mL	Agarose g	Voltage mV	Running time h (approx.)
1.7	75	1.28	100	1.2
1.0	75	0.75	80	2

a)1 x TAE buffer: 40 mM Tis-acetate, 1 mM EDTA, (Sambrook and Russel 2001)

Table 7 illustrates which PCR amplification conditions were applied to which DNA extraction approach. Due to the limited volume of the biofilm suspension and as a

consequence the limited volume of DNA extracts, it was not possible to test every extract with every PCR conditions.

Tab. 7: PCR conditions applied to the DNA extracts of the different preparation attempts. In every PCR the primers 616 F and 1525 R were used. fix.: formaldehyde fixed biofilm suspension

DNA preparation	**PCR-program**	**Applied sample**
inhibition and adsorption chapter 3.3.1.1	prog. 2 57°C annealing, 35 cycles	M IV (fix.): undiluted 1+5 µL, dilution 1:10 to 1:10^6 1 µL each BWB III: 1 µL of undiluted and each dilution with and without control DK 79
alkaline lysis chapter 3.3.1.2	prog. 2 57°C annealing, 35 cycles	M IV (fix.): undiluted 1+5 µL, dilution 1:10 to 1:10^6 1 µL each
ethanol precipitation chapter 3.3.1.3	prog. 2 57°C annealing, 35 cycles	M IV (fix.): undiluted. 1+5 µL, dilution 1:10 to 1:10^6 1 µL each
isopropanol precipitation chapter 3.3.1.3	prog. 2 57°C annealing, 35 cycles	M IV: undiluted. 1+5 µL, dilution 1:10 to 1:10^6 1 µL each
BSA chapter 3.3.1.4	prog. 2 57°C annealing, 35 cycles	BWB III: undiluted and 1: 10 to 1:10^6, 1 µL each with BSA in different concentrations with and without spiked *E. coli*
Fast Prep chapter 3.3.2.1	prog. 2 57°C annealing, 35 cycles	BWB V, BWB VII, M X: 0.5, 1, 5,17.5 µL undiluted. extract
Fast Prep diluted chapter 3.3.2.1.2	prog. 3 52°C annealing, 40 cycles	M VIII, RBI PE, BWB VI: dilution 1:10 to 1:1000, MVIII=10µL each, RBI PE=10 µL each BWB VI=1, 10, 17.5 µL each
CTAB chapter 3.3.2.2	prog. 3 52°C annealing, 40 cycles prog. 2 57°C annealing, 35 cycles	*A. citratiphilum* spiked: 1, 8, 17.5µL *A. citratiphilum* spiked: 4 parallels, 1, 10 µL
QIAamp DNA Mini Kit chapter 3.3.2.3	prog. 1 60°C annealing, 35 cycles	BWB III fix., 1. eluat, 1, 10, 17.5 µL
Genomic tips 20 chapter 3.3.2.4	prog. 2 57°C annealing, 35 cycles	BWB VI, *E.coli* (fix., not fix.), lysozym lysis, alk. lysis, alk. lyis combined with precipitation, all undiluted, 1 + 10 µL

2.4.3.6.2 Measurement of DNA

To determine small amounts of double-stranded DNA the sensitive fluorescent nucleic acid stain PicoGreen was used (PicoGreen dsDNA Quantitation kit P-7589, Molecular Probes Europe BV, Leiden, Netherlands). With this fluorescent dye it is possible to determine as little as 25 pg/mL of dsDNA. The preparation of standards and samples is described in detail in the product information sheet. It is possible to prepare two standard curves depending on the expected DNA concentration of the in-

vestigated sample. One curve ranges from 1 ng/mL to 1 µg/mL the other from 25 pg/mL to 25 ng/mL. Measurement was done with a Hitachi Fluorescence Spectrometer F-4500 equipped with a Xenon lamp.

2.4.4 Sequencing of bacterial 16S rDNA

2.4.4.1 Alkaline lysis

Bacterial isolates incubated on agar plates or in liquid medium were transferred to a 1.5 mL centrifugal tube (pelleted and washed with 1 x PBS). Bacterial cells were resuspended in 20 to 30 µL alkaline lysis solution (0.25 % SDS, Sigma; 50 mM NaOH, Merck, filtered through a 0.2 µm Minisart-Filter, Satorius) and heated at 95°C (Thermomixer 5436, Eppendorf) for 15 min under permanent agitation. Afterwards the DNA was diluted 1:10 in AE buffer (Qiagen, Germany) and usually the concentration and the DNA/Protein ratio determined at 260 and 280 nm (1 OD_{260}= 50 µg DNA/mL). The DNA concentration was usually adjusted to 10 to 100 ng in the PCR reaction.

2.4.4.2 Sequencing reaction

For phylogenetic identification selected isolates obtained from different drinking water habitats were sequenced as follows. One hundred µL total PCR reaction mixture contained 70 µL diluted DNA extract (see above) and 30 µL master mix. The 30 µL master mix contained 1x reaction buffer, 1.5 mM $MgCl_2$, 200 mM dNTP (Promega, Germany), 1µM each primer, and 2.5 U Taq (no. 1647687, Roche Diagnostics GmbH, Germany). Samples were initial denaturated at 96°C for 1 min 30 sec followed by 28 cycles of 96°C for 30 sec, 57°C for 2 min 30 sec, 72°C for 3 min 30 sec, and a final extension cycle at 72°C for 10 min in a Biometra cycler (Biometra, Germany). The 16S rRNA genes were amplified with the universal primer pairs 616F (5`-AGA GTT TGA TYM TGG CTC AG 3`) and primer 1492R (5`-CGG YTA CCT TGT TAC GAC-3`) or 63F (5`-CAG GCC TAA CAC ATG CAA GTC-3`) and 1387R (5`-GGG CGG WGT GTA CAA GGC-3`) (Lane 1991; Marchesi et al. 1998). Sequence analysis was performed with an ABI Prism 310 sequencer (Perkin-Elmer Applied Biosystems Deutschland GmbH, Weiterstadt, Germany) using an Applied Biosystems Big Dye Terminator

Ready Reaction Mix Kit according to manufacturer's instruction. Cycle sequencing was done in a Gene Amp PCR System 9700 (Perkin-Elmer Applied Biosystems, Germany) and additionally to the above mentioned primers the following were used to amplify the hole double stranded 16S rRNA gene: 699R (5`-RGG GTT GCG CTC GTT-3`); 610R (5`-ACC GCG GCT GCT GGC AC-3`), 610F (5`-GTG CCA GCA GCC GCG GT-3`), nonEUB (5`-ACT CCT ACG GGA GGC AGC-3`).

2.4.5 RFLP analysis of the 16S rRNA gene of isolates

Pure cultures of the selected isolates were harvested from plates (Sly et al. 1999), washed with sterile 1 x PBS (Sambrook and Russel 2001), resuspended in 20 µl 50 mM NaOH and 0.25 % SDS, and heated for 15 min at 94°C. PCR amplification of the 16S rRNA gene fragment was done with the primers 9F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1512R (5'-ACG GCT ACC TTG TTA CGA CTT-3') targeting most bacteria (Martiny et al. 2003). The following thermal cycling program was applied: 94°C for 5 min, followed by 25 cycles of 94°C for 45 sec, 52°C for 45 sec and 72°C for 2 min and a final extension step at 72°C for 10 min. The enzymes *Rsa*I and *Msp*I were used separately to restrict the amplified 16S rDNA fragments to increase resolution power. Six U of *Rsa*I and 8 U of *Msp*I (New England BioLabs, Beverly, Mass., USA) were employed and the mixtures were incubated at 37°C for 2 h. A third enzyme BstUI was applied to check the resolution power of the technique. Six U of BstUI was used and the mixture was incubated at 60°C for 2 h. The resulting restricted product was separated on a 1.7 % agarose gel. The software package containing GeneScan and GeneTools (Syngene, Cambridge, U.K.) was used to obtain and compare the resulting band patterns. The band pattern was checked manually to control for restriction fragments at the same position and short fragments resulting in bands of low intensity. Isolates with the same band pattern were considered an operational taxonomic unit (OTU) and the abundance was scored. Novel OTUs were identified when fragment sizes differed more than 5 % and bands below 50 bp were not scored due to the low fluorescence intensity in the gel following the recommendations by Vaneechoutte and Heyndrickx (Vaneechoutte and Heyndrickx 2001).

2.4.6 Phylogenetic analysis

2.4.6.1 Pipe sample isolates

Sequences of the 112 isolates of the pipe samples were aligned by using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/). The aligned sequences were admitted to the MEGA 4 software package to group the pipe isolates of this study and to detect identical isolates (sequence difference <1 %). The distance matrix was calculated (neighbour-joining, pairwise deletion, p-distance, Transitions+Transversions) and OTUs determined. Representatives of each OTU were chosen and the closest 16S rRNA sequence (clone or isolate) and the closest described relative (only published isolates selected) matching the sequence in the Genbank database were identified by the BLASTN tool of the NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The closest sequence and described relative has to show the maximum number of compared bases and lowest gaps.

2.4.6.2 Representative reactor sample isolates obtained from RFLP analysis

Sequence analysis of the OTU representatives obtained from the RFLP anaylsis was performed with an ABI Prism 310 sequencer as described in chapter 2.4.4. 16S rDNA sequences were aligned with the ARB software package (Strunk et al. 1999) and manually corrected for errors. The new sequence was analyzed against the phylogenetic tree containing all sequences in the ARB database (6spring2001) using the maximum parsimony “quick add” tool to get a first estimate of the affiliation of new bacterial species. The family of each strain was identified and a new tree was reconstructed using sequences from all species within the corresponding family as described in Bergey’s Manual (Bergey`s Manual Trust 2001). Strains were identified using a consensus based on the neighbour-joining, maximum parsimony and fastdna maximum likelihood algorithms and the bacterial nomenclature described in the latest edition of Bergey’s Manual. A base frequency filter was generated based on the selected sequences excluding all positions different in more than 70 % of the strains to enable a comparison of homologous positions.

2.5 Statistical analysis

The statistical analysis was performed either with the statistic tools of the Excel 2000 program for simple descriptive statistics or the program STATISTIKA 7.1 (StatSoft, Inc. 2005, www.statsoft.com). The samples were tested for normal distribution by the Kolmogoroff-Smirnoff test with Lilliefors correction and the Shapiro-Wilk test. If the values showed no normal distribution they were log transformed and 1 was added to all values to avoid negative log values. If not further noted, non-transformed data were used. As a consequence of the results parametric or non parametric test statistic for dependent or independent samples was chosen. The chosen tests are described in the result chapters. The error probability alpha was 0.05 for all tests.

3 Results

3.1 Pipe samples taken from the distribution systems in Berlin and the Ruhrgebiet

As shown in table 8 in total 18 pipe samples were taken in the drinking water distribution systems of the Ruhrgebiet and in Berlin by the working groups of Prof. Dr. H.-K. Flemming of the Universität Duisburg or the working group of Prof. Dr. Szewzyk at the TU Berlin in the years 1999, 2000 and 2001. The pipe samples were assigned with the internal code M II to XII for the samples in the Ruhrgebiet and BWB I to VII for pipes taken from the distribution system of the Berliner Wasserbetriebe. Most of the pipes consisted of the materials PVC, cement and the metallic materials grey cast, cast iron and Tyton. Two exceptional materials are a cast iron pipe with an inline material and a tin coated steel pipe. Thirteen of the sampled pipes had a diameter of DN 100, in single cases it was DN 200, 150, 125 and 50. The time of exposition in the drinking water system varied from 12 to 34 years for the PVC pipes and from 8 to 20 years for cement pipes. The metallic materials showed a greater range of exposition time from 24 to 99 years. Another parameter of interest for the pipe samples is the scraped inner pipe surface. For 15 of the 18 pipe samples more than 2500 cm² were scraped (min. 2540 cm², max. 5511 cm²). For the 10 year old cement pipe the minimum inner pipe surface scraped was 298 cm². The pH of the scraped biofilm suspensions ranged between 7.9 and 8.2 for the PVC pipes and between 5.7 and 7.7 for the metallic materials. Values above pH 9 were measured for M XI and M XII due to the material cement. Two further interesting parameters for the description of the pipe samples are disinfection and temperature of the supplied drinking water. These parameters were measured in the bulk water after it had flown through the pipe and before the pipe section was cut out of the distribution system. In the drinking water distribution system of the Ruhrgebiet the water was generally disinfected this resulted in a free chlorine content of the bulk water phase between ≤ 0.01 mg/mL and 0.13 mg/mL[3] (Flemming 2003). The Berlin drinking water was normally not disinfected except the tin coated steel pipe where a locally limited chlorination was carried out. Temperature of the free water at the sampling day ranged between a minimum of 6°C for a 24 years old grey cast iron pipe and a maximum value of 16.4°C measured in a 20 years old cement pipe.

[3] The free chlorine conc. was determined by the N,N-diethyl-p-phenylenediamine colorimetric method on site by the working group in Duisburg.

Tab. 8: Pipe samples taken from the drinking water distribution systems in Berlin and the Ruhrgebiet.

Sample/ date	Pipe material	Sampling site	Age in years	Scraped inner pipe surface	pH	Free water phase	
						free chlo-rine mg/mL	Temperature °C
M VI 19/10/99	PVC DN 100	Dorsten-Holsterhausen Rhade	12	3142	7.9	n. dis.	n.d.
BWB II 02/02/00	PVC DN 200	Berlin Lichtenberg	24	5511	7.9	n. dis.	8.2
BWB IV 17/04/00	PVC DN 150	Berlin Lichtenberg	24	2779	8.0	n. dis.	10.9
M II 08/06/99	PVC DN 100	Duisburg Rheinhausen	28	3110	8.0	<0.01	n.d.
M VII 09/11/99	PVC DN 100	Duisburg Friemersheim	34	3456	8.2	<0.01	n.d.
M XI 11/09/01	Cement DN 100	Duisburg Ungelsheim	8	3272	9.2	0.05	12.7
BWB I 23/06/99	Cement n.d.	Berlin Lichterfelde	approx. 10	298	n.d.	n. dis.	n.d.
M XII 09/10/01	Cement DN 100	Duisburg Wanheim	20	3343	9.1	0.13	16.4
BWB III 29/02/00	grey cast iron DN 100	Berlin Lichtenrade	24	1860	7.2	n. dis.	6
M V 14/09/99	grey cast iron DN 100	Duisburg Neudorf	30	3340	6.7	<0.01	n.d.
M III 13/07/99	grey cast iron, cement DN 125	Mülheim (Ruhr) Raadt	37	3244	7.7/7.3	<0.01	n.d.
M IV 10/08/99	grey cast iron DN 100	Oberhausen	99	3202	5.7	0.01	n.d.
M X 06/03/01	cast iron DN 100	Duisburg Buchholz	62	2540	6.4	0.02	6.6
BWB V 05/06/00	cast iron DN 100	Brandenburg	approx. 73	2942	7.3	n. dis.	14.8
M IX 17/10/00	Tyton (spheroidal graphite) DN 100	Duisburg Wanheimerort	26	3189	6.7	0.01	13.9
M VIII 17/10/00	Tyton (spheroidal graphite) DN 100	Duisburg Bissingheim	27	3810	6.4	<0.01	13.8
BWB VI 10/10/00	tin coated steel DN 50	Berlin Schönerlinde	approx. 10	976	7.2	0.13	15
BWB VII 23/04/01	cast iron with inline material DN 100	Berlin Grunewald	2	3009	7.5	n. dis.	9.9

n. dis.: no disinfection n.d.: not determined

3.1.1 Macroscopic description of deposits on the inner pipe surface

The deposits on the inner pipe surface showed appreciable differences regarding the macroscopic structure. The 99 years old grey cast pipe M IV or the approximately 73 years old cast iron pipe BWB V (figure 3) had voluminous coatings on the inner pipe surface which irregularly extended 3 to 5 cm in the pipe interior (figure 3). Remarkable are the different brown colours from yellow-brown and red-brown to dark-brown or nearly black which are an indication for different deposits of iron and manganese. The counterexample are the less voluminous coating on the inner pipe surface of the 24 years old grey cast iron pipe (figure 3) or the PVC and cement pipes (not shown) Here the deposits only extend approximately 1 to 3 mm in the pipe interior . Nevertheless, these coatings often showed light-brown and dark-brown areas.

Fig. 3: Deposites on the inner pipe surface of two iron pipes. On the left side approx. 73 years old cast iron pipe, on the right side 24 years old grey cast iron pipe.

3.1.2 Total cell counts and culturable bacteria on the different pipe materials

For the statistical analysis the 18 pipe samples were arranged in groups according to the material of the inner pipe surface (table 9). The materials are PVC, cement, and the metallic materials grey cast iron, cast iron, and Tyton. As described above the pipes also differ in age, inner pipe diameter, and scraped inner pipe surface (see table 8). Table 9 summarizes the results of total cell counts and colony counts of the three different media of the laboratory of the Universität Duisburg and the Technische Universität Berlin.

Tab. 9: Total cell counts and colony counts on different media for the pipe samples.

Sample/ date	Pipe material	Sampling site	Age in years	Scraped inner pipe surface cm²	TCC[a)] cells/cm² 1. lab	TCC[a)]* cells/cm² 2. lab	R2A+T[b)] KBE/cm² 7 d	R2A[c)]* KBE/cm² 7 d	GDWR[d)]* 20°C KBE/cm²	GDWR[d)]* 36°C KBE/cm²
M VI 19/10/99	PVC DN 100	Dorsten-Holsterhausen Rhade	12	3142	1.03×10^6	1.30×10^6	3.65×10^2	2.8×10^3	4.4×10^1	2.4
BWB II 02/02/00	PVC DN 200	Berlin Lichtenberg	24	5511	1.60×10^7	1.60×10^7	5.44×10^3	7.8×10^3	7.2×10^2	2.1×10^2
BWB IV 17/04/00	PVC DN 150	Berlin Lichtenberg	24	2779	1.66×10^7	7.40×10^6	1.02×10^3	3.1×10^3	8.7×10^2	1.1×10^1
M II 08/06/99	PVC DN 100	Duisburg Rheinhausen	28	3110	1.80×10^5	4.80×10^5	1.52×10^2	6.3×10^2	0.3	1.2
M VII 09/11/99	PVC DN 100	Duisburg Friemersheim	34	3456	1.73×10^6	1.90×10^6	1.43×10^1	3.0×10^1	0.4	0.1
M XI 11/09/01	Cement DN 100	Duisburg Ungelsheim	8	3272	n. d.	8.00×10^5	n. d.	1.2×10^1	1.4	3.9
BWB I 23/06/99	Cement	Berlin Lichterfelde	approx. 10	298	n.d.	n. d.	2.41×10^2	n. d.	n. d.	n. d.
M XII 09/10/01	Cement DN 100	Duisburg Wanheim	20	3343	n. d.	3.00×10^5	n. d.	1.5×10^2	0.5	4.1
BWB III 29/02/00	grey cast iron DN 100	Berlin Lichtenrade	24	1860	3.08×10^9	n.d.	6.10×10^4	n.d.	n.d.	n.d.
M V 14/09/99	grey cast iron DN 100	Duisburg Neudorf	30	3340	4.94×10^5	1.10×10^7	3.05×10^4	3.8×10^5	8.8×10^1	1.6×10^2

Sample/ date	Pipe material	Sampling site	Age in years	Scraped inner pipe surface cm²	TCC[a)] cells/cm² 1. lab	TCC[a)]* cells/cm² 2. lab	R2A+T[b)] KBE/cm² 7 d	R2A[c)]* KBE/cm² 7 d	GDWR[d)]* 20°C KBE/cm²	GDWR[d)]* 36°C KBE/cm²
M III 13/07/99	grey cast iron, cement DN 125	Mülheim (Ruhr) Raadt	37	3244	7.17×10^6	6.10×10^6	5.54×10^4	2.0×10^5	3.9×10^2	4.2×10^2
M IV 10/08/99	grey cast iron DN 100	Oberhausen	99	3202	3.30×10^7	2.60×10^7	6.64×10^3	1.3×10^4	2.3×10^1	9.1×10^1
M X 06/03/01	cast iron DN 100	Duisburg Buchholz	62	2540	n. d.	2.10×10^7	n. d.	1.0×10^5	1.0×10^1	1.2
BWB V 05/06/00	cast iron DN 100	Brandenburg	approx.. 73	2942	n.d.	n.d.	2.45×10^4	1.4×10^5	3.2×10^2	4.8×10^2
M IX 17/10/00	Tyton (spheroidal graphite) DN 100	Duisburg Wanheimerort	26	3189	n. d.	6.70×10^6	n. d.	1.5×10^4	1.5×10^3	1.1×10^3
M VIII 17/10/00	Tyton (spheroidal graphite) DN 100	Duisburg Bissingheim	27	3810	n. d.	2.40×10^7	n.d.	5.0×10^4	6.9×10^1	1.3×10^2
BWB VI 10/10/00	tin coated steel DN 50	Berlin Schönerlinde	approx.. 10	976	8.24×10^5	2.00×10^8	n. d.	1.2×10^5	1.4×10^2	3.5×10^1
BWB VII 23/04/01	cast iron with inline material DN 100	Berlin Grunewald	2	3009	3.10×10^4	4.90×10^7	n. d.	9.9×10^3	1.6×10^1	2.3

*results determined by the laboratory of the Universität Duisburg a)TCC: total cell counts determined by DAPI staining b)R2A+T: Tween 80 modified R2A

c)R2A: standard R2A d)GDWR: cultivation according to German drinking water regulation

To ascertain whether the total cell counts (TCC) determined with DAPI staining differ in the two laboratories of Duisburg and Berlin, statistical anaylsis was done. The results in table 10 show that with the two applied non-parametric statistical tests for paired variables no significant difference was detected between the counts of the two laboratories (p=0.173 and p=0.505 at alpha=0.05).

Comparison of the arithmetic mean of the total cell counts of the two laboratories with the four different cultivation approaches (R2A, R2A+Tween, GDWR 20°C and 36°C) showed a significant difference between the TCC and each cultivation (p=0.0003 to 0.008, at alpha=0.05, table 10). Culturable counts were always below TCC.

The statistical analysis of the cultivation data resulted in significant differences of the cultivation data on different media (R2A, GDWR 20°C and 36°C) or media modifications (R2A+Tween, R2A). In contrast to this no statistically significant difference was verified between the different incubation temperatures postulated by incubation according to GDWR (blood agar basis, GDWR 20°C, GDWR 36°C).

Tab. 10: Comparison of microbiological data of the pipe samples. TCC: total cell counts determined by DAPI staining; R2A: plate counts on standard R2A; R2A+Tween: plate counts on Tween 80 modified R2A; GDWR 20°C and 36°C plate counts according to German drinking water regulation 1990

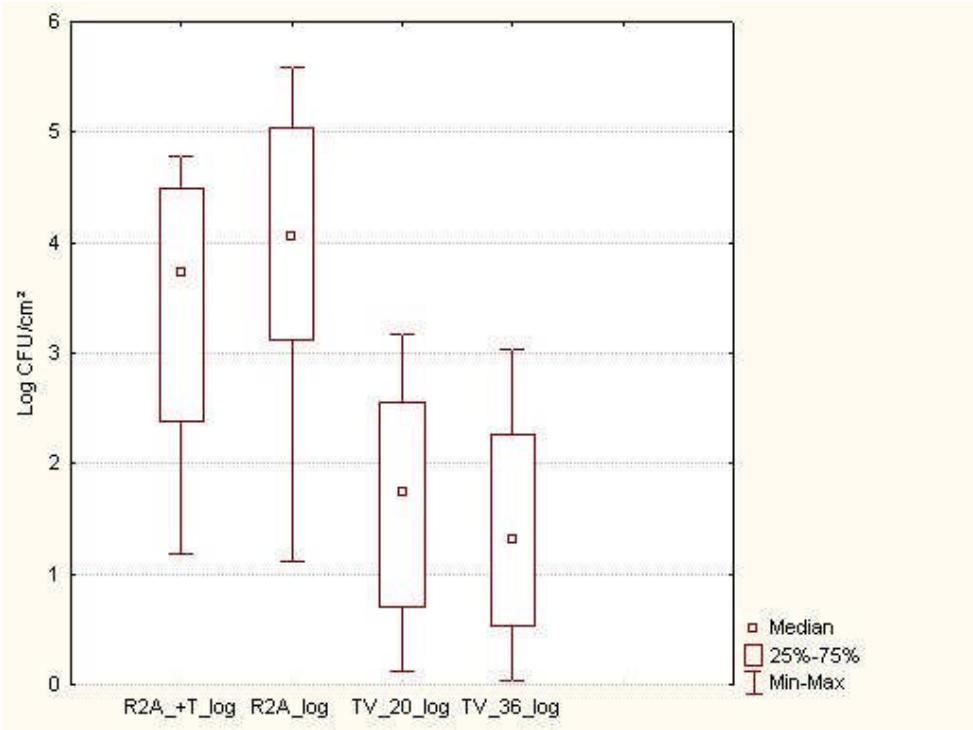
Compared parameters alpha= 0.05	**p-level Wilcoxon-test**	**p-level Sign-test**	**Sample size**	**Result**
TCC laboratory 1 & TCC laboratory 2	0.173	0.505	10	no significant difference
TCC & different cultivations	0.0007 to 0.008	0.0003 to 0.008	9 to 15	significant difference
R2A+Tween & R2A	0.008	0.008	9	significant difference
R2A+Tween & GDWR 20°C	0.008	0.008	9	significant difference
R2A+Tween & GDWR 36°C	0.008	0.008	9	significant difference
R2A & GDWR 20°C	0.0004	0.0002	16	significant difference
R2A & GDWR 36°C	0.0004	0.0002	16	significant difference
GDWR 20°C & GDWR 36°C	0.379	0.803	16	no significant difference

p-level: probability level alpha: error probability

Furthermore, it was of interest whether more bacteria grow on standard R2A or on the modified medium with Tween 80 instead of soluble starch. As described in table 10 there is a significant difference between the colonie counts on standard R2A and the modified medium with Tween 80. The comparison of the median of the data of the two colony counts shows that there are more counts on standard medium than on the modified version (3.89 and 3.73, respectively, transformed). The smallest counts were detected on nutrient agar (median GDWR 20°C=1.85, median GDWR 36°C=1,67, transformed). These results are illustrated in the boxplot in figure 4. As a consequence the analysis allows the following ranking of CFU on the used media and used incubation conditions:

R2A +starch > R2A +Tween > GDWR 20°C and 36°C

Fig. 4: Boxplot of heterotrophic plate counts on different media in biofilm samples of the pipes. R2A_log: standard R2A, R2A_+T_log: R2A modified with Tween 80, TV_20_log and TV_36_log: medium GDWR incubated at 20 and 36°C.



The last item of interest was the difference between the three material groups PVC, cement and the metallic materials as shown in table 11. For comparison of the three material groups the Kruskal-Wallis analysis of variance by ranks showed that there were significant differences between the materials for TCC (p-level=0.0392) standard R2A (p-level=0.0054), and Tween 80 modified R2A (p-level=0.0232). No difference

was found in the materials for cultivation according to GDWR at 20 and 36°C (p-level=0.246 and 0.1223). For PVC and the metallic materials the result was verified by the Mann-Whitney U test for two samples (alpha=0.05, table 11). In most cases the results confirmed the results of the Kruskal-Wallis analysis except for the TCC. Here no significant difference was found with the Mann-Whitney test between the material groups PVC and metallic materials (p-level=0.062). This allows the conclusion that only the cement pipes are different to the other material groups.

Tab. 11: Statistical analysis of the microbiological data with focus on material differences.

Compared parameters alpha= 0.05	**Compared material groups**	**Sample size**	**p-level Kruskal-Wallis ANOVA by ranks**	**Result**
$TCC_{lab1\&lab2}$	P, C, M	5, 2, 7	0.0392	significant difference
R2A	P, C, M	5, 2, 7	0.0054	significant difference
R2A+Tween	P, C, M	5, 1, 7	0.0232	significant difference
GDWR 20°C	P, C, M	5, 2, 7	0.2466	no significant difference
GDWR 36°C	P, C, M	5, 2, 7	0.1223	no significant difference

Compared parameters alpha= 0.05	**Compared material groups**	**Sample size**	**p-level Mann-Whitney U test**	**Result**
$TCC_{lab1\&lab2}$	P, M	5, 7	0.062	no significant difference
R2A	P, M	5, 7	0.005	significant difference
R2A+Tween	P, M	5, 7	0.009	significant difference
GDWR 20°C	P, M	5, 7	0.57	no significant difference
GDWR 36°C	P, M	5, 7	0.074	no significant difference

p-level: probability level alpha: error probability P: PVC C:cement M: metallic materials
GDWR: German drinking water regulation

Application of the non-parametric Spearman Rank correlation and the Kendal Tau correlation (table 12) showed a significant correlation between standard R2A and modified R2A. Furthermore, the colony counts on GDWR 36°C correlated with all of the other cultivations (standard R2A, R2A+Tween, GDWR 20°C). No apparent rela-

tionship was evident between the total cell counts and the cultivations. Due to the limited number of samples no analysis of the kind of correlation was made.

Tab. 12: Correlation of the different microbiological parameters.

Compared parameters alpha= 0.05	Spearman rank correlation coefficient	Kendall Tau correlation coefficient	Sample size	result
TCC & different cultivations	0.26 to 0.53	0.18 to 0.39	9	no significant correlation
R2A+Tween & R2A	0.98	0.94	9	significant correlation
GDWR 36°C & R2A+Tween	0.73	0.65	9	significant correlation
GDWR 36°C & R2A	0.61	0.51	9	significant correlation
GDWR 36°C & GDWR 20°C	0.82	0.70	9	significant correlation

alpha: error probability high correlation: coefficient=1, no correlation: coefficient=0
GDWR: German drinking water regulation TCC: total cell counts determined by DAPI staining

3.1.3 Phylogenetic bacterial groups detected in the pipe samples

The phylogenetic analysis of 112 isolates resulted in the identification of 65 different OTUs (chapter 3.2.5.1). A sequence error of 1 % was assumed. Consequently sequences with a similarity ≥ 99 % were supposed to be identical (Stackebrandt and Ebers 2006). The pie chart in figure 5 represents the number of phyla and classes detected by isolation on modified R2A incubated at 20°C. Within the 112 isolates the phylum Proteobacteria represents the greatest group with 18, 19 and 12 % for *Alpha*-, *Beta*- and *Gammaproteobacteria*, respectively. Only two isolates were affiliated to the *Bacteroidetes*. A relatively high portion of the gram-positive phyla *Actinobacteria* (28 %) and *Firmicutes* (21 %) was found. The results of the BLASTN search of the representatives of each OTU are shown in table 13. The closest described 16S rRNA sequence (clone or isolate) and the closest described relative of each representative (only published isolates selected) are listed. The 16S rRNA sequence of some pipe sample isolates closely matched with a variety of *Alphaproteobacteria* with a similarity between 95 and 100 % (analyzed bases: min: 380, max: 986) including *Sphingomonas*, *Caulobacter*, *Phyllobacterium*, *Hyphomicrobium*, *Bradyrhizobium*, *Chelatococcus*, *Sphingopyxis*, *Blastobacter*, and *Kaistobacter*. Other pipe sample isolates grouping into the *Betaproteobacteria* class showed phylogenetic affinities with the genera *Acidovorax*, *Comamonas*, *Hydrogenophaga*, *Aquaspirillum*, *Vario-*

vorax, *Ramlibacter*, *Undibacterium*, and *Herminiimonas* (similarity between 95 and 99 %, analyzed bases: min: 434, max: 452). The isolates sorted to the class *Gammaproteobacteria* grouped with the genus *Pseudomonas* and *Rhodanobacter* (similarity between 96 and 99 %, analyzed bases: min: 455, max: 474). The last gram-negative group found included two different members of the genus *Chryseobacterium* of the phylum *Bacteroidetes* (similarity between 95 and 96 %, analyzed bases: min: 455, max: 471). The sequences of the gram-positive isolates closely aligned with the *Actinobacteria* and *Firmicutes*. The *Firmicutes* included the genera Bacillus and *Paenibacillus*. In contrast to this, the *Actinobacteria* found are more versatile and show closest described relatives sorted to the genera *Mycobacterium*, *Nocardia*, *Nocardioides*, *Cellulomonas*, *Streptomyces*, *Kocuria*, *Agrococcus*, and *Microbacterium* (similarity between 93 and 100 %, analyzed bases: min: 419, max: 1039). The identified isolates separated in nearly as much gram-positive bacteria (49 %) as gram-negative (51 %, figure 5).

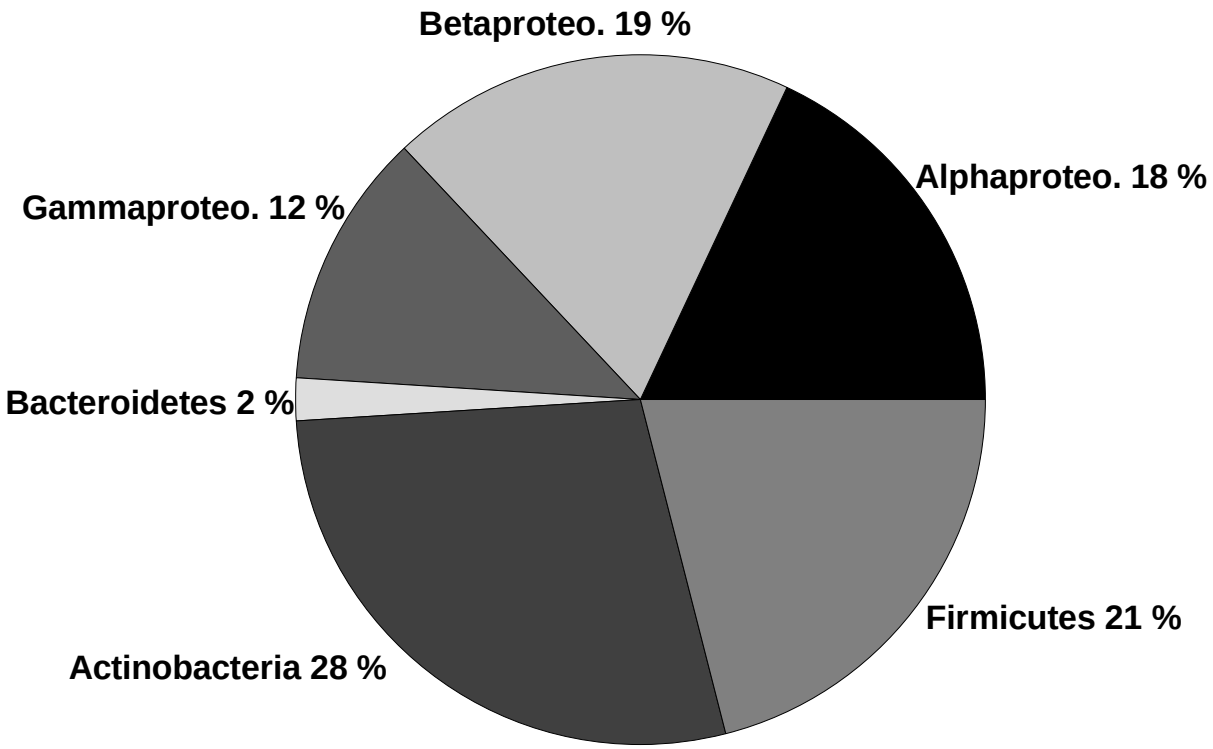


Fig. 5: Number of phyla and classes detected by isolation on modified R2A. Total number of investigated isolates 112.

Tab. 13: Phylogenetic affiliation of bacterial isolates harvested from modified R2A medium (7d/20°C) inoculated with biofilms suspensions of different drinking water pipe samples. Last update january 2010.

isolate	Taxonomic group/ Phylogenetic affiliation Phylum/class	Closest 16S rRNA sequence (acc. no.)	% Similarity (analysed bases)	Closest described relative (acc. no.)	% Similarity (analysed bases)	Pipe material/ years	Comment Closest 16S rRNA sequence, closest described relative
BWB IV 21	Alphaproteobacterium	Bacterium gwi41 DQ335922	98 (415/423,5G)	Caulobacter sp. FWC21 AJ227767	97 (403/414,1G)	PVC/24	desert sand grains, phylogenetic study
M III 8	Alphaproteobacterium	Uncultured Alphproteob. clone SI-2F_H03 EF221413	99 (419/421,0G)	denitrifying bacterium W49b AB162081	99 (420/421,0G)	grey cast iron, cement/37	arctic terrestrial habitat, subsurface upland soil
BWB IV 4	Alphaproteobacterium	Alphaproteob. BAL239 DQ063175	100 (382/382,0G)	Phyllobacterium myrsinacearum AM285008	100 (382/382,0G)	PVC/24	baltic sea ,soil
BWB IV 13	Alphaproteobacterium	Uncultured bacterial clone C19 EF590014	97 (394/403,3G)	Hyphomicrobium sp. P-48 AM411913	96 (380/394,2G)	PVC/24	nitrobenzene contaminated soil, soil
M VI 8	Alphaproteobacterium	Afipia massiliensis NW-12 AY568510.1	99 (425/426,0G)	Bradyrhizobium sp. ORS135 AJ301630	99 (425/426,0G)	PVC/12	spacekraft assembly facility, rhizo-bium-legume symbiosis
BWB I 13	Alphaproteobacterium	Deinococcus sp. 4B6 EU029132	97 (975/1005,15 G)	Chelatococus asaccharovorans AJ871433	97 (977/1006,13G)	cement/10	radiation-resistant soil bacte-ria,Biological soil crust
M II 4	Alphaproteobacterium	Uncultured bacterium clone 290 DQ158105	98 (998/1014,6G)	Sphingopyxis witflariensis AJ416410	97 (993/1014,8G)	PVC/28	phenol-degrading soil commu-nity,activated sludge

isolate	Taxonomic group/ Phylogenetic affiliation Phylum/class	Closest 16S rRNA sequence (acc. no.)	% Similarity (analysed bases)	Closest described relative (acc. no.)	% Similarity (analysed bases)	Pipe material/ years	Comment Closest 16S rRNA sequence, closest described relative
M VI 2	Alphaproteobacterium	Sphingomonas taejonensis EM0331 EF473286	99 (423/425,0G)	Sphingomonas sp. R1_7 AJ876688	100 (424/424,OG)	PVC/12	aphaprot. in Korea, chromium contaminated wastewater
M II 2_2	Alphaproteobacterium	Sphingomonas sp. D31C2 AY162145	97 (983/1013,7G)	Blastobacter sp. SMCC BO477 U20772	97 (984/1014,9G)	PVC/28	bottled water, aromatic contaminated deep subsurface
M VI 4	Alphaproteobacterium	Uncultured bacterium clone SSmCB08-42 AB176206	100 (424/424,0G)	Sphingomonas sp. MK346 D84521	99 (421/424,0G)	PVC/12	hydrothermal system of a seamount, isolates from the plant family Gramineae
BWB I 11	Alphaproteobacterium	Sphingomonadaceae bacterium PB323 AB220146	99 (994/998,0G)	Kaistobacter koreensis PB180 AY769084	98 (986/999,2G)	cement/10	terrestrial and freshwater environments, phylogenetic study
BWB IV 25	Alphaproteobacterium	Uncultured clone WD290 AJ292616	98 (406/413,4G)	Sphingomonas mali Y09638	97 (401/410,0G)	PVC/24	polychlorinated biphenyl-polluted soil, phylogenetic study
BWB IV 10	Alphaproteobacterium	Uncultured Sphingomonadaceae Elev-659 EF019415	95 (408/428,12G)	Sphingomonas suberifaciens MP11 AY521009	95 (407/428,12G)	PVC/24	soil microbial diversity associated with trembling aspen, soil
M II 5	Alphaproteobacterium	Alphaproteobacterium IMCC1753 DQ664250	100 (424/424,0G)	Sphingomonas sp. HTCC500 AY584571	98 (417/425,0G)	PVC/28	artificial pond, freshwater crater lake
BWB V 9	Alphaproteobacterium	Uncultured clone ML-3-45.2 DQ520160	98 (404/411,3G)	Sphingomonas sp. HTCC503 AY584572	98 (404/411,3G)	cast iron/73	hypertrophic freshwater lake, freshwater crater lake

isolate	Taxonomic group/ Phylogenetic affiliation Phylum/class	Closest 16S rRNA sequence (acc. no.)	% Similarity (analysed bases)	Closest described relative (acc. no.)	% Similarity (analysed bases)	Pipe material/ years	Comment Closest 16S rRNA sequence, closest described relative
BWB V 3	Betaproteobacterium	Uncultured Betaproteobacterium clone nsc093 DQ211445	99 (444/446,0G)	Acidovorax sp. R-25075 AM084109	99 (442/444,0G)	cast iron/73	wastewater bioreactor, nitrifiying bacteria from activated sludge
M VI 10	Betaproteobacterium	Uncultured Delftia sp. Clone DGR2_p1f03 EF033504	99 (468/471,3G)	Comamonas sp. WT OTU1 AY965248	99 (468/471,3G)	PVC/12	denitrifying dispersed-growth reactor, rhizospheric and soil bacterial communities in mycorrhizal symbiotic experimental microcosms
BWB IV 5	Betaproteobacterium	Uncultured clone 242ds5 AY212690	97 (450/461,5G)	Hydrogenophaga defluvii BSB 9.5T AJ585993	97 (449/461,5G)	PVC/24	equine fecal contamination, activated sludge
BWB IV 23	Betaproteobacterium	Aquatic bacterium R1-B18 AB195750	98 (461/466,2G)	Aquaspirillum delicatum AF078756	96 (452/466,2G)	PVC/24	lake, phylogenetic study
BWB IV 6	Betaproteobacterium	Uncultured clone HDBW-WB14 AB237677	98 (459/468,7G)	Variovorax sp. P-34 AM411934	95 (446/469,9G)	PVC/24	fault-bordered aquifer, soil
BWB IV 14	Betaproteobacterium	Uncultured Comamonadaceae clone M10Ba46 AY360635	97 (452/464,6G)	Ramlibacter sp. HTCC332 AY429716	96 (452/466,8G)	PVC/24	oxic rice field soil, trichloroethene-contaminated groundwater
M III 5	Betaproteobacterium	Uncultured clone LaC15L125 EF667521	98 (467/472,3G)	Denitrifying bacterium W125 AB162105	97 (453/465,4G)	grey cast iron, cement/37	rhizosphere bacteria, subsurface upland soil
BWB IV 15	Betaproteobacterium	Uncultured soil clone L1A.13E10 AY989480	98 (453/460,6G)	Undibacterium pigrum CCUG 49009 AM397630	96 (445/463,12G)	PVC/24	soil, drinking water
BWB V 14	Betaproteobacterium	Uncultured proteobacterium clone Hmd12B14 EF196963	99 (437/439,2G)	Herminiimonas fonticola S-99 DQ011678	98 (434/439,2G)	cast iron/73	bottled mineral water, holocene sediment from archaeological site

isolate	Taxonomic group/ Phylogenetic affiliation Phylum/class	Closest 16S rRNA sequence (acc. no.)	% Similarity (analysed bases)	Closest described relative (acc. no.)	% Similarity (analysed bases)	Pipe material/ years	Comment Closest 16S rRNA sequence, closest described relative
BWB IV 12	Betaproteobacterium	Uncultured clone SLB530 DQ787709	98 (457/466,6G)	Beta proteobacterium C23 AM179884	95 (446/468,8G)	PVC/24	sediments of a temperate lake, intestine of rainbow trout
M IV 5	Gammaproteobacterium	Uncultured clone AKAU3567 DQ125572	98 (468/475,4G)	Rhodanobacter thiooxydans AB286179	96 (463/479,8G)	grey cast iron/99	uranium reduction and reoxidation, biofilm on sulphur particles
BWB IV 22	Gammaproteobacterium	Pseudomonas sp. BWDY-40 DQ200853	100 (459/459,0G)	Pseudomonas mendocina PC1 DQ178219	99 (457/459,0G)	PVC/24	river estuary, phenol- and p-cresol degrading Pseudomonas species
BWB IV 19	Gammaproteobacterium	Uncultured clone C1S DQ856512	98 (466/475,6G9)	Pseudomonas migulae S57 AY972432	98 (466/474,4G)	PVC/24	crab intestine, activated sludge
M VI 3_1	Gammaproteobacterium	Pseudomonas sp. OPS1 AF368760	98 (469/473,4G)	Pseudomonas putida IA2YCDA AY512612	99 (470/473,3G)	PVC/12	new eugenol degrading Pseudomonas sp., BTEX contam. industrial site
M VI 3_2	Gammaproteobacterium	Pseudomonas fluorescens gene AB259171	99 (473/476,2G)	Pseudomonas mandelii AF058286	99 (474/476,2G)	PVC/12	demethyl disulfide degrading P. fluorescens, mineral water
BWB IV 1	Gammaproteobacterium	Shewanella putrefaciens X81623	93 (455/487,20G)	Shewanella sp. W3-18-1 CP000503	93(455/487,20G)	PVC/24	facultatively iron-reducing bacterium, complete sequence
M III 2	Bacteroidetes, Falvobacteria	Uncultured clone CCTR211152… EF409289.1	96 (455/471,5G)	Chryseobacterium indologenes LMG 12856 AY468481	96 (455/471,4G)	grey cast iron, cement/37	child-care facility, diseased aquatic animals
BWB IV 2	Bacteroidetes, Falvobacteria	Uncultured cloneC3D DQ856545	95 (446/467,13G)	Chryseobacterium haifaeyi H38 EF204450	95 (444/466,11G)	PVC/24	intestine of a crab, raw milk

isolate	Taxonomic group/ Phylogenetic affiliation Phylum/class	Closest 16S rRNA sequence (acc. no.)	% Similarity (analysed bases)	Closest described relative (acc. no.)	% Similarity (analysed bases)	Pipe material/ years	Comment Closest 16S rRNA sequence, closest described relative
M II 8	Actinobacteria	Mycobacterium chelonae CIP 104535T AY593980	99 (1042/1047,0G)	Mycobacterium massiliense AY593980	99 (1039/1047,0G)	PVC/28	phylogenetic study, sputum of patient with hemoptoic Pneumonia
M VII 6	Actinobacteria	Mycobacterium septicum ATCC 700731 AY457070	98 (462/467,2G)	Mycobacetrium septicum D13 AY772166	99 (463/467,2G)	PVC/34	phylogenetic study, sputum of hemoptoic pneumonia patient
M VII 7	Actinobacteria	Uncultured clone B4M69D3 AY957948	99 (453/456,1G)	Mycobacterium sp. O228YA DQ372728	99 (454/456,1G)	PVC/34	chlorinated drinking water biofilm, soil at illegal dumping site and landfills in Japan
M V 4	Actinobacteria	Mycobacterium aichiense JS618 AF498656	99 (449/453,2G)	Mycobacterium sp. K128Y DQ372730	99 (448/450,2G)	grey cast iron/30	soil at illegal dumping site and landfills in Japan, vinyl chloride contaminated soil
M V 9	Actinobacteria	Uncultured actinobacterium clone DOK_BIODYN_254 DQ827954	99 (454/457,1G)	Mycobacterium tusciae JS617 AF498655	99(452/456,1G)	grey cast iron/30	soil, vinyl-chloride-assimilating bacteria from contaminated sites
M VI 11	Actinobacteria	glacial ice bacterium SB12K-2-16 AF479359	99 (441/445,3G)	Mycobacterium aurum X55595	98 (446/454,7G)	PVC/12	bacteria from glacial and subglacial environments, phylogenetic study
M IV 10	Actinobacteria	Uncultured clone tpb-16-IJ-F04 DQ407394	97 (451/461,4G)	Nocardioides sp. JS614 AF498652	97 (456/467,5G)	grey cast iron/99	mixed waste contamination, vinyl chloride-assimilating bacteria from contaminated sites
BWB IV 11	Actinobacteria	Nocardioides sp. 2_4V EF540473	97 (424/433,11G)	Nocardioides sp. CF8 AF210769	97 (419/431,7G)	P´VC/24	solid waste from oil-shale chemical industry, alkane-utilizing strain
M VI 1	Actinobacteria	Nocardia sp. TFS 285 EF216367.1	98 (423/431,2G)	Nocardia salmonicida DSM40472 AF430050	98 (419/426,2G)	PVC/12	shallow water sediments of Trond-heim fjord, phylogenetic study

isolate	Taxonomic group/ Phylogenetic affiliation Phylum/class	Closest 16S rRNA sequence (acc. no.)	% Similarity (analysed bases)	Closest described relative (acc. no.)	% Similarity (analysed bases)	Pipe material/ years	Comment Closest 16S rRNA sequence, closest described relative
M II 7	Actinobacteria	Nocardia sp. TFS 359 EF216366	98 (1019/1032,5G)	Nocardia cummidelens DSM 44490 AF430052	99 (1009/1017,1G)	PVC/28	shallow water sediments of Trondheim fjord,phylogenetic study
M II 1	Actinobacteria	Nocardia cummidelens DSM 44490 AF430052	99 (935/936,0G)	Nocardia cummidelens R89 AF277202	99 (935/936,0G)	PVC/28	both phylogenetic study
M V 5	Actinobacteria	Uncultured clone D_FB066_h2_OTU177 EF175375	99 (454/458,0G)	Cellulomonas composti AB166887	93 (434/463,4G)	grey cast iron/30	acidic nitrate- and uranium-contaminated aquifer, cattle farm compost
BWB I 16_2c	Actinobacteria	Streptomyces amakusaensis NRRL B-3351 AY999781	99 (1018/1027,2G)	Streptomyces inusitatus LMG 19955 AJ781340	99 (1000/1008,1G)	cement/10	both phylogenetic studies
BWB I 12	Actinobacteria	Streptomyces sp. ME02-6979.3a EU080943	99 (1025/1030,5G)	Streptomyces sp. CNR918 PL04 DQ448731	99 (1024/1030,5G)	cement/10	scabby potatoes, marine sediment
BWB I 2	Actinobacteria	Kocuria sp. RM1 EF675625	98 (988/1002,5G)	Kocuria sp. HI-4° DQ205297	99 (983/992,0G)	cement/10	extreme alkaline bauxite residue, microbial diversity in an cavern (Höhle)
BWB I 1	Actinobacteria	Arthrobacter sp. KV-653 AB279890	99 (1028/1031,2G)	Actinobacterium EC5 AY337600	99 (1028/1031,2G)	cement/10	phylogenetic study, study of translational apparatus
BWB I 15	Actinobacteria	Agrococcus citreus IAM 15145 AB279547	99 (1021/1024,0G)	Agrococcus jenensis X92492	99 (1017/1024,0G)	cement/10	taxonomic study, new genus of actinomycetes with special cell wall
BWB I 4	Actinobacteria	Microbacterium sp. BM-12_4 AY635877	99 (1022/1023,0G)	Microbacterium phyllosphaerae DSM 13468 AJ277840	100 (1023/1023,0G)	cement/10	spent mushroom compost, phyllosphere of grasses

isolate	Taxonomic group/ Phylogenetic affiliation Phylum/class	Closest 16S rRNA sequence (acc. no.)	% Similarity (analysed bases)	Closest described relative (acc. no.)	% Similarity (analysed bases)	Pipe material/ years	Comment Closest 16S rRNA sequence, closest described relative
M IV 3	Firmicutes	Marine sediment bacterium ISA-7324 AY911145	98 (476/485,6G)	Bacillus megaterium SUF4 AJ880767	97 (477/487,7G)	grey cast iron/99	sea floor sediment, clean room facilities
BWB I 3A	Firmicutes	Bacillus licheniformis DQ372686	99 (1069/1070,1G)	Bacillus licheniformis M1-1 AB039328	99 (1069/1070,1G)	cement/10	halothermotolerant B. licheniformis from petroleum reservoir, cellulose degradation by mixed-culture system
M IV 8	Firmicutes	Bacillus subtilis E9 DQ474759	97 (473/486,6G)	Bacillus amyloliquefaciens FZB42 CP000560	97 (473/486,6G)	grey cast iron/99	optimization of fermentation, plant growth promoting B. amyl.
M III 1	Firmicutes	Bacillus sp. TUT1217 AB188216	98 (482/489,3G)	Bacillus licheniformis BCRC 12826 EF423608	98 (483/489,3G)	grey cast iron, cement/37	proteolytic bacteria in garbage composters, phylogenetic study of B. subtilis group
BWB I 8	Firmicutes	Bacillus subtilis B3 EF492885	100 (1075/1075,0G)	Bacillus subtilis B-FS01 DQ520955	99 (1074/1075,0G)	cement/10	plant protection, antibiotics producing Bacillus
M IV 7	Firmicutes	Bacterium Te23R AY587810	98 (477/484,6G)	Bacillus pumilus TUT1346 AB308441	98 (478/485,7G)	grey cast iron/99	spoilage bacteria from sausages after shelf-life period, water depend-ent population shift in mesophilic fed batch composting
M II 10	Firmicutes	Uncultured clone Amb_836 EF018569	99 (1065/1066,1G)	Bacillus sp. VTT E-052910 EF093131	99 (1065/1066,1G)	PVC/28	microbial diversity associated with trembling aspen, surfaces of historic scottish monuments
M IV 9	Firmicutes	Bacillus sp. NH14-1 EF690432	99 (487/490,2G)	Bacillus cereus ATCC 25621 AY795568	99 (487/490,2G)	grey cast iron/99	plant growth promoting bacteria, phylogenetic study
BWB I 7	Firmicutes	Bacillus sp. M14-10 EF690435	100 (1028/1028,0G)	Bacillus simplex WN579 DQ275178	100 (1028/1028,0G)	cement/10	plant growth promoting rhizobacteria, B. endospores isolated from granite

isolate	Taxonomic group/ Phylogenetic affiliation Phylum/class	Closest 16S rRNA sequence (acc. no.)	% Similarity (analysed bases)	Closest described relative (acc. no.)	% Similarity (analysed bases)	Pipe material/ years	Comment Closest 16S rRNA sequence, closest described relative
M IV 3	Firmicutes	Uncultured clone KSC2-10 DQ532282	97 (477/487,7G)	Bacillus megaterium KL-197 AY030338	97 (477/487,7G)	grey cast iron/99	clean-rooms where spacecraft are assembled, clean-room facilities
M VI 7	Firmicutes	Bacillus endophyticus Mali 49 AY211143	98 (481/489,4G)	Bacillus sp. BacB10 AF497256	98 (476/483,4G)	PVC/12	aerosolized bacteria from desert dust, rhizosphere of transgenic potatoes
M V 6	Firmicutes	Paenibacillus sp. HPC251 AY305001	99 (411/412,0G)	Paenibacillus lactis MB 1871 AY257868	99 (410/413,0G)	grey cast iron/30	antibiotic sensitive isolate, raw and heat-treated milk
BWB I 10	Firmicutes	Bacillus sp. SGE2 AY556409	99 (1062/1071,4G)	Bacillus chitinolyticus AB021183	99 (1063/1071,4G)	cement/10	enrichment of chitinolytic microorgan-isms, phylogenetic study
BWB I 9	Firmicutes	Paenibacillus sp. M10-6 EF690424	99 (991/995,2G)	Paenibacillus sp. MI-61a DQ180952	97 (984/1010,0G)	cement/10	plant growth promoting rhizobacteria, cultural bacteria in an cavern in Arizona

acc. no: genbank accession no. analysed bases: analysed bases from query and genbank sequence G: gaps in alignment between query and genbank sequence

The class *Betaproteobacteria* was of special interest because their members *Aquabacterium commune* and *A. parvum* were described as dominant in young drinking water biofilms grown on PE in the Berlin drinking water system (Kalmbach et al. 1997b). Therefore, the phylogenetic relationship of the *Betaproteobacteria* found in this study and the *Aquabacteria* of the investigation by Kalmbach was assayed. To achieve an improved phylogenetic analysis of the *Betaproteobacteria* of this study, the *Aquabacteria* and their next relatives described by Kalmbach were loaded into the ARB program, aligned by the aligner tool and a similarity matrix was calculated as shown in table 14. The calculation resulted in similarities of 89 to 92 % for M III 5 as the closest isolate to the *Aquabacteria* and their next relatives. The similarities of the rest of the betaproteobacterial isolates varied between 81 and 89 % similarity to the *Aquabacterium* cluster.

Tab. 14: Phylogenetic relationship of isolates of different pipe samples of this study and *Aquabacterium commune*, *A. citratiphilum*, *A. parvum* and their next relatives. The calculation was done with the ARB software package, database SS_jan04_corr_opt.arb.

Species	***Ideonella sp.***	***L. cholodnii***	***L. mobilis***	***L. discophora***	***A. citratiphilum***	***A. commune***	***A. parvum***
genbank	**AB049107**	**X97070**	**X97071**	**L33975**	**AF035050**	**AF035052**	**AF035052**
BWB IV 5	89	87	88	89	88	88	89
BWB IV 6	86	83	84	86	86	85	85
BWB IV 12	86	85	84	83	87	87	86
BWB IV 14	89	85	86	88	88	88	87
BWB IV 15	84	81	83	82	85	84	82
BWB IV 23	87	85	85	87	89	87	85
BWB V 3	85	84	84	86	83	84	87
BWB V 14	84	83	83	82	85	85	84
M III 5	91	91	92	89	91	91	89
M VI 10	85	83	83	88	84	84	87

L.:*Leptothrix* A.:*Aquabacterium* for details of the isolates BWB and M see table 13 chapter 3.1.3

3.2 Results reactor system Berlin

3.2.1 Operation of the Berlin reactor system

The flow conditions in figure 6 show that at the first 134 days of operation the median flow rate was higher compared to flow rates after day 134, 5 m^3/h (range 0.5 to 45 m^3/h) and 0.4 m^3/h (range 0.05 to 3 m^3/h) respectively. The enhanced flow rates in first operation phase aimed to reduce the contamination with *P. aeruginosa*. This contamination was also the reason for the flushing operations up to day 110. As chlorination should be avoided and because the disinfection with H_2O_2 did not solve the contamination, enhanced flushing seemed to be a possibility with less chemical impact to the system.

The median temperature in the outlet was 13.1°C (range 7.0 to 20.8°C). A slight seasonal tendency is indicated with higher values in August 2000 and 2001 around day 200 and 560 and lower temperatures in February (around day 20 and 400) of both years. The statistical analysis of the temperatures measured at the inlet and outlet during the flow operation mode showed that there was a very slight warming of the water during passage through the system (n=42, alpha=0.05, p=0.03, Wilcoxon matched pairs test). In contrast to this, there was a distinct significant warming of the water during stagnation experiments at the inlet and outlet sampling point (n=34, alpha= 0.05, $p<0.001$, Wilcoxon matched pairs test).

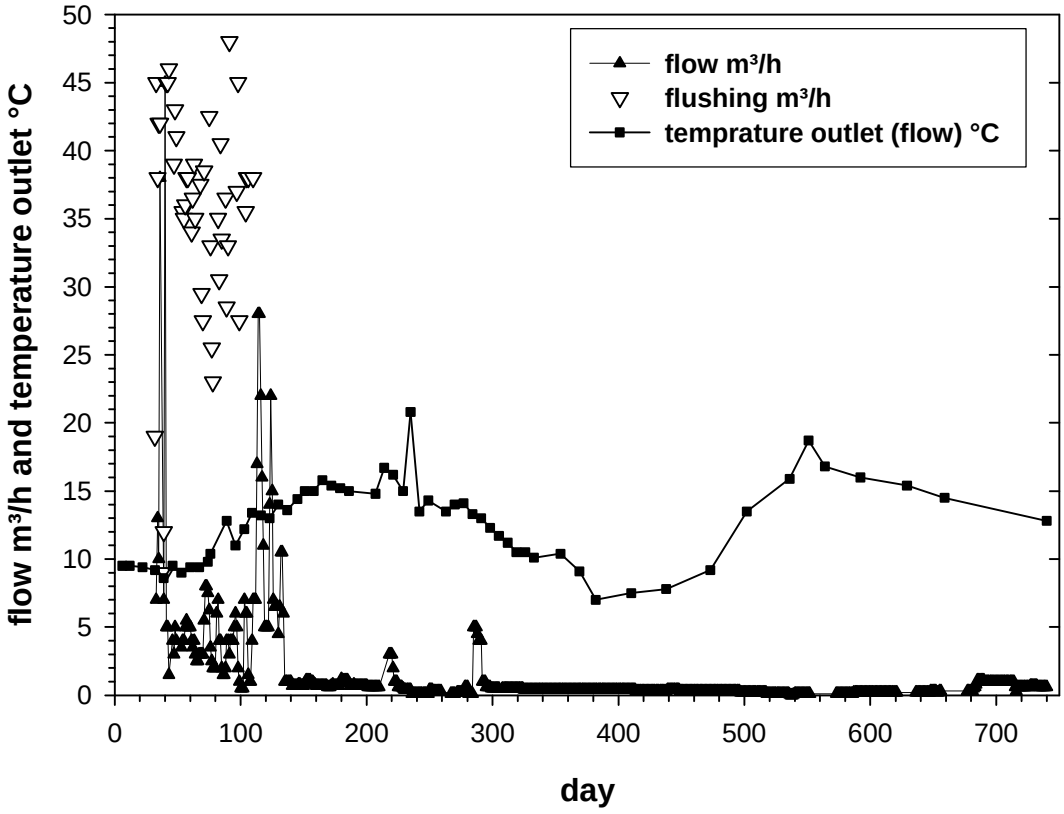


Fig. 6: Flow conditions and temperature during flow at the outlet in the reactor system during 740 days of operation. In addition to the flow operation mode, flushing operation mode (flushing) up to day 110 with an enhanced flow rate is documented.

3.2.2 *P. aeruginosa* in the bulk water phase of the reactor system in Berlin

The reactor system was first started in November 1999 after cleaning and disinfection with H_2O_2. A first sample taken of the bulk water phase after some hours of operation showed an indication for a *P. aeruginosa* contamination (specific odour of the colonies on the agar plates, laboratory of the Berliner Wasserbetriebe). The problem of the *P. aeruginosa* contamination arose again 15 and 21 days after start and disinfection of the system. Because different flushing operations, depletion and partial drying of the system did not solve the *Pseudomonas* contamination the system was stopped. The system was cleaned and disinfected once again and additionally the whole reactor system was disinfected with H_2O_2 (active conc. approx. 10^4 mg/L determined at three different sites of the system) to preclude a contamination during the set up of the system. Subsequent to these actions the system was started again. The

bulk water phase was regularly investigated for heterotrophic plate counts (GDWR 1990, chapter 2.3.2) and *P. aeruginosa* (DIN EN 12780, chapter 2.3.4). The *P. aeruginosa* specific cultivation method DIN EN 12780 showed that the organism was still present in the system. After conference with the project partners it was decided to run the system with *P. aeruginosa* as a real contamination event and to study its progress. According to the cooperation project rules the coupons were sampled every 6 months. As a consequence it was not possible to investigate if *P. aeruginosa* was able to establish in the biofilms on the coupons at this time.

3.2.2.1 Specificity of the DIN EN 12780

The DIN EN 12780 for specific detection of *P. aeruginosa* is based on cultivation of the targeted organism under formation of typical coloured colonies (blue-green, pyocyanin-production). Typically coloured colonies are accepted as *P. aeruginosa* without any further characterization. (chapter 2.3.4) To verify the specificity of the standard method the specificity was checked by application of molecular techniques on isolates obtained from the Berlin reactor system. From the *P. aeruginosa* positive isolates 14 were randomly chosen and the 16S rRNA gene was sequenced. All isolates could be phylogenetically ranged as *P. aeruginosa* with the ARB-Software package (chapter 2.4.6). The maximum difference was 1.3 % to *P. aeruginosa* (ATCC 25330). This result confirms the EN standard 12780 as a specific cultivation technique for *P. aeruginosa* in the habitate of this study.

3.2.2.2 Growth of isolated P. aeruginosa on modified R2A medium

The 14 *P. aeruginosa* isolates obtained from the bulk water phase of the reactor system were plated on Tween 80 modified R2A (chapter 2.3.1) and incubated at 36°C in the dark. After 14 h of incubation the plates were examined and the typical blue-green or yellow-green colonies were seen on the plates.

3.2.2.3 Detection of P. aeruginosa in the bulk water phase of the reactor system

As described above the *P. aeruginosa* contamination in the reactor system was observed by the *P. aeruginosa* specific cultivation method DIN EN 12780. At the end of the first run the *P. aeruginosa* colony counts were 563 CFU/100 mL. Because of this high CFU value the system was cleaned once again and additionally the whole reactor system was disinfected with a solution of H_2O_2 (active conc. approx. 10^4 mg/L at three different sites of the system). Despite this treatment the contamination was not removed. The contamination was observed up to 355 days. At 59 days 184 bulk water samples from different sites of the reactor system were analysed. Figure 7a shows the progression of the contamination at the first 96 days of operation in the outlet after the above mentioned disinfection. On one hand results of samples taken during water flow through the system can be seen. On the other hand bulk water samples were taken after a stagnation time of 4 to 6.5 h (from day 235 stagnation times of 16.5 to 19.5 h, data not shown) to determine the regrowth potential of *P. aeruginosa* in the system. In the beginning of the second run (figure 7a) the colony counts of *P. aeruginosa* in the flowing water of the outlet were reduced to 4, 7 and 1 CFU/100 mL at days 5, 14 and 18. In contrast, 563 colonies were counted at the end of the first run of the system (data not shown). A slight increase to the maximum of 17 CFU/100 mL in the flowing water was detected at day 22 before the stagnation experiments were started. In the first stagnation experiment after a stagnation time of 5.5 h at day 22 a value of 200 CFU/100 mL was detected before the water flow was started again. At day 32 the CFU were reduced to 4 in the flowing water and 44 CFU/100 mL after stagnation. In the following weeks the colony count in the flowing water continuously decreased. After 67 days of operation *P. aeruginosa* was generally not detected by the DIN EN 12780. In two exceptional cases (out of 47 samples taken) 1 and 2 CFU/100 mL were detected. An extended stagnation time from day 235 (16.5 to 19.5 h) resulted not in higher colony counts. The last samples were analyzed at day 355.

Figure 7b shows the colony counts of *P. aeruginosa* in comparison with the temperature of the bulk water. Until day 74 when *P. aeruginosa* was detected in the bulk water phase the temperature was below 15°C, and despite an increase after day 74 no enhanced colony counts for *P. aeruginosa* were observed (figure 7 b).

The addition of the values for the flow rates (m^3/h) during flushing operation mode and normal flow (figure 7c) show that a reduction in the normal flow in combination with periodic flushing experiments did not result in an increase of culturable *P. aeruginosa* in the bulk water. Moreover resulted termination of the flushing operations and another drawdown of the flow to less than or equal to 1 m^3/h not in enhanced *P. aeruginosa* counts (data not shown).

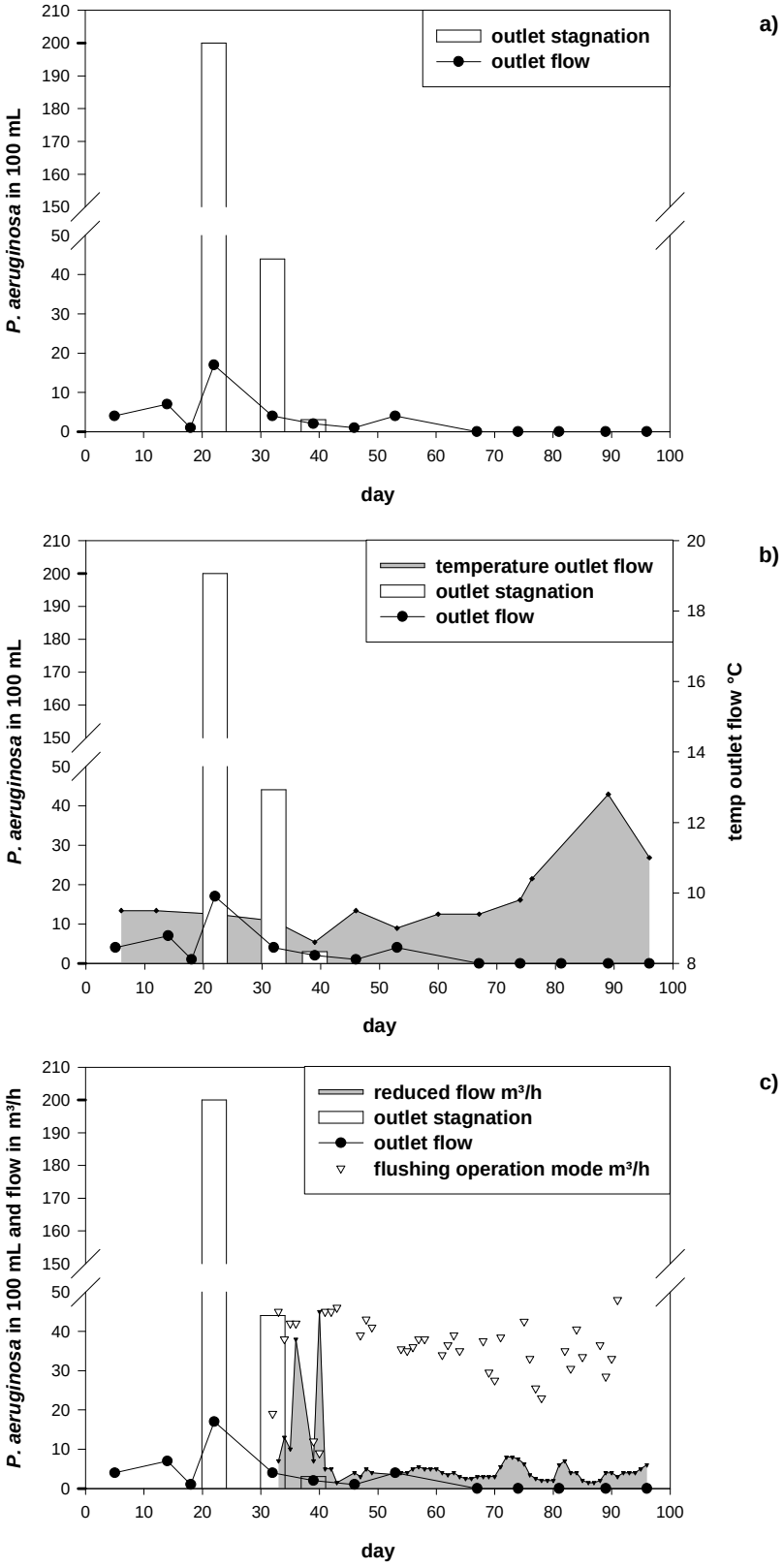


Fig. 7: Detection of *P. aeruginosa* (DIN EN 12780) in bulk water samples of the reactor system outlet in the flow and stagnation mode a) compared to temperature b) and flow conditions (reduced flow and flushing operation mode) c) during the first 96 days of operation of the Berlin reactor system.

In addition to the outlet the bulk water phase of the inlet was regularly investigated. The samples taken at the inlet till day 67 showed values of zero to 33 CFU/100mL at the flow and zero to 13 at the stagnation operation mode and decreased to zero afterwards (data not shown). In the flow mode the maximum at the inlet (33 CFU/100 mL, day 39) is clearly higher than the maximum of the outlet (17 CFU/100 mL, day 22). Differing from this the stagnation sample of the outlet showed a maximum of 200 CFU/100 mL (day 22) and the inlet a maximum of 53 CFU/100 mL (day 13). Extended stagnation times (16.5 to 19.5 h) from day 235 showed no influence to *P. aeruginosa* detection at any of the sampling points. The investigation of *P. aeruginaosa* terminated at day 355.

3.2.3 Further microbiological investigations of the bulk water phase

3.2.3.1 Heterotrophic bacteria in the bulk water phase

To estimate the development of the heterotrophic population in the bulk water phase of the Berlin reactor system during the running time of 740 days colony counts according GDWR 1990 (German drinking water regulations 1990) were performed. At 86 sampling days 264 samples of the inlet, outlet or sampling points at the reactors were analysed. Figure 8a shows the heterotrophic plate counts (HPC) of 355 days in the flow operation mode. During the first 109 days a strong irregular fluctuation can be seen in the inlet and outlet samples with maxima at 460 and 490 CFU/mL, respectively. Later the values decreased about one log unit to 10-30 CFU/mL and the curve fitting of inlet and outlet approximates. Extended stagnation times after day 235 (16.5 to 19.5 h) resulted not in distinct higher colony counts. An outier at day 165 can be seen. As can be seen in figure 8b and 8c higher temperatures, termination of flushing experiments and drawdown of normal flow to less than or equal to 1 m^3/h resulted not in an increase of HPC bacteria.

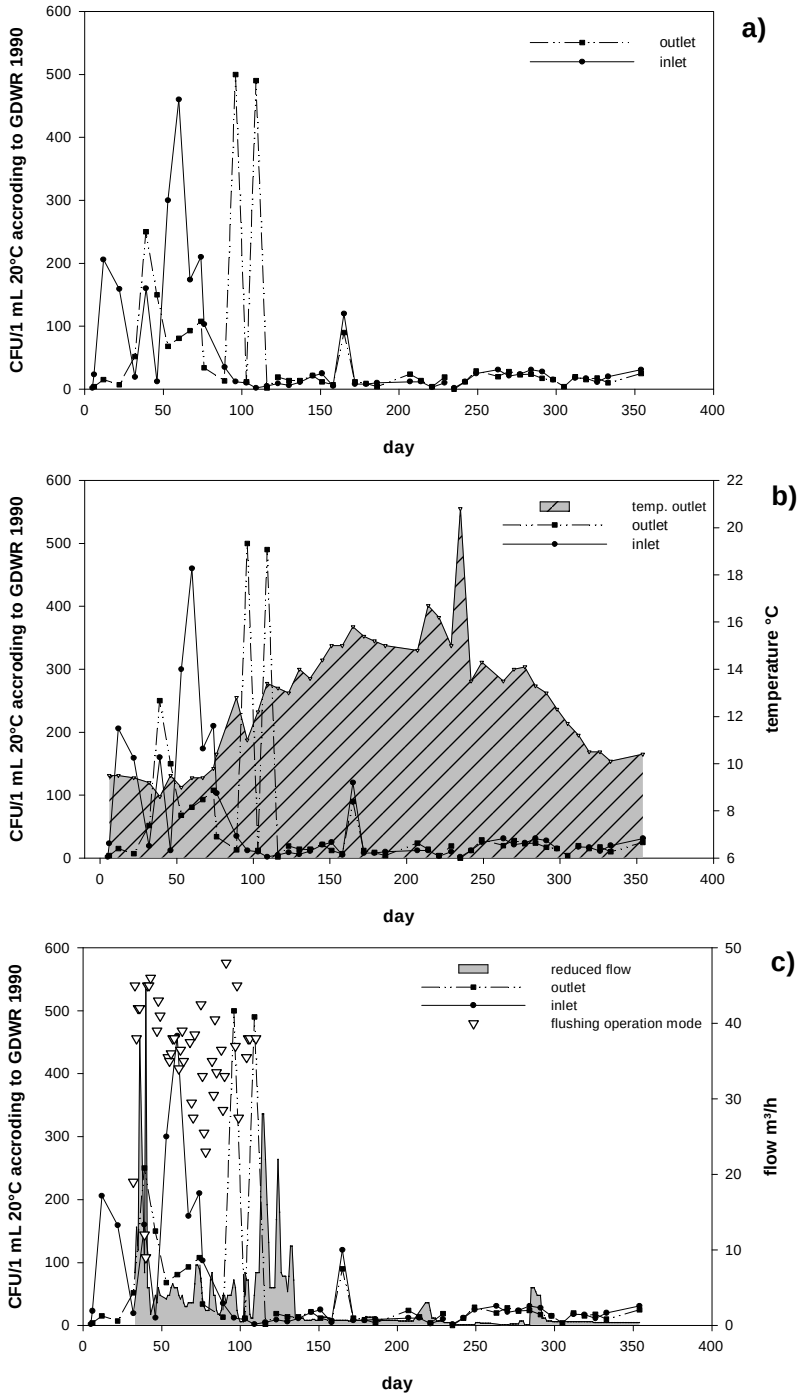


Fig. 8: Heterotrophic plate count (HPC) bacteria in the free water phase of the reactor system in the flow operation mode cultivated according to GDWR 1990. HPC bacteria grown at 20°C until day 355 in fig. a) and compared to temperature in fig. b) and flow conditions fig. c).

The data of the inlet and outlet in the stagnation mode display a comparable trend (data not shown). Until day 46 the data also fluctuate irregularly but show higher maxima (>5000 CFU/1mL) as detected in the flow samples. After the initial phase the values were reduced one log unit and the curve fitting of the inlet and outlet approximates as described for the flow operation mode. In contrast to the flow operation mode extended stagnation times resulted temporarily in higher colony counts at day 236 (outlet) and 243 (inlet). Again an outlier at day 285 was detected.

3.2.3.2 Statistical analysis of the data from the Berlin reactor system

Because there were enough data for statistical analysis the culturable bacterial population of the bulk water phase according to GDWR 1990 was tested as described in chapter 2.5. The tests for normal distribution showed that most of the natural and the transformed (log, +1) data were not normal distributed. As a consequence non parametric test statistics were chosen to find out differences or consistency between the parameters. The non parametric Wilcoxon matched pairs test for dependent samples was applied at an error probability of 5 % to answer the following questions:

Question 1: Is there a quantitative difference between the culturable bacteria of the bulk water phase incubated at 20°C or 36°C?

The test statistics showed that there are significant higher numbers of bacteria detected at the incubation temperature of 20°C. Values were compared at the inlet and outlet for flow and stagnation operation mode ($n=41$ to 57, $p< 0.05$).

Question 2: Are there countable differences on the medium according to GDWR 1990 between flow and stagnation operation mode?

The results for this analysis are not consistent and are shown in table 15. The analysis of the data showed no difference between the stagnation and flow operation mode for the samples taken at the inlet and incubated at 36°C and the outlet incubated at 20°C. In contrast to this, samples from the inlet incubated at 20°C and the outlet at 36°C showed higher values after stagnation.

Tab. 15: Statistical analysis (Wilcoxon matched pairs test) of bacterial counts in the bulk water phase of the Berlin reactor system according to GDWR 1990. Flow versus stagnation operation mode.

sample	**p-value***	**result**	**comment**
20°C_inlet_flow & 20°C_inlet_stag	0.006	different	20°C_inlet_flow < 20°C_inlet_stag
36°C_inlet_flow & 36°C_inlet_stag	0.119	equal	
20°C_outlet_flow & 20°C_outlet_stag	0.562	equal	
36°C_outlet_flow & 36°C_outlet_stag	0.019	different	36°C_outlet_flow < 36°C_outlet_stag

*alpha=0.05 n=41 to 57

Question 3: Is there a regrowth potential for the culturable bulk water population during flow through the reactor system?

The analysis showed that there is no regrowth potential for the bacterial population incubated at 20°C. In contrast to this a regrowth potential with significant higher values at the outlet than at the inlet could be seen for the 36°C incubated culturable bacteria.

Question 4: Is there a difference in the number of culturable bacteria detectable in the outlet and in the inlet during stagnation?

The comparison of the colony counts at 20°C for inlet and outlet showed no difference during stagnation mode. Contrary to this the cultivations at 36°C resulted in higher values in the outlet than in the inlet.

Question 5: Are there any correlations between flow, temperature and/or the cultivation data according to GDWR?

Tab. 16: Correlation of colony counts according to GDWR 1990 with flow and temperature (Spearman rank order correlations).

colony counts at (n=34)	flow rate	temperature
20°C_inlet_flow	n. c.	-0.45
20°C_outlet_flow	n. c.	-0.35
36°C_inlet_flow	+0.46	n. c.
36°C_outlet_flow	+0.46	n. c.

n. c.: no correlation high positive or negative correlation: ±1

During flow operation mode the inlet and outlet colony counts at 20°C showed a slight negative correlation with temperature and no correlation with the flow conditions. Opposite to this colony counts at 36°C showed no correlation with temperature but a slight positive correlation with the flow rate through the reactor. (Spearman rank order correlations)

The appropriate stagnation values showed no significant correlation with temperature.

3.2.3.3 E. coli and coliform bacteria according to DIN 38 411 K 6

During the total operation of the system no *E. coli* or coliform bacteria were detected in the bulk water samples taken at the outlet, inlet, and at the reactors during flow and stagnation mode of the system tested with the method DIN 38 411 K 6. In total 264 samples from different sampling points were analyzed during 740 days of operation.

3.2.4 Bacterial population on material coupons of the reactor systems in Germany

To access the total number of bacteria including culturable and non culturable species total cell counts with the DNA stain DAPI were performed directly on the material coupons of three different reactor systems. The systems in Berlin Lichterfelde (B) and Gladbeck (G) were located in the distribution system. In contrast to this the system in Dorsten-Holsterhausen (DH) was situated near to the drinking water treatment plant. The coupons were made up of glass, PE-HD, PVC, Cu, and steel (V2A in Berlin, V4A in Gladbeck and Dorsten-Holsterhausen). The exposition time to drinking water was 6, 12, 18, and 24 months.

3.2.4.1 Total cell counts (TTC) on material coupons

Tables 17 and 18 show the total cell counts determined by DAPI staining on the material coupons exposed to drinking water in the reactor systems of Berlin Lichterfelde (B), Gladbeck (G), and Dorsten-Holsterhausen (DH). Bacterial cells were counted in scraped biofilm suspensions table 17 and directly on coupons of different materials table 18. The comparison of values determined in the biofilm suspension with the corresponding value directly counted on the coupon shows that for every value pair the counts in the suspension were below the direct counts on the coupons. Values are of the same magnitude or are one to two orders of magnitude higher. Due to the small number of values a statistical analysis has not been done.

Tab. 17: Total cell counts (DAPI) detected on coupons of different materials. Coupons were exposed for 6, 12, 18, and 24 months. Counts were determined in the scraped biofilm suspension tab.17) and directly on material coupons tab. 18).

Months*	**Total cell counts in biofilm suspension**														
	PE			PVC			Cu			V2A	V4A		glass		
	B	G	DH	B	G	DH	B	G	DH	B	G	DH	B	G	DH
6	8.02×10^5	6.43×10^5	6.82×10^5	8.18×10^5	8.94×10^5	1.2×10^6	1.02×10^5	n.d.	3.12×10^7	5.02×10^5	3.21×10^5	9.71×10^5	3.88×10^5	n.d.	2.89×10^6
12	1.75×10^4	2.30×10^5	2.12×10^5	5.47×10^4	n.d.	n.d.	n.d.	3.51×10^6	n.d.	n.d.	3.77×10^4	1.45×10^5	9.47×10^3	n.d.	n.d.
18	1.31×10^5	1.71×10^6	3.70×10^5	5.41×10^5	2.89×10^5	1.23×10^5	1.17×10^6	2.08×10^6	n.d.	2.76×10^5	1.10×10^6	2.67×10^5	3.22×10^5	n.d.	n.d.
24	n.d.	n.d.	2.81×10^6	n.d.	n.d.	2.24×10^6	n.d.	n.d.	3.46×10^7	n.d.	n.d.	1.61×10^6	n.d.	n.d.	n.d.

*months coupons were exposed to drinking water before sampling reactor systems in Berlin Lichterfelde (B), Gladbeck (G), and Dorsten-Holsterhausen (DH)

Tab. 18: Total cell counts directly determined on material coupons.

Months*	**Total cell counts directly on material coupons**														
	PE			PVC			Cu			V2A	V4A		glass		
	B	G	DH	B	G	DH	B	G	DH	B	G	DH	B	G	DH
6	2.2×10^6	1.3×10^6	6.0×10^6	1.7×10^6	1.8×10^6	1.9×10^6	n.d.	1.5×10^7	3.7×10^7	3.1×10^6	5.1×10^5	1.3×10^6	1.6×10^6	n.d.	3.2×10^6
12	$6. \times 10^6$	6.0×10^6	1.2×10^7	5.1×10^6	3.3×10^6	8.3×10^6	n.d.	9.6×10^6	1.6×10^7	9.9×10^6	7.2×10^5	1.6×10^6	6.1×10^5	1.2×10^6	4.2×10^6
18	4.4×10^6	8.0×10^6	1.3×10^7	1.1×10^7	2.6×10^6	1.2×10^7	6.0×10^6	9.8×10^6	1.2×10^7	4.2×10^6	1.2×10^6	9.2×10^6	n.d.	2.2×10^6	5.4×10^6
24	8.3×10^6	n.d.	1.2×10^7	4.5×10^6	n.d.	7.2×10^6	1.4×10^7	n.d.	1.3×10^6	n.d.	n.d.	1.3×10^6	3.6×10^6	n.d.	8.9×10^6

*months coupons were exposed to drinking water before sampling reactor systems in Berlin Lichterfelde (B), Gladbeck (G), and Dorsten-Holsterhausen (DH)

The above mentioned comparison of counts directly determined on material coupons and in biofilm suspension suggests that the direct counts are more consistent to the real counts. This is the reason why in the following only the counts directly counted on the material coupons are diagrammed.

In both diagrams of figure 9 no considerable difference can be seen in between the five materials or the four different expositions times. Total cell counts scatter around $5.6x10^6$ (median all). But two tendencies are recognized. On one hand there are slightly increased values at the copper coupons after 6 and 12 months of exposition. On the other hand total cell counts of all materials appear to converge with increased exposition time (6 to 24 months). At 18 and 24 months (figure 9) values range approximately between one order of magnitude from $1,2x10^6$ (RM_VI_G18 steel) to $1,3x10^7$ (RM_V_DH18_PE) counts/cm².

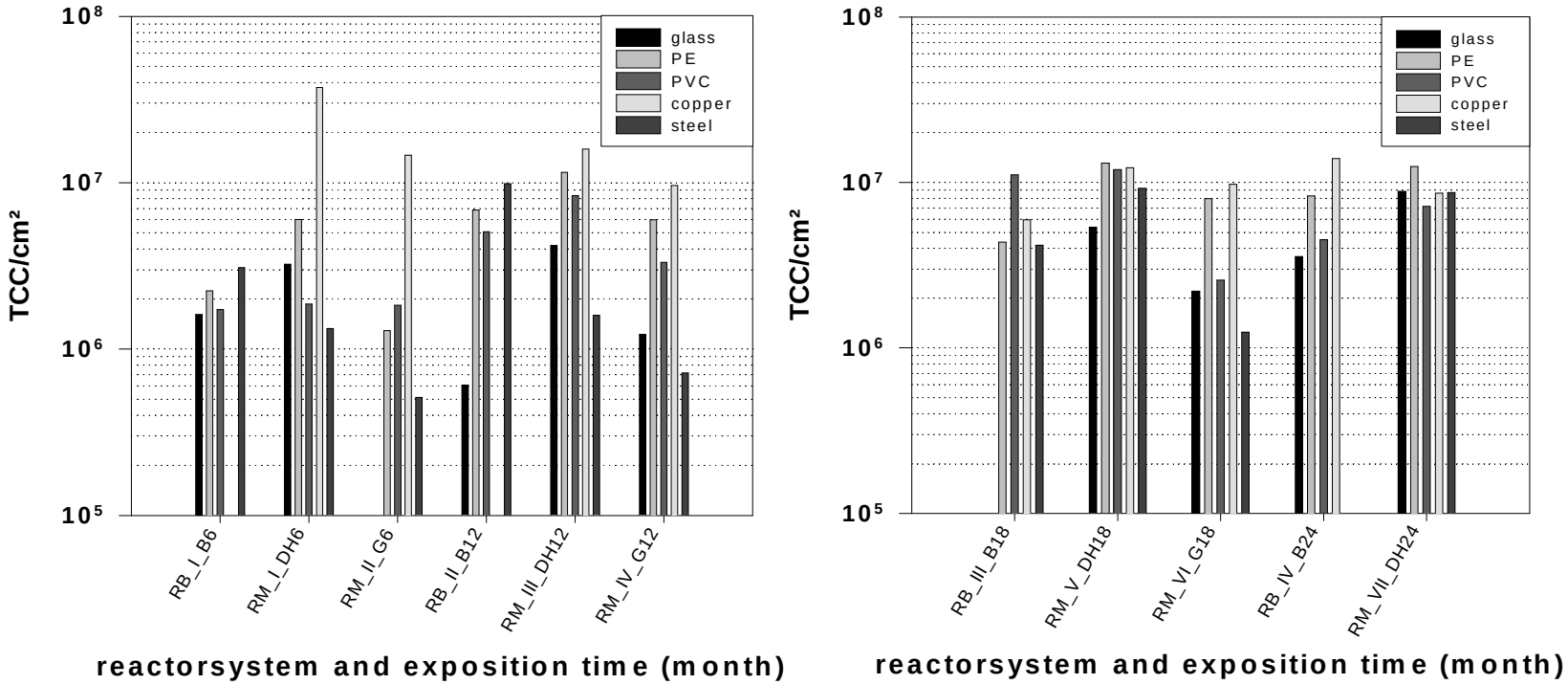


Fig. 9: Total cell counts (DAPI) detected on coupons of different materials exposed in the reactor systems in Berlin (B) and the Ruhr-gebiet (DH, G). Coupons were exposed to drinking water for 6, 12, 18, and 24 months.

3.2.4.2 Culturable bacteria on six months exposed material coupons

To get an insight in the culturable bacterial population biofilms of six months exposed coupons of different material were scraped off the surface under sterile conditions, suspended in sterile drinking water and cultivated on Tween 80 modified R2A agar as described in chapter 2.3.1. Colonies were selected by their colony morphology after 7 days of incubation at 20°C.

Comparison of the percentage of culturable bacteria in the two distribution system situated reactor systems in Berlin and Dorsten yielded the following results. The culturability in the Berlin system was higher than in the system in Dorsten (range 0.02 to 2 % in Berlin compared to a range of <0.01 to 0.11 % in Dorsten). Highest percentages of culturable bacteria were found on PE, PVC, and V2A coupons of the Berlin system.

Tab. 19: Culturable bacteria on modified R2A agar (+Tween 80) and total cell counts (TCC) on the 6 months exposed coupons of the reactor system in Berlin Lichterfelde and Dorsten-Holsterhausen.

Coupon material	**Berlin Lichterfelde**		**Culturable bacteria (%)[a)]**	**Dorsten-Holsterhausen**		**Culturable bacteria (%)[a)]**
	TCC/cm²	CFU/cm²		TCC/cm²	CFU/cm²	
PE	2.2×10^6	4.36×10^4	2	6.0×10^6	4.39×10^2	< 0.01
PVC	1.7×10^6	4.14×10^4	2	1.9×10^6	2.3×10^3	0.04
Cu	n.d.	3.71×10^4		3.7×10^7	6.93×10^2	< 0.01
V2A	3.1×10^6	3.0×10^4	1	n.d.	n.d.	
V4A	n.d.	n.d.		1.3×10^6	1.45×10^3	0.11
glass	1.6×10^6	3.01×10^2	0.02	3.2×10^6	3.95×10^3	0.1

n.d.: not determined a)percent of culturable bacteria based on total cell counts (DAPI)

3.2.4.3 Characterization of the biofilm isolates with specific oligonucleotid probes

The obtained biofilm isolates were subjected to a formaldehyde fixation and afterwards hybridized with 3 probes of different specificity (chapter 2.4.2). The first hybri-

dization was done with a genus specific probe which targets the most true pseudomonads (Schleifer et al. 1992). Isolates that showed a positive signal were hybridized again with a *P. aeruginosa*-specific probe (Hogardt et al. 2000) and a probe targeting *P. aeruginosa* and closely related pseudomonads (this study).

3.2.4.3.1 Verification of the new oligonucleotid probe

The newly developed *P. aeruginosa* specific oligonucleotide probe had to be tested for their specificity. For this purpose the probe was tested against type strains and environmental isolates from biofilm samples of the reactor system of this study. Table 20 shows the results of the hybridization with type strains. Both the *P. aeruginosa* specific and the genus specific probes showed a positive hybridization signal with the type strain of *P. aeruginosa*. With *P. fluorescens*, *P. putida*, and *P. stutzeri* only the genus specific probe hybridized positive. As expected non of the probes provided a positive signal with the negative controls *A. commue* and *E. coli*.

Tab. 20: Hybridization of probes targeting the genus *Pseudomonas* at different phylogenetic levels.

Type strain	**GCT GGC CTA GCC TTC** **true pseudomonads[a)]** **FA 35 %**	**TCT CGG CCT TGA AAC CCC** ***P. aeruginosa*[b)]** **FA 35 %**	**CCC ACC CGA GGT GCT GG** ***P. aeruginosa*[c)]** **FA 50 %**
P. aeruginosa *ATCC 15442*	+	+	+
P. fluorescens *DSM 50090*	+	–	–
P. putida *DSM 291*	+	–	–
P. stuzeri DSM 5190	+	–	–
A. commune DSM 11901	–	–	–
E. coli ATCC 11229	–	–	–

FA: formamide concentration a)Schleifer et al. 1992 b)Hogardt et al. 2000 c)this study

To access the *in situ* specificity four isolates from different material coupons that showed a positive signal with the genus specific probe and no signal with the *P. ae-*

ruginosa specific one, were sequenced. All isolates could be arranged in the genus *Pseudomonas* (table 21) and the results confirm the *in situ* specificity of the probes.

Tab. 21: Phylogenetic affiliation of selected isolates of the biofilm coupons of the Berlin reactor system.

Origin of isolate	**Nearest neighbour**	**Percent 16S rDNA sequence homology**
steel	*Pseudomonas mandelii*	99.8
PVC	*P. aureofaciens* *P. veronii*	99,1 98,9
PE	*P. aureofaciens* *P. veronii*	99,0 98,9
glass	*P. libaniensis* *P. cedrella*	99,6 99,2

3.2.4.3.2 Identification of isolates grown on material coupons in the Berlin reactor system

To detect *P. aeruginosa* in the culturable biofilm population yielded from modified R2A medium (chapter 2.3.1) 134 isolates from the material coupons (steel, PVC, PE, glass) were hybridized with the genus and the strain specific probes (see above). Of the total isolates 26 % could be identified as pseudomonads but non of them provided a positive signal with both *P. aeruginosa* specific probes. The culturable biofilm population grown on glass showed the highest fraction of pseudomonads (57 %), copper the lowest (3 %).

Tab. 22: Detection of pseudomonads and *P. aeruginosa* by *in situ* hybridization among the isolates of different material coupons exposed for 6 months to drinking water in the Berlin reactor system.

probe sequence (5`-3´)	**specificity**	**steel (V2A)**	**PVC**	**Cu**	**glass**	**PE**
GCT GGC CTA GCC TTC	most true pseudomonads[a)]	7 26 %	11 28 %	1 3 %	12 57 %	4 29 %
TCT CGG CCT TGA AAC CCC	*P. aeruginosa*[b)]	0	0	0	0	0
CCC ACC CGA GGT GCT GG	*P. aeruginosa*[c)]	0	0	0	0	0
total number of hybridized isolates	—	27	40	32	21	14

a)Schleifer et al. 1992 b)Hogardt et al. 2000 c)this study

3.2.5 Culturable biofilm population of the two reactor systems in Germany and Denmark

The following investigations were done in cooperation with Adam C. Martiny of the working group of Søren Molin at the BioCentrum-DTU and Environment and Resources of the Technical University of Denmark in DK-2800 Lyngby. The RFLP analysis and most of the phylogenetic analysis was done in Denmark, sequencing and a first phylogenetic sorting of the sequenced isolates has been carried out in Germany. Fifty-seven strains could not be recultured and were therefore discarded from the analysis, a problem previously observed (Kaeberlein et al. 2002).

3.2.5.1 Pre-screening of drinking water isolates by RFLP analysis

To have a less time consuming and less cost intensive method for the characterization of drinking water isolates based on 16S rDNA the 342 isolates of the two pilot plants were screened by RFLP analysis and sorted in OTUs (operational taxonomic units) according to their RFLP band pattern. Subsequent representatives of the resulting OTUs were selected, sequenced (single strand > 1400 bases), and the phylogenetical affiliation determined. For each RFLP pattern (OTU) consisting of more than one isolate, 16S rRNA genes of one to eight isolates were sequenced, resulting in a total of 55 complete 16S rDNA sequences. In cases where a specific OTU was detected in more than one microhabitat (bulk/biofilm or Denmark/Germany), one isolate from each sample was selected for subsequent sequence analysis. Sequence analysis was performed with an ABI Prism 310 sequencer (PE Deutschland GmbH, Weiterstadt, Germany) using an Applied Biosystems Big Dye Terminator Ready Reaction Mix Kit according to manufacturer`s instruction as described in chapter 2.4.4. 16S rDNA sequences were aligned with the ARB software package and manually corrected for errors. The new sequence was analyzed against the phylogenetic tree containing all sequences in the complemented ARB database (6spring2001) and allowed the identification of the families and genera in most cases. (chapter 2.4.6) Table 23 summarizes the results of RFLP sorting, phylogenetic affiliation, and origin of the isolates.

Tab. 23: RFLP sorting, phylogenetic affiliation, and origin of the culturable bacteria of the pilot plants in Denmark and Germany.

Phylogenetic affiliation					**RFLP sorting**			
					Biofilm		Bulk	
		Different OTUs	**Sequenced strains**	**Genotypes 16S RNA**	**DK**	**Germany**	**DK**	**Germany**
Sphingomonadaceae	*Sphingobium*	1	1	1	-	2	-	-
	Novosphingobium	1	2	1	-	-	-	7
	Sphingopyxis	1	1	1	-	2	-	-
Caulobacteraceae	*Brevundimonas*	4	8	4	-	-	5	36
Beijerinckiaceae	*Beijerinckia*	1	1	1	-	1	-	2
Bradyrhizobiaceae	*Afipia*	1	1	1	-	-	2	-
Rhodobiaceae	*Rhodobium/Devosia*	1	2	1	75	-	-	-
Total α-*Proteobacteria*					75	5	7	45
Oxalobacteraceae	*Duganella*	2	2	1	-	-	4	-
	deeply rooted[a)]	1	1	1	-	-	1	-
Alcaligenaceae	*deeply rooted*[a)]	1	3	1	-	-	-	6
Comamonadaceae	*Hydrogenophaga*	3	6	3	-	-	14	3
	Aquabacterium	2	2	2	-	-	8	2
	Rhodoferax	2	2	2	-	-	1	1
	Acidovorax	1	1	1	-	-	2	1
Neiserriaceae	*Iodobacter*	1	1	1	-	-	2	1
	Formivibrio	1	1	1	-	-	-	2
Rhodocyclaceae	*Rhodocyclus*	1	2	2	-	-	3	-
Total β-*Proteobacteria*					0	0	35	16
Xanthomonadaceae	*Luteimonas*	1	2	1	24	-	-	-
Pseudomonadaceae	*Pseudomonas*	3	8	5	-	47	1	1
Morexellaceae	*Acinetobacter*	1	1	1	-	-	3	-
Alteromonadaceae	*Shewanella*	1	2	1	-	1	-	5
Aeromonadaceae	*Aeromonas*	1	2	1	-	2	-	2
Total γ-*Proteobacteria*					24	50	4	8
Flavobacteriaceae	*Flavobacterium*	2	2	2	-	2	2	-
Bacillaceae	*Bacillus*	1	1	1	-	2	-	-
Others					0	4	2	0
Unknown					1	18	26	22
Total			55		100	77	74	91

a)no clear phylogenetic relationship with any genus

OTUs containing more than one isolate were investigated by sequencing. To check the specificity of the RFLP analysis more than one representative of an OTU was sequenced if the OTU contained more than six isolates. In general the sequence analysis validated the RFLP sorting but in three cases OTUs were heterogeneous by 16S rDNA sequencing. After application of a third enzyme (BstUI) only two heterogeneous OTU in the *Rhodocyclus* and the *Pseudomonas* group remained (table 23). In these cases one OTU contained more than one 16S rRNA genotype. In contrast, it was also found that strains from several OTUs were affiliated to one genus for example the *Hydrogenophaga* group (table 23). Only once in 34 analyzed OTUs strains from the same RFLP group clustered in different genera (*Iodobacter* and *Duganella*, sequence similarity 11 %).

3.2.5.2 OTUs detected on biofilm and in bulk water

Table 24 gives a review of the found OTUs on the biofilm and in the bulk water of the two pilot plants. For the amount of 77 investigated isolates of the biofilms of three materials (steel, PVC, PE) in Germany the initial RFLP analysis resulted not in differences in the microbial communities grown on the three materials. As a consequence the data were pooled for further analysis. The lowest number of OTUs (three) was found in the Danish biofilm were the highest number of isolates was analysed by RFLP analysis. In contrast to this the 77 isolates of the German biofilm resulted in 21 OTUs. In this study the sampled 100 mL of bulk water of the inlet in Denmark and the outlet in Germany showed a greater number of OTUs (37 and 35 OTUs for 74 and 91 isolates, respectively). None of the bacteria isolated from the Danish inlet water were detected in the biofilm, whereas three isolates cultivated from the Berlin outlet water were also isolated from the biofilm. Despite the geographical separation, five OTUs were detected in both systems.

Tab. 24: Distribution of OTUs in biofilm and bulk water of the two pilot plants.

Sampling site		No. of analyzed isolates	Total no. of OTUs	Sequenced strains
Denmark	Biofilm [a)]	100	3	4
	bulk	74	37	19
Germany	Biofilm [b)]	77	21	12
	bulk	91	35	20

a)steel b)steel, PVC, PE

3.2.5.3 Phylogenetic groups detected in the two reactor systems in Denmark and Germany

The 342 isolates obtained by cultivation of biofilm or bulk water on R2A were analysed by RFLP. The subsequent 16S rDNA sequencing of representatives of the resulting OTUs allowed the identification of phylogenetic affiliation. Seventy nine percent of the analyzed isolates were grouped to the phylum *Proteobacteria*. These can be subdivided in 49 % *Alphaproteobacteria*, 19 % *Betaproteobacteria*, and 32 % *Gammaproteobacteria*. Of all 342 isolates only two gram-positive *Bacillus* (phylum: *Firmicutes*) and four *Flavobacteriaceae* (phylum: *Bacteroidetes*) were found. (table 23) For a total of 67 isolates (67 from 342 in total) no clear phylogenetic relationship could be found.

Under the terms of the isolation conditions used in this study *Alphaproteobacteria* were detected in biofilm and bulk water of the pilot systems in Denmark and Germany. This class represented by members of the family *Rhodobiaceae* (Genus: *Rhodobium* and *Devosia*) was most frequently found in the biofilm of Denmark. Another huge amount was detected as *Caulobacteraceae* (genus: *Brevundimonas*) in the German bulk water. The families *Sphingomonadaceae*, *Beijernickiaceae*, and *Bradyrhizobiaceae* were less frequently found. The class *Betaproteobacteria* was neither isolated from the Danish nor from the German biofilms. In the Danish bulk water the genus *Hydrogenophaga* and *Aquabacterium* (both *Comamonadaceae*) were detected in slightly higher amounts. Furthermore the families *Oxalobacteraceae*, *Neiserriaceae* and *Rhodocyclaceae* were identified. The representatives of two OTUs showed no clear affiliation to any family. Gammaproteobacteria were found throughout both pilot systems in biofilm and bulk water. The German biofilm was dominated by the family *Pseudomonadaceae*. In the German biofilm and bulk water also the genera *Shewanella* and *Aeromonas* were identified. From the Danish biofilm only the family *Xanthomonadaceae,* in detail the genus *Luteimonas,* was isolated. (table 23).

3.3 DNA extraction methods

Regarding the DNA extraction the general problem of a limited amount of biofilm suspension of the different pipe samples had to be handled. The biofilm preparation in the laboratory (chapter 2.2) limited the length of the pipe sample to about one meter. Another challenge was the expected small number of bacterial cells in the biofilm samples of this habitat. It did not seem reasonable to dilute the biofilm suspension much as this might result in a low cell number for extraction. Furthermore, the sample had to be split up between the participants of the BMBF cooperation project. This reduced the available volume of biofilme suspension for this study. These are the reasons why the different methods or preparations were only applied to selected samples. After first results showed that amplification of bacterial DNA from biofilm suspensions after simple lysis and extraction procedure did not succeed, some approaches were made to get an idea of the problems caused by the old drinking water biofilm samples in DNA extraction and amplification. Afterwards, more complex DNA extraction methods were applied.

3.3.1 Inhibition and limitation of PCR by sample quality and preparation

3.3.1.1 Does sample composition, preparation or concentration effect PCR effectivity?

First it was of interest whether the sample composition e.g. diverse ions, limescale, cement, or residues of the pipe material inhibit PCR. Second it was important to know whether there was an influence of sample preparation and used solutions on the molecular reactions. The third point was to find out if this interference was concentration dependent.

For these reasons the extracted biofilm suspensions of the pipe samples BWB III (non-fixed) and the formaldehyde fixed M IV were spiked with PCR positive DNA of the drinking water isolate DK 79 in the PCR approach (chapter 2.4.3.1.1). These pipe samples were selected as they seemed to be the hardest for extraction because of the great amount of inorganic matter. Table 25 shows that the detection of spiked DNA of DK 79 is depending on the concentration of the lysed sample added in the

PCR approach. Moreover, there is no great difference between both biofilm samples. The slight divergence might be due to systematic failures as small differences in pipetting procedure. Amplification of the concentrated suspension and the six dilutions without addition of control DNA of DK 79 resulted in no band in the agaraose gel. PCR with control DNA of DK 79 without biofilm suspension confirmed that amplification basically functioned.

Tab. 25: PCR interference caused by sample composition and/or preparation of M IV (fixed) and BWB III (non-fixed) biofilm suspensions of the pipe samples. 1 µL biofilm suspension was added in 25 µL total PCR volume. DK 79 DNA: control DNA of bacterium DK 79

Dilution biofilm suspension	**Formaldeyhde fixed M IV**		**Non-fixed BWB III**	
	1. PCR	2. PCR	1. PCR	2. PCR
Concentrated biofilm suspension	–	–	–	–
10^{-1}	–	–	–	n.d.
10^{-2}	–	–	**(+)**	–
10^{-3}	–	+	+	+
10^{-4}	+	+	+	+
10^{-5}	+	+	+	n.d.
10^{-6}	+	+	+	+

– : no PCR band (+) : very weak PCR band + : PCR band

3.3.1.2 Does alkaline lysis in formaldehyde fixed biofilm suspensions interfere with PCR amplification?

This question was important because most of the initial biofilm samples were formaldehyde fixed. This procedure was performed since the first method of choice to describe the population of the biofilm suspension was FISH which usually supposes formaldehyde fixed bacteria (chapter 2.4.2). The biofilm suspension of M IV was

lysed with the alkaline buffer and diluted in AE buffer before applied to the PCR. In the PCR approach 1 µL (all dilutions) and 5 µL (only non diluted) of the lysed biofilm suspension were added in 25 µL PCR volume. (chapter 2.4.3.6.)

Alkaline lysis procedure seems to slightly reduce the inhibition. While the results in table 25 show a reliable positive PCR result at a dilution of $10^{-3}/10^{-4}$ in this approach a positive result was obtained at 10^{-2} (table 26). Under the used conditions amplification of total bacterial DNA in alkaline lysed biofilm suspensions of formaldehyde fixed material did not succeed.

PCR with formaldehyde fixed *E. coli* (DSM 5695) showed that it is in general possible to amplify DNA from formaldehyde fixed bacteria after alkaline lysis.

Tab. 26: Influence of alkaline lysis on PCR amplification of bacteria in formaldehyde fixed biofilm suspensions. M IV: biofilm suspension of the pipe sample. 1 µL biofilm suspension was added in 25 µL total PCR volume. DK 79 DNA: control DNA of bacterium DK 79

Dilution biofilm suspension	**Formaldeyhde fixed M IV**	
	alkaline lysis	alkaline lysis + DK 79 DNA
concentrated biofilm suspension	–	–
10^{-1}	–	–
10^{-2}	–	+
10^{-3}	–	+
10^{-4}	–	+
10^{-5}	–	+
10^{-6}	–	+

– : no PCR band + : PCR band

3.3.1.3 Do different DNA concentration methods combined with enhanced sample volume effect DNA amplification?

To clarify whether low DNA concentration was the reason for the lag of a positive PCR result, the volume of the biofilm suspension was enhanced and the DNA concentrated by ethanol or isopropanol precipitation. The combination of alkaline lysis, enhanced sample volume, and a precipitation step allowed for testing of interference of this combination of methods with PCR.

Neither enhanced sample volume nor concentration of DNA by ethanol precipitation made an amplification of *in situ* DNA possible in the formaldehyde fixed biofilm suspensions of M IV (table 27). In the PCR approach 1 µL (all dilutions) and 5 µL (only non diluted) of the lysed biofilm suspension were added in 25 µL PCR volume. Ethanol precipitation did not influence amplification in general because the control DNA of DK 79 was still detectable at the same level as without ethanol precipitation (table 26).

Tab. 27: Influence of enhanced sample volume and ethanol precipitation on PCR success. 1 µL biofilm suspensions was added in 25 µL total PCR volume. In addition one approach with 5 µL of concentrated suspension was made. M IV: biofilm suspension, DK 79 DNA: control DNA of bacterium DK 79

Dilution biofilm suspension	**Formaldehyde fixed M IV**	
	alkaline lysis 3 times enhanced sample volume **ethanol precipitation**	alkaline lysis 3 times enhanced sample volume **ethanol precipitation** **+ DK 79 DNA**
concentrated biofilm suspension	–	–
10^{-1}	–	–
10^{-2}	–	+
10^{-3}	–	+
10^{-4}	–	+
10^{-5}	–	+
10^{-6}	–	+

– : no PCR band + : PCR band

The second method used to precipitate DNA was the addition of isopropanol combined with sodium acetate. In addition the sample volume of M IV was increased from three to five caps. Table 28 shows that there is no positive PCR result after increase of extracted sample volume and isopropanol precipitation in the PCR approach without seeded control DNA of DK 79. When DK 79 DNA was seeded there was a weak band at a higher dilution (10^{-5}) compared to the ethanol precipitation (table 27). Positive PCR control (DK 79 DNA + PCR H_2O) yielded the expected result. As a consequence the precipitation procedure was done with slight modifications. *E. coli* was used and isopropanol precipitation was extended to 15 min. This resulted in positive results in PCR.

Tab. 28: Further increase of biofilm suspension volume and precipitation by isopropanol. 1 µL biofilm suspension was added in 25 µL total PCR volume. In addition one approach with 5 µL of concentrated suspension was made. M IV: biofilm suspension, DK 79 DNA: control DNA of bacterium DK 79

Dilution biofilm suspension	**Formaldeyhde fixed M IV**	
	alkaline lysis **isopropanol precipitation** 5 times enhanced sample volume	alkaline lysis **isopropanol precipitation** 5 times enhanced sample volume **+ DK 79 DNA**
Concentrated biofilm suspension	–	–
10^{-1}	–	–
10^{-2}	–	–
10^{-3}	–	–
10^{-4}	–	–
10^{-5}	–	+
10^{-6}	–	**(+)**

– : no PCR band + : PCR band

3.3.1.4 Does bovine serum albumine (BSA) reduce the effect of inhibitory agents?

Comparison of the PCR results for BWB III with BSA (band at 10^{-2} ,table 29) and without (band at 10^{-2}/10^{-3}, table 26) showed that there is no evident positive effect by

adding BSA to the amplification reaction. As controls *E. coli* (DSM 5695) + BSA, *E. coli* only and a water control were prepared and gave the expected results. Interpretation of table 29 resulted neither in a general positive effect of BSA (positive PCR result at 10^{-2} as before) nor a concentration dependent effect (bands always in the same range from 10^{-2} to 10^{-6} at different BSA concentrations).

Tab. 29: Influence of bovine serum albumine (BSA) on inhibition caused by the biofilm suspension of BWB III (non-fixed). 1 µL biofilm suspension was added in 25 µL total PCR volume. BWB III: biofilm suspension

BSA added to BWB III serial diluted up to 10^{-6}	**Addition of *E. coli* DSM 5695 DNA**	**PCR result as band in agarose gel**
BSA 0.1 µg/µL	yes	band from 10^{-2} a) up to 10^{-6}
	no	no band
BSA 0.3 µg/µL	yes	band from 10^{-2} a) up to 10^{-6}
	no	no band
BSA 0.6 µg/µL	yes	band from 10^{-2} a) up to 10^{-4} b)

a)at 10^{-2} only weak band b)further dilutions not determined

3.3.2 Complex DNA extraction methods

As the more simple extraction procedures mentioned above were not applicable, more complex methods were tested for their suitability.

3.3.2.1 FastDNA Spin Sample Kit for soil

The biofilm suspensions with the large amount of inorganic matter looked more like soil than drinking water samples. Therefore, an extraction method optimized for soil samples was used.

3.3.2.1.1 Concentration of DNA obtained

Two to four DES eluates of the FastDNA Spin Sample Kit for soil were pooled and afterwards precipitated with isopropanol (chapter 2.4.3.2). The suspension was directly extracted from the biofilm suspensions or frozen at –20°C until use for extraction. Table 30 summarizes the results of PCR amplification.

Tab. 30: PCR results of pooled and precipitated FastDNA Spin Sample Kit extracts of different biofilm samples.

Biofilm sample	Biofilm suspenison applied to test µL	PCR result
BWB V	150	–
BWB VII	80	–
BWB VII	120	–
M X	130	–

For the precipitated extracts of BWB V, BWB VII, and M X 0.5, 1, 5, and 17 µL were added in 25 µL PCR volume. All PCRs showed no band in the agarose gel except the control DNA of *E. coli* DSM 5695. This confirms that the PCR reaction functioned in general.

3.3.2.1.2 Dilution of DNA

A missing band in the PCR might also be generated by a high DNA concentration which is inhibiting the amplification. To test this possibility the FastDNA Spin extracts of M VIII, BWB VI, and RBI PE (reactor system sample) were diluted 10^{-1}, 10^{-2}, and 10^{-3}. For M VIII and RB I PE 10 µL for BWB VI 1, 10, 17.5 were applied to 25 µL PCR volume but no band appeared in the agarose gel. Because there was only a limited volume of the extracted DNA in this approach different biofilm samples were appointed.

3.3.2.1.3 Evaluation of extraction efficiency

As there was no extraction success with the above applied methods the following questions appeard:

How effective is the extraction of total DNA by the FastDNA Spin Sample Kit for soil?

And as consequence is a loss of DNA during the extraction procedure the reason for the missing amplification success in the PCR?

To test this, the extraction procedure (chapter 2.4.3.2) was spiked with a defined concentration of *Aquabacterium citratiphilum* (DSM 11900) DNA. This bacterium had been detected by FISH as one of the dominant bacteria in young drinking water biofilms (Kalmbach et al. 1997b). Therefore, it seemed to be the appropriate organism for evaluation of a suitable method for extraction of DNA from drinking water biofilms. To determine the loss of DNA the amount of DNA was measured again after extraction by the PicoGreen dsDNA Quantitation kit (chapter 2.4.3.6.2). Additionally, to access the loss of amplifiable DNA by the mechanical-chemical lysis the extraction was repeated without the kit recommended lysis.

Tab. 31: Recovery of DNA after extraction with the FastDNA Spin Sample Kit with and without mechanical-chemical lysis.

Culture	**Spiked DNA amount ng**	**DNA amount after extraction ng**	**Recovery %**
A. citr. [a)] culture A (mech.-chem. lysis[b)])	713.3	29.6	4.1
A. citr. [a)] culture B (mech.-chem. lysis[b)])	815.8	55.0	6.7
A. citr. [a)] culture C (without mech.-chem. lysis[b)])	708.8	22.2	3.1
A. citr. [a)] culture D (without mech.-chem. lysis[b)])	678	19.1	2.8

a)*A. citr.*: *Aquabacterium citratiphilum* DSM 11900 b)mech.-chem. lysis: kit recomended mechanical-chemical lysis

The DNA amount applied to the extractions with mechanical-chemical treatment was higher than that of the procedure without the lysis. As a consequence the DNA amount found after extraction was as well higher. To access the efficiency independent of the applied DNA the recovery was calculated as percentage DNA amount after extraction compared to DNA applied to the extraction (table 31). The extraction with mechanical-chemical lysis showd a slight increased recovery and a higher coefficient of variation (coef. var.= 34 %) than the less effective procedure without lysis (coef.

var.=7.5 %). As there seems to be no drastic disadvantage caused by the mechanical-chemical lysis, the values were calculated as parallel approaches. (table 32) The mean recovery of all approaches was 4.2 % (coef. var. =42 %).

Tab. 32: Average DNA recovery after extraction with the FastDNA Spin Sample Kit with and without mechanical-chemical lysis. number in bracket: standard deviation

DNA preparation with FastDNA Spin Sample Kit	**Mean recovery %**	**Coefficient of variation %**
with mech.-chem. lysis [a)]	5.4 (±1.8)	34.0
without mech.-chem. lysis [a)]	3.0 (±0.22)	7.5
all	4.2 (±1.8)	42.2

a)mech.-chem. lysis: kit recomended mechanical-chemical lysis

3.3.2.2 CTAB based DNA extraction

The DNA extraction method modified according to Ogram et al. 1998 (chapter 2.4.3.3) is also a method normally used for soil. It was suitable for soil with an organic carbon content of 1.5 % and 15 %. In the case of inorganic matter not being the problem in the extraction but organic carbon, this method might solve the problem. Beside CTAB as the main polysaccharide and protein binding agent further purification and concentration is done with a chloroform-isoamyl alcohol mixture, PEG 8000 and ethanol precipitation. An advantage of this method for environmental samples with low bacterial content is the relatively large amount of 5 g of soil that can be appointed.

The main modification made, compared to the original protocol of Ogram et al., is the substitution of the lysozym lysis with the alkaline lysis (chapter 2.4.4.1). This lysis method was used successfully to lyse most of the biofilm isolates of this investigation for the sequencing approach (chapter 2.4.4). The DNA content was again measured by the PicoGreen dsDNA Quantitation kit (chapter 2.4.3.6.2).

To establish this method four main optimization steps were made:

1.) Extraction of DNA with complete protocol (chapter 2.4.3.3)

Sample: DNA preparation of *A. citratiphilum* (DSM 11900) with defined content. Biofilm suspension of M III formaldehyde fixed.

Result: DNA concentration beyond the detection limit of 250 pg/mL

The first extractions were made with a biofilm suspension of the pipe sample M III and a DNA preparation of *A. citratiphilum* with the defined amount of 30-40 ng DNA (4 parallels). For the DNA preparation the first sodium phosphate buffer purification steps were left out to avoid loss of free (not cell bound) DNA and the protocol was directly started at step 3 with the alkaline lysis (chapter 2.4.3.3).

2.) Extraction of DNA with complete protocol (chapter 2.4.3.3.1)

Sample: DNA preparation of *A. citratiphilum* with enhenced defined DNA content.

Result: DNA concentration beyond the detection limit of 250 pg/mL

As it was not possible to detect DNA in the first approach the DNA content was enhanced 15 to 30 fold to 430 to 490 ng dsDNA but the DNA was still not detectable after extraction.

3.) Extraction of DNA without alkaline lysis (chapter 2.4.3.3.2)

Sample: DNA preparation of *A. citratiphilum* with enhanced defined content.

Result: DNA concentration beyond the detection limit of 250 pg/mL

As a consequence of approach 1.) and 2.) the DNA mass was increased again to 1200 to 6600 ng. Additionally the alkaline lysis step as a possible source of DNA loss and shear force was left out. The lysis is not necessary for the DNA preparation because the DNA is already free in solution.

4.) Extraction of DNA without alkaline lysis and without PEG precipitation

Sample: DNA preparation of *A. citratiphilum* with enhenced defined DNA amout.

Result: DNA detectable

In literature the PEG precipitation step is discussed as a reason for high DNA loss (Lee et al. 1996). Because there is another precipitation step with 99.8 % ethanol in combination with a high salt concentration (chapter 2.4.3.3) the PEG precipitation seemed not to be necessary. Another modification with a practical background was storage of the extractions over night at -20°C after addition of 99.8 % ethanol (chapter 2.4.3.3.2) for extractions no. 3. and 4., approaches 1. and 2. were not stored over night.

1. approach: 14.6 % of spiked DNA detectable

2. approach: 3 % of spiked DNA detectable

3. approach: 2.9 % of spiked DNA detectable

4. approach: 8.2 % of spiked DNA detectable

The recovery shows a great variety between 2.9 and 14.6 this spreading is reflected in the high coefficient of variation (76.9 %) for the four parallels (table 33).

Tab. 33: DNA measurement before and after extraction of DNA preparations without alkaline lysis and PEG precipitation. Storage of extraction no. 3 and 4 at -20°C over night after addition of 99.8 % ethanol in step 12. number in bracket: standard deviation

DNA preparation No.	**Spiked DNA amount ng**	**DNA amount after extraction ng**	**Recovery %**
1	1992	290	14.6
2	4162	126.0	3.0
3	3530	103.5	2.9
4	2914	238	8.2
		coefficient of variation 76.9 %	**average recovery** 7.2 % (± 5.53 %)

3.3.2.3 Extraction and purification by the QIAamp DNA Mini Kit

In this kit the DNA is lysed, bound to a silica-gel membran, washed, and eluted. There is a minimum number of steps as potential source of DNA loss. To test the influence of the alkaline lysis solution (chapter 2.4.4.1) on the binding to the columns an *E. coli* DSM 5695 was lysed with the alkaline procedure and applied to the columns. The pipe sample BWB III (formaldehyde fixed) was lysed according to the kit

protocol. As described before the formaldehyde fixed sample was taken because of the limited volume of sample material.

The results in table 34 show that there is no intensive negative influence of the alkaline lysis method when a pure culture like *E. coli* is applied to the extraction procedure. For a formaldehyde fixed biofilm suspension like the pipe sample BWB III this extraction method was not suitable under the used PCR conditions because no band appeared in the agarose gel.

Tab. 34: Extraction and purification of DNA of *E. coli* liquid culture and biofilm suspension of fixed BWB III with the QIAamp DNA Mini Kit.

Sample	**Lysis**	**Result (band in gel after PCR)**
E. coli DSM 5695	alkaline lysis	band[a)]
BWB III formaldeyde fixed, 6 caps	alkaline lysis	no band[b)]

a) 17 and 5 µL in 25 µL total PCR volume b) 1, 10, 17 µL in 25 µL total PCR volume

3.3.2.4 Extraction and purification by the Qiagen Genomic-tips 20

In the FastDNA Spin Sample Kit for soil (chapter 2.4.3.2) and the QIAamp DNA Mini Kit (chapter 2.4.3.4) silica matrixes requiring specific conditions were used to bind DNA. To see whether another binding matrix with different conditions might enhance DNA binding the Qiagen Genomic-tips with an anion exchange resin were tested.

The system is described as an optimized buffer system for careful lysis of cells followed by binding of genomic DNA to anion exchange resin under appropriate low salt and pH conditions. Impurities are removed by a medium-salt wash and the genomic DNA is eluted in a high-salt buffer and concentrated and desalted by isopropanol precipitation. Because SDS and other anionic detergents interfere with the binding of nucleic acids to the column resin by competing for binding to the anion exchange groups, SDS was removed by potassium acetate precipitation in some of the alkaline lysed samples.

The extraction of DNA from the liquid culture of *E. coli* DSM 5695 with initial lysozyme or alkaline lysis (the last including SDS) showed that there is no complete saturation of the anion exchange groups by SDS (table 35 experiment 1 and 2), because both

resulted in a band in the agarose gel. To avoid any loss of DNA a potassium acetate precipitation of SDS directly after the lysis was tested with the *E. coli*. (table 35, experiment 3). Once again a positive result in the PCR was obtained. So the DNA of the pipe sample BWB VI was extracted with the alkaline lysis followed by SDS precipitation and genomic-tip purification. No positive result was obtained in the PCR for the not fixed biofilm suspension BWB VI despite the parallel extracted *E. coli* liquid culture showed a positive PCR result. Experiment 4 illustrates that under the experimental conditions of this DNA preparation extraction of formaldehyde fixed *E. coli* did not succeed.

Tab. 35: Extraction and purification of DNA by the anion-exchange resin system of the Qiagen Genomic-tips 20.

Experiment	**Lysis procedure**	**SDS precipitation after lysis**	**Amplification of DNA[d)]**
1. *E. coli DSM 5695*, liquid culture	lysozyme[a)]	no	band
	alkaline[b)]	no	band
2. *E. coli DSM 5695*, liquid culture	lysozyme[a)]	no	band
	alkaline[b)]	no	band
3. *E. coli DSM 5695*, liquid culture	alkaline[b)]	no	band
	alkaline without dilution[c)]	yes	band
4. *E. coli DSM* 5695, formaldehyde fixed	alkaline[b)]	no	no band
	alkaline without dilution[c)]	yes	no band
5. BWB VI, not formaldehyde fixed	alkaline without dilution[c)]	yes	no band

a)kit recommended lysozym lysis b)alkaline lysis and final dilution with AE buffer c)alkaline lysis without final dilution with AE buffer d)PCR amplification with subsequent agarose gel

4 Discussion

4.1 Bacterial populations in mature drinking water biofilms

4.1.1 Investigated drinking water systems

In the present study, biofilms grown in reactor systems and in pipes of the distribution system were investigated. The reactors in Berlin and the Ruhrgebiet were built up to investigate middle aged biofilms. The pipes provided old drinking water biofilms. (chapter 2.1 and 2.2) Despite the aim of the study to meet criteria of the actual distribution system the reactor system takes up an intermediate position between distribution system and house installation. This is caused by the used coupon materials copper and stainless steel and the temporary enhanced temperatures (median 13.1°C, range 7°C to 20.8°C) on one hand and construction setup, flow conditions, and used material for construction on the other hand. To the author`s knowledge, concerning distribution system pipes, it is the first time that two urban centers were tested in such a broad range regarding pipe material, age, and investigated inner pipe surface.

4.1.2 Phylogenetic composition of the culturable population of the middle aged and old biofilms

In total 454 isolates (including 174 isolates form the Danish system) grown on modified R2A were phylogenetically analysed. 342 isolates from the reactor systems with the middle aged biofilms in Germany and Denmark, and 112 from the old biofilms of the pipe samples in Germany. (chapter 3.1.3 and 3.2.5)

It has to be pointed out that in this study, as a result of the problems with molecular techniques in this habitat (described in chapter 4.2), predominantly the culturable bacterial population was investigated. Moreover, regarding a better comparability of data and tracking the fate of the genus *Aquabacterium*, modified R2A was used as in the comprehensive studies of Kalmbach et al. in the Berlin drinking water system (Kalmbach et al. 1997a; Kalmbach et al. 1997b; Kalmbach 1998; Kalmbach et al. 1999). From the statistical point of view relatively small sample size (number of pipes and coupons with the same growth conditions) biases the quantitative analysis. Furthermore, as described more detailed in the introduction, comparison with previous

studies are hampered by differences e. g. in drinking water source, biofilm exposition equipment, exposition time, and analyzing methods. Despite this, some interesting details were found.

The analysis of the culturable bacteria from the middle aged biofilm samples (approx. six months exposed) of the German and Danish reactor system by RFLP analysis clustered in the phyla *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. The results were dominated by 79 % of *Proteobacteria*. Only two gram-positive *Bacillus* and four *Flavobacteriaceae* were identified. In addition, 20 % could not be assigned to a phylogenetic phylum reflecting the limitations of the restriction method. On the other hand sequencing of 55 instead of 342 isolates saves resources and time. (table 23, chapter 3.2.5.1) Despite the biases, RFLP analysis in combination with sequencing of representative isolates is a useful tool for a first characterization of bacterial communities in drinking water.

In contrast to the middle aged biofilm population the piechart of culturable bacteria of old biofilms (pipe samples, figure 5, chapter 3.1.3) leaps out that there were nearly as much gram-positive (49 %) as gram-negative (51 %) bacteria. The culturable gram-negative bacteria are dominated by *Beta*- and *Alphaproteobacteria* (19 % and 18 %, respectively) and a relatively high fraction of *Gammaproteobacteria* (12 %). Gram-positive bacteria separated in 28 % *Actinobacteria* and 21 % *Firmicutes*. (figure 5, chapter 3.1.3)

Several investigations in literature and the investigated reactor systems of this study showed a clear predominance of gram-negative bacteria in young and middle aged biofilms detected by cultivation or FISH (Kalmbach et al. 2000; Martiny et al. 2005; Percival et al. 1999; Schwartz et al. 1998). Supporting the observations in old distribution system pipe biofilms of this study, in different studies a dominance of gram-positive bacteria was observed. Pepper et al. detected a shift to gram-positive bacteria following the water in the free water phase from the wells to the distribution system, to the tap by HPC on Tryptic Soy Agar (35°C, 24-48 h) (Pepper et al. 2004). Even though the water phase indicates not the population composition in the biofilm, the water is assigned as one source of the gram-positive bacteria and their spores. After settling in the biofilm the ability to persist and multiply is determined by parameters in the biofilm like nutrients, physico-chemical parameters, and competition of mi-

croorganisms. Norton et al. also reported a shift to gram-positive bacteria in the water column and traced it back to the better survival of chlorination (Norton and LeChevallier 2000). In the present investigation this can be excluded as cause of the shift for the Danish and German reactor system, and the German pipe samples because non of the systems was chlorinated. Only the reactor system and pipes in the Ruhrgebiet were fed by chlorinated drinking water which might have produced a shift to gram-positive bacteria for these samples (table 8, chapter 3.1). Furthermore, Norton and co-workers detected that in contrast to the water column biofilms were generally dominated by gram-negative bacteria but on cPVC surfaces after conventional water treatment a shift to 54 % gram-positive bacteria was detected (Norton and LeChevallier 2000). Another reason for the dominance of gram-positive bacteria might be a systematic bias caused in the sample preparation and homogenization (chapter 2.1.1.2, 2.2.2). Gram-positive bacteria like *Mycobacterium* with a more resistant cell wall or spore forming *Bacillus* are expected to be more tolerant to physical forces like scraping and homogenization. It has been shown in this study for material coupons of the reactor that there is a reduction in total cell counts (DAPI) caused by the scraping and homogenization procedure (chapter 3.2.4.1). Furthermore, More et al. reported that it takes a more regide combined methode of SDS and bead mill homogenization to reduce culturable *Bacillus* endospores to 2 % (More et al. 1994). Consequently, bacteria that are sensitive to sample preparation might be reduced in numbers and bacteria or spores less susceptible to the preparation might dominate the culturable population.

The *Proteobacteria* of the investigated German and Danish middle aged biofilms and bulk water were dominated by 49 % *Alphaproteobacteria* followed by 32 % *Gammaproteobacteria* and 19 % *Betaproteobacteria* (of total *Proteobacteria*). The major part of the *Alphaproteobacteria* was isolated from the biofilm of the Danish system and grouped in the genera *Rhodobium* and *Devosia* (both *Rhodobiaceae*). In the German bulk water also a relatively high amount of *Alphaproteobacteria* was found that grouped in the family *Caulobacteraceae* and the genus *Brevundimonas* (table 23, chapter 3.2.5).

The relatively high amount of *Gammproteobacteria* in the middle aged biofilms of the Berlin reactor system has to be assessed together with the *P. aeruginosa* contamina-

tion of the free water in the starting phase of the reactor system and will be discussed later (chapter 4.1.3). This group is dominated by the high amount of pseudomonads isolated from the German biofilm. Other studies also detected high amounts of *Gammaproteobacteria* (27 to 41 % of total counts) by FISH on biofilms grown 4 days on PE-HD, PVC, and steel exposed at a house branch connection in a chlorinated system (Schwartz et al. 1998). In addition, Percival et al. showed an increase of culturable (R2A) *Pseudomonas* spp. with increasing flow velocity in about one year old biofilms on stainless steel (Percival et al. 1999). In the old biofilms of this study *Gammaproteobacteria* still represent 12 % of total biofilm isolates and there were still some *Pseudomonas* spp. identified (figure 5 and table 13, chapter 3.1.3).

Betaproteobacteria were not cultivated from the middle aged biofilms of the German and Danish reactor systems. These findings are in contrast to the results by Kalmbach et al. in the Berlin drinking water system. They detected *Betaproteobacteria* and in particular the genus *Aquabacterium* as dominant in the young (14 days exposed) biofilms grown on PE-HD by FISH analysis. (Kalmbach et al. 2000) Schwatz et al. also detected the *Betaproteobacteria* very frequently by FISH in young biofilms of metallic coupons (steel, copper) (Schwartz et al. 1998). Despite *Aquabacterium* was not detected in the biofilm by cultivation it was present in the bulk water phase of both systems of this study, reflecting the presence of the bacterium in a culturable state in the German and Danish reactor system. In contrast to the findings of Kalmbach et al. and Schwartz et al. in this study *Aquabacterium* was not able to establish in a R2A culturable state in the middle aged and old biofilms. Although modified R2A medium and cultivation parameters were used as recommended by Kalmbach and co-workers, and PE was among the biofilm material of the German system. Kalmbach et al. suggested that bacteria from oligotrophic water systems are present in distinct physiological states: (i) culturable, (ii) metabolic active but incapable of undergoing the sustained cellular division required for growth on artificial medium, (iii) nonculturable and metabolic inactive. (Kalmbach et al. 1997b) In addition, it has been reported for *E. coli* to be present in drinking water biofilms in an active but nonculturable state (Bjergbaek and Roslev 2005; Juhna et al. 2007). Therefore, one reason for the absence of *Aquabacterium* and other *Betaproteobacteria* may be the change into the physiological states (ii) and (iii) were they could not be detected by the cultivation approach. This hypothesis is supported by further investigations of the Danish sys-

tem by Martiny et al.. With a cultivation independent cloning and sequencing approach they detected the genus *Aquabacterium* in 1 to 256 day old biofilms grown on stainless steel. (Martiny et al. 2003) These findings support the consensus of the scientific community that molecular genetic methods allow detection of culturable and nonculturable bacteria and that detection mainly depends on an intact amplification target that may be located in viable or dead cells (Knight 2000).

Compared to the middle aged biofilms of this study, the culturable bacteria of the old biofilms scraped form pipes of the distribution system showed a higher fraction of *Betaproteobacteria* (19 % of total cultured bacteria, figure 5, chapter 3.1.3). To achieve an improved insight into the phylogenetic clustering of the *Betaproteobacteria* of this study, Aquabacteria and their next relatives were compared to the *Betaproteobacteria* of this study. Table 14 (chapter 3.1.3) shows that out of 10 islolates the isolate M III 5 with similarities of 89 to 92 % was the closest to the *Aquabacterium* genus. This indicates that there is no close relationship of the *Betaproteobacteria* of this study with the *Aquabacterium* and closest realative cluster. In further investigations with improved molecular genetic methods the fate of the uncultured *Betaproteobacteria* and the genus *Aquabacterium* has to be elucidated.

In this study, for the biofilm population no qualitative difference at genus level could be detected between the different materials of the pipe samples or the reactor samples. This might reflect a systematic bias or natural conditions and has to be asertained in further investigations with higher numbers of samples with the same material and age. The above mentioned phyla and classes of middle aged and old drinking water biofilms have been deteceted in previous studies in freshwater or drinking water habitats (Eichler et al. 2006; Hahn et al. 2003; Kalmbach 1998; Martiny et al. 2003; Norton and LeChevallier 2000; Olson et al. 1981; Percival et al. 1999; Schwartz et al. 1998; Zwart et al. 2002).

Because drinking water is of direct health relevance for humans it is of interest if there are bacteria with pathogenic potential among the cultured bacteria. Bacterial pathogens in the drinking water habitat are comprehensively summarized by different authors (Leclerc et al. 2002; Szewzyk et al. 2000; WHO 2000). Szewzyk et al. 2000 distinguished between bacteria with fecal origin and those that have their origin in water or soil. Pathogens with fecal sources are for instance *Campylobacter* species,

enterohemorrhagic *Escherichia coli*, *Salmonella* species, *Vibrio cholerae*, *Yersinia enterocolitica*, *Helicobacter pylori*. On the other hand there are pathogens that inhabit water or soil and are transported from these habitats into drinking water. Here they are able to grow if parameters are getting convenient. Representatives of the later are *Legionella* species, *Pseudomonas aeruginosa*, *Aeromonas* species, *Acinetobacter* species, and environmental Mycobacteria. In addition, toxins of Cyanobacteria may play an important role if surface water is the source for drinking water supply. (Edberg and Allen 2004; Pavlov et al. 2004; Percival et al. 2004; Röske and Uhlmann 2005; Stelma et al. 2004; Szewzyk et al. 2000) Furthermore, it has been shown for some of these pathogens or opportunistic pathogens that they are able to survive in drinking-water biofilms. Lehtola et al. detected survival of culturable *Mycobacterium avium* and *Legionella pneumophila* in 2 to 4 week drinking water biofilms (Lehtola et al. 2007b). Rogers et al. cultivated *Legionella* sp. out of up to 28 days old drinking water biofilms (Rogers et al. 1994). Also *Campylobacter* species have been shown to survive in tap water supplied biofilms for up to 26 days in culturable forms and up to 42 days by FISH (Buswell et al. 1998). In addition, it was shown that also a pathogenic *E. coli* was able to survive and grow in a monospecies biofilm of a gram-positive drinking water isolate continuously supplied by artificial fresh water medium and a drinking water supplied pilot plant. (Fass et al. 1996; Szewzyk et al. 1994) The meaning of *P. aeruginosa* in drinking water biofilms will be discussed in detail in chapter 4.1.3. In this study, some of the above described genera with pathogenic potential were identified but not tested for virulence factors. In the bulk water phase of the reactor systems the genera *Acinetobacter* and *Aeromonas* and *P. aeruginosa* were found. Additionally, in the biofilms of the Berlin reactor system *Aeromonas* and in the pipes *Mycobacterium* was detected. (RFLP analysis or sequencing, chapter 3.2.5.1)

4.1.2.1 Heterotrophic plate count (HPC) bacteria in the bulk water phase of the Berlin reactor system

Comparison of different HPC methods used by authorities and scientists showed that inconsistencies in the method often bias the comparison of different studies. The detection characteristics of HPC bacteria in the bulk water phase suggests that the first

running phase of the reactor system is highly influenced by an exhaustible nutrient source and flow regime. Varying impact of physico-chemical parameters (water temperature, flow regime) to HPC bacteria in the bulk water phase cultivated according to GDWR propose the existence of different populations at 20°C and 36°C.

An excellent overview about different HPC methods is given by Allen et. al. and Reasoner (Allen et al. 2004; Reasoner 1990). They remarked that it is important to be aware of the fact, that HPC methods in general only detect a fraction or subpopulation of the total heterotrophic bacteria that are present in the habitat under consideration. Furthermore, they stated that investigators should keep in mind, that the overall composition of bacterial populations is not known and that there is always a fraction of presently nonculturable bacteria. The culture methods that have been used by water authorities and scientists differ in medium composition (high-nutrient versus low-nutrient), incubation temperature (high-temperature, 35-37°C versus low-temperature, 20-28°C), incubation time (34-48h versus 5-7 days), and the means of medium inoculation (pour-plate, spread-plate, membrane filtration). Regarding the different medium inoculation protocols, Reasoner *et al.* (2004) found the R2A-spread-plating-method according to Reasoner and Geldreich (Reasoner and Geldreich 1985) to be more sensitive than methods based on the pour-plating-method like EN ISO 6222 (DIN EN ISO 6222 1999). Reasoner also demonstrated, that any variation in the mode of plating may result in significant differences in the plate counts (range <1 to approx. $4x10^3$ CFU/mL) and genera detected, what makes a comparison of the different methods difficult (Reasoner 1990). Nevertheless, heterotrophic plate counts are considered a useful parameter to assess drinking water treatment efficiency and changes in bacterial water quality during storage and distribution (Allen et al. 2004; Bartram et al. 2004b).

The cultivation protocol according to GDWR (1990) with 44 h of incubation used in this study predominantly provides the fast growing heterotrophic plate count bacteria of the culturable bacterial population. It was reported that a high-temperature (35-37°C) and short-time (34-48 h) incubation favours the growth of bacteria from animal and human origin. Low-temperature (20-28°C) and long-time (5-7 d) incubation, in contrast, supports the enrichment of water-based bacteria (Allen et al. 2004). The population obtained by incubation with a nutrient rich medium (according to GDWR

1990) is regarded to be different from the one harvested from a nutrient poor medium like R2A. This assumption for example is supported by the investigations of Carter and colleagues. They have shown that the HPC determined on the nutrient-rich medium TSA-SB (standard method: 9215C APHA, 35°C, 48h)[4] was not correlated with the HPC determined on the nutrient-poor R2A medium (standard method: 9215 C APHA, 25°C, 7d). In this case the difference might be reinforced by discrepancies in temperature and time of incubation. (Carter et al. 2000)

In the free water phase of the Berlin reactor system high HPC values were detected in the first days of operation (figure 8, chapter 3.2.3.1). This may indicate a high availability of bacterial nutrients - especially carbon source - and/or other suitable growth parameters. Nutrients might have been imported during the installation of the system or have been released by the hardware composition. Even though only material was used which is routinely implemented by the Berliner Wasserbetriebe and reactors were cleaned before installation (chapter 2.1.1.1). Furthermore, routinely used rubber-like sealing was replaced with Teflon sealing if possible, and sampling taps free of rubber-like sealing were installed. One possible source of nutrients, however, may have been the rubber-like sealing in the rubber coated valves used. The latter could not be replaced and were also routinely used in the distribution system. Although, the rubber coated valves are standard in the Berlin distribution system, it is not usual to install as much valves on such a short pipe distance as it was practised in the reactor system of this study. A similar rubber coated valve has been investigated by Schmeisser *et al.* and has been shown to harbour a bacterial population capable of utilizing a huge amount of complex nutrients. The authors proposed the additives within the rubber coating as a possible carbon source. (Schmeisser et al. 2003) These additives may also be one of the factors supporting bacterial growth in the system investigated in this study. Furthermore, Uhl *et al.* have shown in laboratory batch experiments regarding nutrients described as AOC (assimilable organic carbon), that a duplication of AOC from 30 to 60 µg/L resulted not only in higher (two times) HPC counts (R2A) but also in much faster growth (Uhl and Schaule 2004). In addition, Van der Wende *et al.* made a statement regarding the relationship of hy-

[4] TSA-SB: tryptic soy agar with 5 % sheep`s blood, APHA: American Public Health Assosiation

draulic residence time and bacterial replication: “when the hydraulic residence time in the system is significantly shorter than the generation time of the planktonic cells, replication of planktonic cell numbers in the system is negligible” (van der Wende et al. 1989). Therefore, the high HPC values of the free water phase of this study could have been generated by a rapid growth of the bulk water population.

Another possible source of heterotrophic plate count bacteria in the water phase of the drinking water system in this study is the biofilm. Experiments by Lehtola *et al.* in PE and copper pipes with established biofilms (2 months) showed that stagnation times > 4 h resulted in significant higher HPC (R2A, 22°C, 7d) in the bulk water. In contrast, in stagnation experiments (16 h) without biofilm contact no significant increase in HPC of the water phase occurred. (Lehtola et al. 2007a) This finding is supported by Zacheus *et al.* who addessed deposits at the inner pipe surface to be a major source for regrowth in the water phase (Zacheus et al. 2001). Moreover, Van der Wende *et al.* and later on Stoodley *et al.* have demonstrated that a major origin of planktonic bacteria is detachment of cells from biofilms (Stoodley et al. 2001; van der Wende et al. 1989). Regarding the influence of the flow regime on stability of established biofilms, Lehtola *et al.* reported that below a threshold of 0.3 m/s, the biomass in biofilms increased with increasing water flow velocity (Lehtola et al. 2006). Velocities of 0.5 m/s detached bacteria from the surface but were not high enough to dislodge the biofilms completely (Lehtola et al. 2007a). For higher sloughing of biofilms Cloete *et al.* described 3 and 4 m/s as suitable (Cloete et al. 2003). Based on observations in systems with low surface-to-volume ratios (at large pipe diameters), Boe-Hansen *et al.* suggested, that planktonic growth may be a significant factor of bacterial regrowth. They also described the planktonic bacteria as being more active and culturable than their sessile counterparts. (Boe-Hansen et al. 2002)

Further investigations revealed that phosphate is another important parameter of bacterial growth in the free water phase of drinking water systems. Besides the impact of the drinking water source, the concentration of phosphorous compounds is additionally influenced by the chemicals used in water treatment, for example liming agents to adjust pH. (Miettinen et al. 1997; Sathasivan et al. 1997) Since the Berlin reactor system is not located near to the treatment plant and because there are no general regrowth problems on the water`s way to the reactor system, these factors

are negligible. Another source of phosphorous compounds has been described by Lehtola et al.. They detected leaching of phosphorus from new PE pipe material to the water (Lehtola et al. 2004). Because the Berlin reactor system is mainly built from PE pipes, this parameter may also contribute to the high HPC found in the starting time of the reactor system of this study. In addition to the effects on planktonic bacteria reported this side up, Rubulis et al. showed a positive correlation between phosphate of the inlet water and HPC and TCC of biofilms (Rubulis and Juhna 2007).

It has been described that the number of bacteria in the free water phase increases during the water`s way from the source, through the distribution system to the consumer´s tap (Pepper et al. 2004; Zacheus et al. 2001). Anyway, if this circumstance also would have applied to the Berlin reactor system, high HPC values would not have been a serious problem only in the initial phase, but would have been lasted throughout the whole operation time. Despite an increase of HPC on the water`s way to the consumer, both investigators - Lehtola et al. as well as Pepper et al. - stated, that the highest increase of HPC was caused by the house distribution system or water fittings. This supports the above mentioned hypotheses that the rubber coated valves are a major source of bacterial growth and this may be supported by phosphorous leaching of PE material. The consideration that nutrients are one major cause of the enhanced HPC values in the beginning of the experiments of this study is supported by the observation, that subsequent to the decrease of the initially high HPC below the limit of 100 CFU/mL after 109 days, neither higher temperatures, nor extended stagnation times or a termination of the flushing experiments resulted in higher cultivation values (figure 8b, 8c, chapter 3.2.3.1).

One reason for the irregular fluctuations of HPC in this work in the first 109 days of operation (figure 8, chapter 3.2.3.1) may be the population dynamics during the initial adhesion and maturation of the biofilm. In this phase interactions between immigrant and resident bacteria might result in detachment of bacteria from the biofilm (Battin et al. 2007). Furthermore, there might have been interference by the flushing operations during the first 110 days which resulted in enhanced sloughing of bacteria from the biofilm. As already mentioned above, higher sloughing of drinking water biofilms was observed at flow velocities of 3 and 4 m/s (Cloete et al. 2003). Horn *et al*. also reported that detachment of wastewater biofilms in a tube reactor occurred rapidly after

an increase of shear stress (Horn et al. 2003). In contrast, Melo *et al.* proposed that at higher flow velocities - combined with a turbulent flow and low substrate concentrations - biofilms were more compact and stable and consequently are more resistant to detachment (Melo and Vieira 1999). These inconsistent findings suggest that every test system features very specific parameters and any discussion of results have to consider them. In this study, possible interactions between any biofilm present in the system and the bulk water phase that may have led to the initially strong irregular fluctuations of HPC could not be investigated, because the first coupon sampling was defined in the cooperation project rules after 6 months. As can be seen in figure 8a (chapter 3.2.3.1) after approximately 6 months the amount of heterotrophic plate count bacteria decreased about one log unit to 10-30 CFU/mL, beneath the limit of 100 CFU/mL according to the GDWR 1990. Furthermore, after this time bacterial counts were less irregular, and the counts of inlet and outlet samples approximate. Despite it could not be confirmed by biofilm investigations, it is supposed that there is a take-off phase of the system of this study with a higher nutrient level and enhanced sloughing of bacteria from the biofilm as a result of the hydraulic conditions (stagnation times and flushing operation mode). These parameters may yield a higher level of fast growing heterotrophic plate count bacteria counted as CFU/mL according to GDWR. After the nutrient source has run out or was minimized and the hydraulic force was reduced, heterotrophic plate counts were clearly reduced below the limit of 100 CFU/mL according to GDWR 1990 (figure 8c, chapter 3.2.3.1). Only two outliers were detected (chapter 3.2.3.1). They may be the consequence of an incorrect water sampling. Thes findings of heterotrophic plate counts support the recommendation of different scientists who defined criteria for biologically stable drinking water. They described different regrowth parameters mainly regarding the utilization of nutrients (Uhl and Schaule 2004).

In addition to the above described observations made during the initial phase (first 109 days), the reactor system parameters were measured for the whole operation period of 740 days. The comprehensive sampling of the heterotrophic plate count bacteria of the bulk water phase in the Berlin reactor system and subsequent statistical analysis resulted in the following relationships (chapter 3.2.3.2): Cultivation at 20°C resulted in significantly higher CFU than at 36°C. This doesn’t come as a surprise, since 20°C is more comfortable to bacteria growing at drinking water tempera-

ture and thus most of the bacteria might be adapted to it. The recommended temperature value in Germany is 8 to 12°C (Grohmann et al. 2003). The temperature in the reactor system of this study showed a broader range from 7 to 20.8°C. Testing for correlation of CFU at the two incubation temperatures with the temperature in the bulk water of the reactor (range 7 to 20.8°C) resulted in a weak negative correlation of values counted at 20°C incubation (table 16, chapter 3.2.3.2). This means, that high bulk water temperatures result in reduced CFU, or vice versa, low temperature results in enhanced numbers of heterotrophic plate count bacteria. Contrary to this, counts at 36°C incubation showed no correlation in the investigated temperature range. Thus, heterotrophic plate count bacteria that grow at 20°C may be less temperature tolerant than bacteria growing at 36°C in the investigated bulk water temperature range of 7 to 20.8°C. Additionally, the counts of the population kept at 36°C showed a weak positive correlation with the flow rate. In future experiments it has to be investigated, whether this is a consequence of the presence of a bulk-water-adapted population that is growing well at higher flow conditions - or a result of higher sloughing of biofilm bacteria due to higher flow rates as described above.

Furthermore, statistical analysis indicates that if the heterotrophic plate count bacteria of bulk water during flow and stagnation mode are not equal, flow values are always smaller than stagnation values (figure 15, chapter 3.2.3.2). This might be the consequence of a higher dilution during flow and/or an enhanced migration of biofilm bacteria into the bulk water during stagnation, combined with a higher growth at higher stagnation temperatures as described above.

Concerning the regrowth potential of the heterotrophic plate count bacteria during flow of the water through the reactor it was found to be zero for the populations incubated at 20°C. In contrast, the cultivations at 36°C showed a significant regrowth potential. Taking into account the investigations of the bulk water, it is not possible to distinguish between regrowth caused by bacterial growth or sloughing of biofilms. Additionally, the difference between inlet and outlet samples during stagnation mode was investigated. The resulting bacterial counts also showed no difference at the incubation temperature of 20°C, but significant higher values at the outlet at 36°C incubation temperature of the plates. On the one hand, the last two findings might be an evidence for a regrowth potential on the water`s way through the reactor system. On

the other hand, the bacterial counts during stagnation might indicate a local growth potential at the outlet for the population cultivated at 36°C. The free water samples taken during stagnation mode show no correlation of HPC with water temperature for both incubation temperatures of the medium, indicating that in the investigated reactor system temperature is not the main limiting parameter during stagnation. Finally, a positive correlation was detected in analysis of the CFU counted in samples during the flow operation mode at both incubation temperatures of 20°C and 36°C (figure 16, chapter 3.2.3.2). This can be explained in three ways: The first reason might be that one of the culturable populations, 20°C or 36°C, is implicated in the other. Second, one population may depend on physiological parameters of the other population and third, both populations may be independent from each other but may depend on the same third parameter.

4.1.3 Regrowth potential of the opportunistic pathogen *P. aeruginosa* in the Berlin reactor sytem

In this study the DIN EN 12780 was confirmed as specific method for cultivation of *P. aeruginosa*. The persistence of *P. aeruginosa* in the bulk water phase is assumed to be linked to the protection in biofilms and the ability of catalase production supported by an exhaustible nutrient source as discussed for the HPC bacteria. It has to be elucidated in further experiments if missing culturable *P. aeruginosa* in the biofilm is caused by maturation of the biofilm and/or transition into viable but nonculturable state.

Beside the population of heterotrophic plate count bacteria in general as described before, the second bacterial population regularly investigated in the bulk water phase of the Berlin reactor system was that of *P. aeruginosa*. A possible contamination with this bacterium was first assumed during routine analysis of HPC bacteria of the bulk water phase according to GDWR 1990. The experienced laboratory assistants of the Berliner Wasserbetriebe detected the specific odour of this bacterium. As a consequence, the standard method for detection of *P. aeruginosa* by membrane filtration and subsequent incubation of the membrane on selective cetrimide-containing medium (chapter 2.3.4) was included in regular investigations (DIN EN 12780 2002).

The opportunistic human pathogen *P. aeruginosa* has the ability to adapt to and to thrive in many ecological niches, ranging from water and soil to plants and animals, including humans (Aramaki and Fujita 1999; Ringen and Drake 1952).

At first the specificity of the DIN EN 12780 was of interest. According to the standard method, blue-green colonies (indicating pyocyanin-production) are accepted as *P. aeruginosa* without further test procedures. The pigment pyocyanin is produced by more than 90 % of *P. aeruginosa* strains (Anderl et al. 2000; DIN EN 12780 2002). To assess the specificity, 14 isolates (from the bulk water sample of the reactor system) featuring this typical colour were phylogenetically grouped by 16S rDNA sequencing. This analysis resulted in a maximum difference of 1.3 % to *P. aeruginosa* ATCC 25330 and confirms the expression of the blue-green colour on cetrimide-containing medium as a specific criterion for identification of *P. aeruginosa* in the reactor system of this study (chapter 3.2.2.1).

Although the author of this thesis is aware of the fact, that an investigation of 14 isolates may not result in a statistically robust assessment of the method, false positive results in the habitat of this study may be a negligible risk. Furthermore, regarding *P. aeruginosa* positive results it should be taken into account that according to DIN EN 12780 the isolates were identified by their phenotypic characteristics and not by virulence factors, thus a *P. aeruginosa* positive result is not inevitable related to a pathogenic impact. A more serious risk to the human health than false positive results is the possibility of false negative results. First of all it should be taken into account, that only the culturable fraction of *P. aeruginosa* is detected by this method. However, it has been described by Binnerup et al. that *P. aeruginosa* is able to form VBNC states (Binnerup and Sorensen 1993). Furthermore, as mentioned above, about 90 % of the *P. aeruginosa* strains produce the blue-green pigment. In other words, at least 10 % of *P. aeruginosa* strains are not detected by the method and thus may entail a health risk for the consumer. Additionally, the membrane filtration method is limited by the filter size of 0.45 µm. It has been described for oligotrophic environments like drinking water, that bacteria may be smaller in size and as a consequence can pass the filter (Morita 1997).

As described in chapter 3.2.2.3, at the end of the first run the huge number of 563 CFU/100 mL of *P. aeruginosa* was detected with the standard method DIN EN 12780.

Since flushing attempts, depletion and partially drying of the reactor system did not remove the contamination, the system was stopped. Subsequent all components were cleaned and disinfected again and after reconstruction, the system was disinfected with a solution of H_2O_2 to avoid contamination during the set up of the system. (chapter 2.1.1) This procedure, however, was not able to solve the problem of contamination but reduced the culturable *P. aeruginosa* to 1 to 17 CFU/100 mL in the free water samples of the outlet during flow operation mode (chapter 3.2.2.3).

A minimum time of 6 months exposition of the material coupons was the convention of the cooperation project. Afterwards biofilm samples were incubated on modified R2A medium. The phylogenetic affiliation of 137 isolates from different material coupons (steel, PVC, PE, glass) was investigated by hybridization and resulted in 26 % pseudomonads of all isolates from the different materials. None of the isolates showed a positive signal with the used *P. aeruginosa* targeting probes. (chapter 3.2.4.3.2)

While considering these results, two questions arise. First: What makes the organism persistent in the free water phase of the system? Second: Why is *P. aeruginosa* not detected in the biofilm after 6 months?

The persistence of *P. aeruginosa* in the free water of the reactor system might be caused by survival of the bacterium in biofilms and subsequent transfer into the free water phase. Other investigators detected *P. aeruginosa* in the culturable state in young drinking water biofilms (Lee and Kim 2003; Moritz et al. 2010). Furthermore, in a model monospecies biofilm grown with citrate minimal medium dispersion of *P. aeruginosa* from the biofilm to the water phase has been reported before (Klausen et al. 2006). One survival mechanism of biofilm bacteria that has been reported by different authors is the 10 to 1000 times greater resistance of biofilm bacteria to antimicrobial agents compared to planktonic cells (Brown and Gilbert 1993; Ceri et al. 1999; Mah and O'Toole 2001; Rodriguez-Martinez and Pascual 2006). Several authors have suggested that different factors contribute to resistance of biofilms to antimicrobial agents in general (Brown and Gilbert 1993; Chambless et al. 2006; Mah and O'Toole 2001; Rodriguez-Martinez and Pascual 2006; Schulte and Flemming 2006; Stewart 2002). Thus, the EPS of the biofilm bacteria may act as a physical diffusion barrier. Contradicting this idea, it has been shown by Stewart *et al.* that diffu-

sion is nearly not limited (Stewart et al. 2001; Stewart et al. 1998). The extracellular polymeric substances therefore may more likely form a reaction barrier, in which components of the EPS adsorb or react with the antimicrobial agents (De Beer et al. 1994). It has been shown for instance, that aldehyde biozides are able to react with amino acid groups of proteins. Negative groups of different components of the EPS react with cationic agents. (Flemming 1995; Schulte and Flemming 2006). Additionally, it was demonstrated by Stewart and co-workers that in single-species biofilms of *P. aeruginosa* the exoenzym catalase converts H_2O_2 (Stewart et al. 2000). For the bacterium *Klebsiella pneumoniae* investigations showed that ß-lactamase degrades ampicillin in biofilms (Anderl et al. 2000). Another mechanism of resistance in biofilm and planktonic cells is slow or no growth. Reduced growth will make cells less susceptible to antimicrobial agents in many cases but depends on the active compound and its reaction mode (Brown and Gilbert 1993). Resistance might also be promoted by a general stress response that results in a higher tolerance of bacteria to heat, cold, unfavourable pH, and oxidizing agents. Furthermore, scientists have observed that biofilm growth of bacteria is accompanied by an up-and-down regulation of specific genes. The regulation of these genes might be induced by substrate limitation, temperature, irradiation or antimicrobial agents and results in the expression of a biofilm specific phenotype. Additonally, it is supposed that this process is significantly influenced by cell-cell-communication (Quorum sensing) (Davies et al. 1998; Mah and O'Toole 2001). A more recent hypothesis on possible resistance mechanisms proposes the existence of so called “persister cells”, a highly protected phenotypic state of the cell. In brief, this hypothesis postulates that certain cells of a population lack the feature of the programmed cell death that kills damaged cells. As a consequence, these cells are able to react immediately in contrast to more time consuming induced processes. The “persisters” can be discriminated from mutant cells by their number, which is too high to be adapted cells compared with the total population. (Ceri et al. 1999; Chambless et al. 2006; Davies et al. 1998; Mah and O'Toole 2001; Schulte and Flemming 2006; Stewart 2002). Another point is the physiological heterogeneity in biofilms. As a consequence of differing physico-chemical conditions throughout the biofilm, bacteria of mono-species or multi-species biofilms react with expression of different physiological phenotypes and therefore are susceptible to antimicrobial agents to a varying degree (Stewart and Franklin 2008). Another survival

mechanism for *P. aeruginosa* is protection by intracellular survival in protozoal hosts like amoeba and their cysts as it has been described by different authors (Brown and Barker 1999; Matz et al. 2008; Thomas et al. 2010). Most authors agree that the combination of resistance mechanisms results in survival of bacteria.

The discussed background information may help to explain the survival of *P. aeruginosa* in the Berlin reactor system. The detection of *P. aeruginosa* in the bulk water phase after cleaning and disinfection may be the consequence of survival of the bacterium in the biofilm. Cochran and colleagues showed in a mono-species biofilm of *P. aeruginosa* that young (24 to 72 h) exponential phase biofilm cells were more resistant to monochloramine and hydrogen peroxide than exponential phase planktonic cells (Cochran et al. 2000). Moreover, Evans and co-workers presented data which suggest that resistance of *P. aeruginosa* is a complex process depending not only on biofilm and planktonic growth, but also on the kind of antimicrobial agent and the growth rate of the bacteria. They observed a growth-rate related effect of the antibiotic ciprofloxacin on biofilm or chemostat grown *P. aeruginosa*. At slow growth rates, biofilm bacteria of *P. aeruginosa* were slightly more susceptible to ciprofloxacin than planktonic cells. In contrast, fast growing biofilm cells of *P. aeruginosa* were more resistant than cells resuspended from the biofilm. And resuspended cells were more resistant than chemostat grown planktonic cells. (Evans et al. 1991) De Queiroz and Day showed that a mixture of sodium hypochlorite and hydrogen peroxide is effective in disinfection and/or removal of 6 day *P. aeruginosa* biofilms grown on aluminium or stainless steel (DeQueiroz and Day 2007). Furthermore, it has been reported by the working groups of Elkins and Stewart that one of the investigated catalases is able to protect biofilm and planktonic *P. aeruginosa* from damage through H_2O_2 (Elkins et al. 1999; Stewart et al. 2000). In detail, *P. aeruginosa* catalase KatA, in contrast to other catalases, has been shown to be present in the periplasmatic and the extracellular compartments and to be highly resistant to proteases and SDS (Shin et al. 2008). It is not clarified for KatA of *P. aeruginosa*, however, if the extracellular detection of this catalase is a consequence of cell lysis or active secretion as described for the plant pathogen *Pseudomonas syringae* (Klotz and Hutcheson 1992; Shin et al. 2008). In previous studies it has been shown, that catalase activity (KatA) is dependent on iron availability and is controlled by quorum sensing (Frederick et al. 2001; Hassett et al. 1999). Moreover, these antioxidant enzymes are detected in most bac-

terial species, including *E. coli* and *Bacillus subtilis*, and protect the cells from the reactive hydroxyl radical by catalyzing the decomposition of H_2O_2 into water and dioxygen (Shin et al. 2008). As a consequence especially catalase may have been an advantage for survival of *P. aeruginosa* during disinfection with H_2O_2 of the reactor system. The hypothesis of survival in the biofilm is supported by the single detection of culturable *P. aeruginosa* (Arginin-Bouillon, MPN method) in 24 months old biofilms grown on PE in the reactor system of this study (Flemming 2003).

Most of the cited investigations used young mono-species biofilms or planktonic cultures and artificial conditions. In contrast, observations of this study focused on middle aged natural mixed-species biofilms and planktonic water phase grown under changing-distribution-system or house-installation-like conditions. Despite these differences, that make a direct comparison difficult, the above described studies reflect the potential of the opportunistic pathogen *P. aeruginosa* to react to any antimicrobial challenge.

A second explanation for the persistence of *P. aeruginosa* in the free water phase and the biofilms may be the presence of an exhaustible nutrient source. Especially the biofilms from parts of the reactor that could not be disassembled may bear a contamination risk for the free water phase in combination with higher nutrient availability in the beginning of the reactor system run. This has been discussed for the reactor system associated HPC bacteria in chapter 4.1.2.1. Furthermore, Sauer et al. demonstrated for *P. aeruginosa* model biofilms grown with minimal medium that dispersion can be induced by an increase of carbon source concentration in the biofilm passing medium (Sauer et al. 2004). In literature, *P. aeruginosa* is discussed as a degrader of rubber like materials which are also included in the reactor system of this study (Linos et al. 2000; Schmeisser et al. 2003). The hypothesis of an exhaustible nutrient source is supported by the detection characteristics of *P. aeruginosa* in the system following the disinfection procedure. After disinfection, the bacterium was detected in reduced numbers but counts increased again. After a while, *P. aeruginosa* positive counts decrease continuously and finally were generally below the detection limit possibly caused by the exhausting nutrient source. (figure 7, chapter 3.2.2.3)

A depletion of the nutrient source may also explain the results of the stagnation experiments (chapter 3.2.2.3). The first stagnation experiment after disinfection at day

22 resulted in a value of 200 *P. aeruginosa* CFU/100 mL following 5.5 h of stagnation. Subsequently, the stagnation values decreased like the flow values. Despite an increase of temperature during stagnation (chapter 3.2.1), enhanced stagnation time after day 235 up to 19.5 h, and a draw-down of flow operation mode to < 1 m^3/h from day 134, no increase of culturable *P. aeruginosa* was observed. Although an exhaustible nutrient source is favoured as causative for the increased detection of culturable *P. aeruginosa* in the free water phase of the reactor system of this study, it has been described by several scientists, that the bacterium is even possible to multiply and persist in low nutrient (oligotrophic) environments including natural mineral water (batch up to 5 years), drinking water (batch up to 150 days) and de-ionized water (batch up to 43 and 48 days) (Botzenhart and Kufferath 1976; Legnani et al. 1999; Van der Kooij et al. 1982). If this was the reason for survival a more or less stable number of culturable *P. aeruginosa* would have been expected and not the high fluctuations detected in this study.

In the experiments of this study culturable pseudomonads were detected in the biofilms after the project defined incubation time of 6 months, but no culturable *P. aeruginosa*. This may be the result of biofilm maturation. Depending on the material, between 3 % and 57 % of cultured bacteria of this investigation were identified as pseudomonads by FISH (table 22, chapter 3.2.4.3). It has been suggested, that - as a consequence of maturation - pseudomonads like *P. fluorescens* and other fluorescent pseudomonads are capable of growing more rapidly in water at low temperatures than *P. aeruginosa,* with its higher temperature optimum, and therefore overgrow *P. aeruginosa* (Van der Kooij et al. 1982). Furthermore, Byrd et al. detected an enhanced growth and viability of a *Pseudomonas* spp. in a batch of sterile drinking water at 25°C (culturable up to day 200) compared to other gram-negative bacteria, i.e. *Klebsiella pneumonia* and *Enterobater aerogenes,* which were culturable for only a few days (Byrd et al. 1991). Moreover, *P. aeruginosa* may convert into a VBNC (viable but nonculturable) state that makes it undetectable by standard cultivation techniques. The potential of *P. aeruginosa* to form such states has been described by Binnerup *et al.*. Their experiments demonstrated that *P. aeruginosa* - normally growing at aerobic conditions and cultivated in the meantime at energy-starved (no O_2 but NO^{3-}, anaerob) conditions - were not able to multiply again under aerobic conditions.

(Binnerup and Sorensen 1993) This may give an answer to the question why *P. aeruginosa* is not detected by cultivation in the biofilm after 6 months.

There is evidence that a combination of the experimental setup of the reactor system, especially nurtrient source, and the properties of *P. aeruginosae* favoured the multiplication of the bacterium during the initial phase of the reactor run.

4.1.4 Total cell counts and heterotrophic plate count bacteria in middle aged and old biofilms

The approximation of total cell counts (TCC) of middle aged biofilms on glass, PE, PVC, copper, and steel with increased expositon time (18 and 24 months) is related to less impact of the materials in the middle aged biofilms. In contrast to this is the long lasting effect of the alkalinity of cement to TCC of the old biofilms. Scraping and homogenization of coupon biofilms reduced TCC. Statistical analysis of heterotropic plate counts of old biofilms resulted in a ranking of colony forming units (CFU) on the used media and incubation conditions: R2A+starch > R2A+Tween > GDWR 20°C and 36°C. Standard R2A and modified R2A are able to detect quantitative differences between the metallic materials, PVC, and cement of the old biofilms. A significant correlation was found between standard R2A and modified R2A.

Determination of total cell counts by DAPI staining is one of the favoured methods in microbial ecology and drinking water habitats (Block et al. 1993; Kalmbach 1998; Lehtola et al. 2006; Pedersen 1990; Percival et al. 1998a; Piriou et al. 1998; Schwartz et al. 1998; Zacheus et al. 2000). Despite it is not often discussed in literature, scientists who routinely use this method know of some biases accompanied by staining and counting with DAPI. For example Schmallenberger et al. found masking effects of sediment to DAPI counts. Moreover, different authors reported a concentration dependent effect of chlorination by sodium hypochlorite (low conc. results in higher fluorescence) and unspecific binding of DAPI to proteins and phospholipids. (Kepner and Pratt 1994; Saby et al. 1997; Schallenberg et al. 1989) At first autofluorescence in the investigated habitate in the range of the used DAPI filter set has to be checked. Second, in oligotrophic habitats very small cells make it sometimes difficult to distinguish between DAPI stained cells and particles of similar size and colour. Al-

though false negative or positive counts can not completely be excluded, they can be reduced by experience of the scientist in counting in general and in the investigated habitate. This investigator bias has been reported by Kepner et al. (Kepner and Pratt 1994). In the present study, this was checked by statistical comparison of the DAPI counts of the same samples by two different laboratories. The analysis revealed no significant difference between the counts of the laboratories indicating a small investigator bias (chapter 3.1.2). Furthermore, if biofilms are scraped and homogenized before staining and counting this procedure is expected to be harmfull to bacterial cells. For example Zacheus et al. detected a reduction of total counts after sonication of the surface for detachement of bacteria (Zacheus et al. 2000). Comparative investigation of DAPI counts in scraped biofilm suspensions and direct counts on untreated material coupons of this study showed that counts are of the same magnitude or one to two orders of magnitude higher on material coupons, concluding a more or less severe damage of bacterial cells. (chapter 3.2.4.1) Additionally, it has to be taken into account that some bacterial cells might not be available for penetration by the colouring agent DAPI because of depositions on their cell envelop like iron incrustrations as it has been described by Hopf for freshwater biofilms (Hopf 2004).

In the present study total cell counts of material coupons and distribution system pipe samples were investigated (table 9, chapter 3.1.2, table 17 and 18, chapter 3.2.4). One critical difference whithin the pipe samples compared to the coupons is the scraped inner pipe surface. Except the pipe samples BWB I, III and VI the investigated pipe surface was always larger 2500 cm^2 (2540 to 5511 cm^2). The surface area investigated from the pipes is much larger than the surfaces from the reactor system of this study (17.25 cm^2 coupon surface) and most of the investigations summarized in the introduction. The appreciable macroscopic variety of the drinking water deposits in this study (chapter 3.1.1) is an evidence for the heterogeneity of biofilms on the microscopic level. The heterogeneity of biofilms has been discussed by Bishop and Rittmann as “spatial differences in any parameter we think is important” (Bishop and Rittmann 1995). These parmeters can be physical and chemical like pH, concentration of nutrients or inhibitory agents, biofilm thickness, density, viscosity, or EPS properties. Furthermore, microbial diversity of species reflecting the physiological and ecologial potential of a biofilm is one of the important parametes. (Bishop and Rittmann 1995) More information of heterogeneity in biofilms as gained by application of

different microscopic techniques is given by Wimpenny et al. (Wimpenny et al. 2000). Because of the high heterogeneity on the microscopic level the author of this study suggests that there is not much difference between 2540 and 5511 cm² homogenized sample of a pipe surface for unspecific quantitative investigations like TCC and colony counts but there may be a difference to clearly smaller areas. In contrast to quantitative investigations like TCC or HPC the analyzed inner pipe surface area is more important for phylogenetic diversity investigations where a scientist may find a bacterial species in 5000 cm² but not in 17.25 cm² investigated square centimetres (reactor coupons of this study) depending on its frequency.

The total cell counts determined on the different material coupons exposed 6, 12, 18, and 24 months in the reactor systems in Berlin and Duisburg scatter around the median of $5.6x10^6$ counts/cm² (chapter 3.2.4.1). As can be seen in figure 9 (chapter 3.2.4.1) TCC of the different materials appear to converge with increasing exposition time. After expositon times of 18 and 24 months values approximately range inbetween one order of magnitude from $1.2x10^6$ counts/cm² on steel to $1.3x10^7$ counts/cm² on PE. Despite the bias of comparability as described in the introduction, the TCC counts of this study are in good accordance with most of the previous investigations described before in this study (Block et al. 1993; Hallam et al. 2001; Kalmbach 1998; Kalmbach et al. 2000; Lehtola et al. 2006; Pedersen 1990; Percival et al. 1998a; Piriou et al. 1998; Schwartz et al. 1998; Zacheus et al. 2000) Even so some counts were done with the fluorescence dye acridine orange instead of DAPI most are in the same range of 10^6 to 10^7 counts/cm² as the counts of the present study. Schwartz et al. found smaller values around 10^5 after distinct shorter exposition times of 8 to 15 d on copper compared to 6 months in this study (figure 9, chapter 3.2.4.1). This is in contrast to the counts between 10^6 and 10^7 detected on copper after exposition times of 6 months and greater in the reactor systems of this study (figure 9, chapter 3.2.4.1). It can be explained in the way that after some days the biofilm bacteria are still in the initial phase of exponential growth and will reach higher numbers after longer exposition time. Another reason for the smaller values detected by Schwatz et al. may be the scraping of the biofilms from the coupons. The loss of detectable cells by a scraping procedure has been shown for the coupons of this study (see above).

The slightly increased values on copper coupons of this study after 6 and 12 months of exposition compared to the other materials can be explained in the way that the steady state for copper is nearly reached but not for glass, PE, PVC, and steel after this time in the biofilms under the used conditions (figure 9, chapter 3.2.4.1). This is supported by the finding that values of all materials converge after increased exposition times of 18 and 24 months and range approximately inbetween one order of magnitude. (figure 9) There are comparable results by other scientists. An effect of approximating TCC values on different materials after extended exposition times as in this study has been detected by Percival et al. on rough compared to smooth stainless steel coupons stained with acridine orange (Percival et al. 1998a). Percival et al. found differences in TCC after 4 and 8 months but no more differences between the material modifications after 12 months of exposition. This was confirmed by a second study with exposition times of 24 months (Percival et al. 1998b). Zacheus et al. detected also no distinctive difference on coupons of PVC or stainless steel or pipes of PVC and PE after a maximum incubation time close to 6 months. These findings support the hypothesis of approximating TCC values on different materials, but the time needed to reach steady state for total cell counts seems to be different and may depend on parameters like material, drinking water resource, experimental setup, retention time, temperature etc..

A non-statistical comparison of the total cell counts of the middle aged (range between 10^6 and 10^7 counts/cm², 5.6 x 10^6 median) and the old pipe samples show no distinctive difference (table 18, chapter 3.2.4.1, table 9 chapter 3.1.2). In most cases the counts of the pipe samples scatters around 10^6 and 10^7 counts/cm². Only in two cases there are values below this range in the magnitude of 10^5 counts/cm² at a metallic and a PVC pipe. Furthermore, one counting result of a metallic pipe with 10^9 counts/cm² is above the prevalent range. Beside these exceptions the findings are in accordance with the order of magnitude detected in biofilms of the reactor system and earlier studies as has been discussed above. At a first glance this is in contrast to the statistical analysis that TCC of the old biofilms of cement in this study differ from that of PVC and metallic pipes (table 11, chapter 3.1.2). One explanation for the smaller values on cement may be that there is a more long lasting effect of the alkaline material cement compared to the exhausting effect of elastomeric or rubber like materials or more inert materials like glass, Teflon, PE, and PVC. Another reason for

the high alkalinity of the biofilm suspension (table 8, chapter 3.1, pH 9.1 and 9.2) may be the increase of alkalinity produced by scraping and homogenizing of the biofilm. This may result in a die off of parts of the bacterial population.

As has been announced before most results of the total cell counts for the middle aged and old biofilm samples support the model of an initial phase of adhesion and exponential growth followed by a stabilization of biofilms at a steady state phase in which they do not change much in numbers if the environmental conditions (at first water quality) are more or less stable.

In addition to the total cell counts by DAPI staining heterotropic plate count bacteria of the biofilm suspensions of different pipe materials were determined by cultivation on R2A and/or according to GDWR (chapter 2.3). Analysis of the different cultivation approaches resulted in significant differences of the data on different media (R2A and GDWR) or media modifications (R2A and R2A+Tween). No statistically significant difference was found for the incubation temperature of 20°C and 36°C on the medium of the GDWR. The statistical testing of culturable counts of all samples on the different media enabled a ranking of the number of CFU detected in the different approaches independent of the pipe material: R2A+starch > R2A+Tween > GDWR 20°C and 36°C. (table 10, chapter 3.1.2)

Testing for differences in CFU between the three material groups of the pipe samples PVC, cement and metallic material resulted in different effects. Cultivations according to GDWR at 20°C and 36°C, respectively, showed no significant difference between materials. The missing difference may reflect a minimum pathogenic potential of the old biofilms of this study. If there were higher numbers of culturable bacteria with pathogenic potential this would result in higher counts at 36°C incubation temperature which is favoured by bacteria of human and animal origin as discussed in chapter 4.1.2.1.

Significant differences between materials could be detected for standard R2A+starch and modified R2A+Tween. Already a non-statistical comparison of the CFU values of the old pipe biofilms on both R2A modifications (table 9, chapter 3.1.2) shows higher colony counts on the metallic materials ($6.64x10^3$ to $3.8x10^5$ CFU/cm²) compared to PVC (14.3 and $7.8x10^3$ CFU/cm²) and cement (12 to 241 CFU/cm²). In addition, Niquette et al. detected densities of bacterial biomass on cement between the

greater densities on grey iron and the lower on plastic-based materials by a method called potential exoproteolytic activity (PEPA)[5] (Niquette et al. 2000). This result of a better detection capacity for biofilm material differences is in good accordance with findings of Kerr et al.. They detected on average 97 % higher viable counts on cast iron than on MDPE or PVC in young and middle aged biofilms (21 days and 10 months) on standard R2A (25°C, 6 d) (Kerr et al. 1999). Two reasons are discussed as the cause of the higher counts on iron containing materials. At first corrosion products are assumed to act as a growth factor supporting heterotrophic growth and second the higher surface area of iron materials is supposed to lead to higher counts (Kerr et al. 1999; Rogers et al. 1994). Furthermore, the investigations of different researchers have shown that on the materials PVC and/or MDPE there was a slower increase and lower steady state counts compared to cast iron (Camper et al. 2003; Holden et al. 1995; Kerr et al. 1999; Rogers et al. 1994; van der Wende et al. 1989). Despite the statistical limitation of this study, results show that detection of quantitative differences between materials is more influenced by variation in the medium than by the temperature of incubation. The results of this study show that R2A (standard or modified) can detect higher counts in biofilm samples than the medium according to GDWR with incubation at 20°C or 36°C. It has already been presented by Reasoner and Geldreich in 1985 that high nutrient media like the GDWR medium or plate count agar and short incubation times are less convenient to drinking water bacteria and than R2A medium with lower nutrient content and longer incubation times of 5 to 7 days (Reasoner and Geldreich 1985).

For the middle aged biofilms (reactor system, 6 months) the cultivation values for R2A+Tween scatter around 10^4 CFU/cm² on PE, PVC, Cu, and V2A, smaller values were counted on glass 3.01×10^2 CFU/mL (table 19, chapter 3.2.4.2). The latter finding allows the conclusion that after 6 months of exposition the materials PE, PVC, Cu, and V2A result in similar quantities of the culturable bacterial population on

[5] potential exoproteolytic activity (PEPA): A non-fluorescent compound is added to a solution in contact with bacteria at a saturating concentration and this compound is hydrolyzed by bacterial exoenzymes which results in a fluorescent product. The PEPA is proportional to bacterial biomass. (Niquette et al. 2000)

R2A+Tween. Only the more inert material glass yielded reduced counts as has been shown by others before on standard R2A (Rogers et al. 1994).

The drinking water studies that have been described in the introduction and in this chapter attract attention regarding two main things. First, the differences in experimental setup, water resource, time of exposition, water temperature, method of total cell count or culturable count detection make a comparison between the studies difficult as has been described above. Second, despite these differences values for heterotrophic plate counts do not reach an order of magnitude considerably above 10^7. These finding may be an indication for a maximum dimension of culturable heterotrophic plate count bacteria on materials like PE, PVC, cement, and metallic materials (steel, cast iron ect.) in drinking water.

Furthermore, it was of interest if significant correlations could be detected between the different cultivation methods. For that purpose parameters were checked by two different non-parametric tests (table 12, chapter 3.1.2). A significant correlation could be detected between standard R2A and modified R2A. Moreover, colony counts on GDWR 36°C correlated with all of the other cultivations (standard R2A, modified R2A, GDWR 20°C). The last finding may be an indication of a population growing on GDWR 36°C as a part of the population growing on the other media. Another explanation may be a dependency on the same parameter for the cultivations. Finally, testing showed no apparent relationship between TCC (DAPI) and the cultivations. The missing relationship reflects the small number of culturable bacteria compared to total cell counts as will be subsequently described.

The last but not really surprising finding was that the total cell counts were higher than culturable counts of the four cultivation approaches on reactor biofilms (R2A+Tween, table 19, chapter 3.2.4.2) or pipe biofilms (R2A+starch, R2A+Tween, GDWR 20°C and 36°C, table 9 and 10, chapter 3.1.2). Different authors pointed out that it is still a challenge for researchers to isolate and culture bacteria (Lye and Dufour 1993; McFeters et al. 1995; Roose-Amsaleg et al. 2001). Kalmbach investigated the Berlin drinking water system in the 1990s and found 0.1 to 1.5 % culturable heterotrophic cells (standard R2A) of total cell counts (DAPI) (Kalmbach 1998). This is in good accordance with the detected culturable bacteria (0.02 to 2 %) in the middle aged biofilms of the Berlin reactor system of this study (table 19, chapter 3.2.4.2).

The smaller range of culturable bacteria in the reactor system of Dorsten-Holsterhausen (>0.01 to 0.11 %, table 19) shows, that despite a comparable experimental setup there are differences caused by other parameters like drinking water source.

4.2 Limitations of molecular techniques in mature drinking water biofilms

Microbial communities which grow under similar limiting conditions as the drinking water biofilms of the pipe and reactor samples of this study in combination with a high impact of inorganic material, have been discussed as critical material for molecular investigations. Ogram et al. discussed low biomass environments and habitats with high iron impact as problematic for extraction of DNA (Ogram 1998). Furthermore, Riffard et al. investigated *Legionella* in groundwater and held inhibitors in the samples of water and biofilms responsible for decrease of sensitivity level of the PCR (Riffard et al. 2001). A general interfering effect of co-extracted metal ions to PCR has been mentioned by Harry et al. (Harry et al. 1999). Additionally, Stein et al. and Harry et al. suppose a chelating effect of metals on negatively charged DNA (Harry et al. 1999; Stein et al. 2001). Despite it was not possible in this study to determine what exactly caused the problems with DNA extraction in the drinking water biofilm samples, there were some indications what might interfere with DNA extraction and downstream analysis.

4.2.1 Effect of sample quality and preparation on PCR

The results show that there is an adsorption of DNA or inhibition of PCR by the biofilm extracts of this study. It is in general possible to extract and amplify bacterial DNA from formaldeyde fixed samples. Despite increased sample volume and concentration procedures it was not possible to amplify bacterial DNA out of a 99 years old grey cast iron pipe.

Despite the difficulties of the habitat as described above and to avoid the biases linked with cultivation techniques as described by different scientists (Amann et al. 1995; McFeters et al. 1995; Roszak and Colwell 1987; Ward et al. 1990), the attempt

was made to extract the bacterial DNA of this problematic material. Low biomass and possible low DNA concentration in starved or nonculturable bacterial cells (Brauns et al. 1991; Ogram 1998) aspired a minimum number of preparation and concentration steps to avoid loss of DNA as outlined by others (Harry et al. 1999). Furthermore, it was of interest if sample quality or preparations influenced the polymerase reaction.

In the first molecular experiments, PCR reactions with increasing dilutions of biofilm suspensions of the pipe samples M IV (grey cast iron, 99 years, formaldehyde fixed) and BWB III (grey cast iron, 24 years, untreated, fridge stored) were prepared and control DNA of the bacterium DK 79 added (table 25, chapter 3.3.1.1). Detection of the control DNA of DK 79 was dependent on the concentration of the added biofilm suspension. This effect might be caused by inhibition of the molecular reaction and/or adsorption of bacterial DNA by compounds solubilized in the extract. Both effects are likely because there were no purification steps performed after alkaline lysis of the biofilm suspensions. Furthermore, both effects were described in literature for clinical, food, and environmental samples and are summarized by Wilson (Wilson 1997). Wilson also gives a good overview of factors inhibiting or facilitating nucleic acid amplification. He pointed out that the effect of a substance as inhibitor or facilitator depends on its reaction environment and concentration. Often not only one substance generates inhibition but a complex impact of different substances with chemical, enzymatic, and physical interactions. Moreover, there may not always be a complete inhibition but only a reduction in sensitivity. (Wilson 1997) In the old drinking water biofilms of this study it is supposed that factors influencing polymerase activity like binding of Mg^{2+}, interference of other divalent cations like Fe^{2+}, and eventually phenolic groups of humic compounds or other organic substances play a role as described by different authors (Ogram 1998; Tebbe and Vahjen 1993; Wilson 1997). Binding of DNA by mineral compounds has been described for glass, sand, and marine sediment (Aardema et al. 1983; Lorenz and Wackernagel 1987; Wilson 1997). Consequently, there may be a binding of DNA to inorganic compounds of the biofilm samples of this study. Lorenz et al. also reported that desorption of DNA was hindered in the presence of detergents like SDS or Tween 80. As a consequence the SDS containing lysis buffer used in this study may even limit desorption of adsorbed DNA. Furthermore, Saeki et al. 2008 showed that noncrystalline Al and Fe oxides in soils are one

major factor of DNA adsorption in soil (Saeki et al. 2008). Therefore, Fe oxides in the biofilm suspensions of this study may also interfere with DNA after direct lysis.

Even though it was not possible to distinguish between inhibition and adsorption the results indicate that further preparation steps were necessary. To reduce inhibiton that might be caused by humic substances, bacterial extracts and proteases, Kreader 1996 proposed to add 0.4 µg/µL BSA (bovine serum albumine) to the PCR assay. Amplification success by addition of BSA has been reported for extracts from feces, marine water, freshwater, and soil (Bennett et al. 1996; Kreader 1996). Amplification results of BWB III seeded with control DNA of *E. coli* and different BSA concentrations (0.1, 0.3, 0.6 µg/µL) resulted neither in a general nor in a concentration dependent effect of BSA. DNA bands for the control DNA appeared in the same range (at sample dilutions > 10^{-2}; table 29, chapter 3.3.1.4) as without addition of BSA.

To lyse the bacterial cells in the biofilm suspensions a hot alkaline lysis was favoured (chapter 2.4.4.1). This lysis procedure has been shown to be very efficient for the isolates of this study. In this study more than 300 isolates of different bacterial Phyla and Classes could be lysed that way and the DNA was applicable for subsequent sequencing procedures (table 13, chapter 3.1.3). Despite it has been reported that gram-positive bacteria are less susceptible to chemical or enzymatic lysis because of their more resistant structures, this procedure was also suitable for *Mycobacteria* and *Bacillus* isolates (table 13) (Frostegard et al. 1999; Lee et al. 1996; Roose-Amsaleg et al. 2001). Additionally, it has to be considered that different authors reported that autochthonous bacteria were more difficult to lyse than inoculated bacteria (Roose-Amsaleg et al. 2001).

The formaldehyde fixed samples were included in the amplification experiments because it was shown in this study that it is in general possible to lyse and amplify DNA from formaldehyde fixed bacteria (tested for *E. coli* prepared in 3.7 % formaldehyde, subsequently washed, and storage at -20°C in PBS/Ethanol solution before applied to PCR, chapter 3.3.1.2). Since not pure cultures but formaldehyde fixed biofilm suspensions should be used it was of interest if this material interferes with the lysis after the storage solution has been displaced by washing and resuspension in 1 x PBS. There was no distinctive sensitivity difference in detection of seeded control DNA by PCR between the fixed biofilm samples of M IV and the non-fixed samples of BWB III

(table 25, chap. 3.3.1.1). In contrast, Johnson et al. reported a strong inhibitory effect of formaldehyde when samples were stored for 6 months at 4°C in 10 % formalin. (Johnson et al. 1995) In Johnson`s investigations permanent storage in higher concentrated formalin might have reinforced the inhibition compared to this study. As a consequence to prevent limitations, in future investigations formaldehyde should not be the preferred preservation agent for subsequent PCR analysis.

Before purification steps were applied, two DNA concentration techniques were tested for their suitability for the sample material used in this study. The precipitation with ethanol or isopropanol has been widely used in molecular microbiology (Becker et al. 2000; Sambrook and Russel 2001; Smith et al. 1989; Tsai and Olson 1991; Yeates et al. 1997). First precipitation with ice cold 96 % ethanol followed by washing with 70 % ethanol was tested. This did not influence PCR efficiency in the lysed formaldehyde fixed sample M IV (table 27, chapter 3.3.1.3). Despite this is a routinely used technique it was the purpose to preclude a reaction of ethanol with coextracted substances. Likewise the three times enhanced extraction volume of the formaldehyde fixed sample M IV in the same approach had no negative influence. The seeded control DNA of DK 79 was still detectable at the same dilution as before (chapter 3.3.1, table 26, 27). The precipitation of DNA by isopropanol and subsequent washing with 70 % ethanol after extraction of the five times enhanced sample volume of fixed M IV showed a reduction of the detection efficiency. The control DNA DK 79 was not detectable at a dilution of 10^{-2} as before but at a higher dilution of 10^{-5}. In this case, it can not be distinguished between a higher inhibition or adsorption generated by the higher sample volume (5 times enhanced) or inhibition caused by the isopropanol precipitation (table 28, chapter 3.3.1.3). As it has been explained in chapter 3.3, the investigations were limited by the sample volume. Because of this the aspects could not be tested separately and exhaustively. Lysis and isopropanol precipitation in a comparable way (enhanced precipitation time up to 15 min, chapter 3.3.1.3) with the liquid culture of *E. coli* (DSM 5695) allowed a detection of the DNA in PCR. Additionally, as mentioned above, it was used in the sequencing procedure for more than 300 isolates of this study. Moreover, it has been shown above that there is a concentration dependent inhibition or adsorption for the biofilm extract of M IV. For these reasons, it seems more likely that the reduction of detection in the isopropanol approach is caused by the increased sample volume of M IV.

4.2.2 Complex DNA extraction methods

With the purpose of better purification to minimize inhibition and adsorption caused by the sample material, different commercially available column systems and one traditional phenol/chloroform extraction were tested for their suitability. Non of the extraction purification procedures was able to supply bacterial DNA detectable by PCR out of selected biofilm samples of this study. Results show that extraction procedures should not only be assessed for their DNA recovery efficiency but also for their reproducibility.

It was reported previously that each step in an extraction procedure is accompanied by a loss of DNA (Roose-Amsaleg et al. 2001). Consequently, a minimum number of extraction steps were combined, lysis followed by column purification. The two commercially available column systems were first tested for their tolerance to the SDS containing lysis solution (this was mentioned by the manufacturer as a critical point) and the tolerance to formaldehyde fixed sample material. Tests showed a slightly negative effect in the SDS loaded assay in the silica gel membrane containing QIAamp DNA Mini Kit (chapter 3.3.2.3, *E. coli* as positive control). Application of a large amount of the formaldehyde fixed sample BWB III was not suitable for PCR. This may indicate an interference of the sample ingredients to the column system or to PCR or a less amount of amplifyable DNA in the sample. The second columns tested were the Genomic-tips 20 with an anion exchange resin for adsorption of DNA (chapter 3.3.2.4). Both the diluted alkaline lysate (standard procedure, chapter 2.4.4.1) and the kit recommended lysozyme lysis showed only a slight interference with the Genomic-tips. To avoid reduction of detection limit of DNA by dilution of the lysate with AE buffer (standard procedure) this step was left out. The consequently higher concentration of SDS in the lysate was supposed to interfere with the anion exchange resin. Because of this, SDS in the undiluted lysate of the *E. coli* liquid culture was precipitated by potassium acetate. This procedure showed no qualitative disadvantage because amplification still resulted in a PCR band. The same procedure with formaldehyde fixed test *E. coli* with and without SDS precipitation yielded no positive result. This approach showed that these columns are not suitable for the use with formaldehyde containing samples. As a consequence a non fixed BWB IV was applied but resulted not in an amplification product. Because the QIAamp silica

gel and Genomic-tips binding resin column systems resulted not in satisfying PCR products generated by sample inherent limitations or unsuitable purification procedure more complex methods with enhanced purification steps were tested below.

It was supposed above (chapter 4.2.1) that low biomass and possible low DNA concentration in starved or nonculturable bacterial cells in this oligotrophic drinking water habitate in combination with inorganic impact were the shortcomings of biofilm samples of this study. Thus loss of DNA during the extraction process was of interest and the recovery of seeded DNA for a commercially available extraction kit and a classical phenol/chloroform extraction was tested.

The DNA of *Aquabacterium citratiphilum* (DSM 11900) was used for recovery tests because this bacterium was detected as dominant in young drinking water biofilms in previous studies (Kalmbach et al. 1997b). In recovery experiments DNA was determined by the PicoGreen dsDNA Quantitation kit before and after extraction process (chapter 2.4.3.6.2). First, the FastDNA Spin Kit for soil was evaluated. Because naked DNA was applied to the procedure it was performed with and without mechanical-chemical lysis. Although the author is aware that the following interpretations of the recovery tests are not based on a statistically valid basis, trends can be seen and have to be verified in further experiments. Surprisingly, the extraction procedure with seeded naked DNA and mechanical-chemical lysis showed a higher mean recovery than without lysis, 5.4 % and 3.0 % respectively. A smaller recovery due to shear effects would have been expected as described for example by Roose-Amsaleg (Roose-Amsaleg et al. 2001). The higher recovery might be due to a stabilizing effect of the kit included lysis buffer. Despite the higher recovery the calculated coefficient of variation of 34 % with mechanical-chemical lysis compared to 7.5 % without, indicates a negative impact of mechanical-chemical lysis on the reproducibility of extraction.

Second, a classical phenol/chloroform extraction was tested which allowed a maximum application of 5 g of sample (chapter 2.4.3.3). After the first problems with the detection limit of dsDNA of *A. citratiphilum* had been solved by applying a higher amount of DNA, the impact of the different extraction and purification steps was tested. After alkaline lysis and PEG precipitation had been left out of the procedure, DNA reached a detectable level at the end of the extraction. Although both, alkaline

lysis and PEG precipitation, might have an impact on recovery it is supposed that PEG has the higher influence because Lee et al. discussed PEG as a reason for high loss of DNA (Lee et al. 1996). Furthermore, the precipitation step with ethanol combined with a high salt concentration seems to make the PEG precipitation substitutable. This optimized extraction purification procedure was seeded with *Aquabacterium citratiphilum* DNA, DNA was measured, and recovery and coefficient of variation calculated. The high coefficient of variation of 76.9 % indicates a bad reproducibility of the procedure. This is inferior to the coefficient of variation of the FastDNA Spin Sample Kit with 7.5 to 34 %. In contrast to this, the average recovery of 7.2 % was slightly higher than that of the FastDNA Kit with 4.2 %. Additionally, the maximum recovery reached 14.6 % in contrast to the maximum of 5.4 % in the FastDNA Kit. The concluding remark for both methods is that the commercially available FastDNA Kit seems to be more optimized according to the reproducibility but the classical phenol/chloroform extraction allows higher recoveries accompanied by a lower reproducibility. DNA extraction with both methods resulted in a recovery of < 10 %. Lee et al. 1996 used a method comparable to the classical phenol/chloroform extraction of this study and detected a recovery of soil DNA of 3.68 % compared to 7.2 % of this study. In literature some recoveries especially for extraction out of soil samples have been described. A wide range of DNA recovery from 95-85 % (Sephadex G50 colums) and 90 % (Sephadex G200 containing PVPP) for soil samples with high organic impact to less than 10 % (Hydroxyapatite combined to CsCl gradient centrifugation) have been reported (Roose-Amsaleg et al. 2001). The author of this study agrees with the outline of others that there is no single experimental setup for extraction of bacterial DNA appropriate for every environmental sample (Harry et al. 1999; Zhou et al. 1996).

Although not every of the extraction or purification methods appeared to be successful, biofilm samples of this study were applied to different combinations of lysis and purification steps. It was reported by Wilson 1997 that nontarget DNA might inhibit PCR, because of this the FastDNA extracts of M VIII, BWB VI, and the reactor biofilm sample RBI PE (6 months exposed, less inorganic impact) were serial diluted up to 1:1000 and 1, 10, and 17.5 µL added in 25 µL PCR assay (chapter 3.3.2.1.2) (Wilson 1997). This option seemed not promising because no diffuse DNA trace in the agarose gel was seen (data not shown). For the used conditions it was not possible to

detect the bacterial DNA. Because excessive DNA was not the reason for failure of DNA detection, the DNA of the FastDNA Spin Kit extracts was concentrated by isopropanol precipitation after pooling of two to four FastDNA Spin Kit eluates of different samples (BWB V, BWB VII, M X, chapter 3.3.2.1.1). Despite concentration the bacterial DNA was stil not detectable in the PCR approach. Because of the higher reproducibility most of the limited non fixed biofilm sample material was used for extraction with the FastDNA Spin Kit.

4.2.3 Methodological Perspective

Future investigations in old and middle aged drinking water biofilms should take into account the following remarks regarding molecular investigations. At first in problematic habitats it is of great importance to have enough sample material to optimize molecular techniques for a certain sample. In this study, direct lysis of bacteria in the habitat has been choosen because it was reported to get a higher and more representative yield of the bacterial DNA present in a habitat (Ogram 1998). It would be promising to test the procedure applied by Frostegard et al. who treated soil samples with RNA for saturation of adsorption sites prior to lysis and extraction (Frostegard et al. 1999). Beside direct lysis, approaches with separation of bacterial cells before lysis should be tested for applicability, despite the accompanying drawback of smaller DNA yield and greater selection. In general, in such problematic habitats recovery efficiency and reproducibility of used DNA extraction methods have to be evaluated, not only for the naked extraction procedure but also for the combination of habitat samples seeded with a known amount of DNA and bacterial cells. This is necessary for the assessment of complex interactions of sample ingredients with molecular techniques. Regardless of the extraction method different polymerases should be applied because it has been reported that polymerases show different susceptibility to various inhibitors (Abu Al-Soud and Radström 1998; Kermekchiev et al. 2009).

4.3 Impact of bacteria with pathogenic potential in the investigated systems

Sooner or later most of the studies, investigations and reviews dealing with drinking water microbiology end up in the question of microbiological safety of the water or calculation of consumer`s health risk. The cognitions and problems of this study are a good reflection of the difficulties and future challenges in drinking water microbiology regarding consumer`s health.

Investigations in the middle aged and old biofilms of this study with culture dependent methods detected no described human pathogen or opportunistic pathogen bacterium. Most of the genera found have been shown to belong to the autochthonous bacterial populations in drinking water. (chapter 3.1.3) Despite this, there are bacteria belonging to the genera *Aeromonas* and *Mycobacterium* with a known pathogenic potential isolated from the biofilms of the Berlin reactor system and the pipes. In the bulk water phase of the reactor systems regarding the health risk the critical genera *Acinetobacter* and *Aeromonas* and the opportunistic pathogen *P. aeruginosa* were found. (chapter 3.2.2.3, 3.2.5.3)

The HPC bacteria and culturable *P. aeruginosa* in the freewater phase of the reactor reflect the impact of an exhaustible nutrient source to the pathogenic potential. This findings support the opinion that a reduction in assimilable nutrient source is beneficial for microbiological safe drinking water. (chapter 4.1.2.1, 4.1.3) Beside the nutrient source, in the case of persistence of *P. aeruginosa* in the initial phase of the reactor system, the biofilm way of life is discussed as an advantage combined with the production of catalase for protection against H_2O_2. (chaper 4.1.3) Despite the findings in the old biofilm, the problems with the *P. aeruginosa* contamination in the free water phase of the reactor system make aware of the health risk in drinking water systems when parameters are getting suitable for growth of pathogenic or opportunistic pathogenic bacteria in combination with their inherent properties.

Furthermore, it has to be taken into account that *P. aeruginosa* is one of the opportunistic pathogens that inhabit water or soil and are transported from these habitats into drinking water as has been described by others (chapter 4.1.3). The risk of fecal contamination of the reactor system is assessed to be low because no *E. coli* and

coliform bacteria were detected according to DIN 38411 K6 in the bulk water. (chapter 3.2.3.3) Additionally, fecal contamination would result in higher counts of GDWR at 36°C compared to the guideline value and cultivation at 20°C, because the higher temperature is favoured by human and animal pathogens (chapter 4.1.4).

The biases and limitations of the methods used for detection of bacteria have another strong impact on the assessment of the health risk. First, it has been detected for the biofilms of the reactor system by DAPI counts that scraping and homogenization results in a more or less severe damage of bacterial cells what will also affect the pathogens (chapter 4.1.3, 4.1.4). Second, it has to be critically mentioned that for HPC bacteria in the biofilm and water phase and *P. aeruginosa* in the water phase, only the culturable fraction was tested and both detected only a fraction of the not known total population of this drinking water system (chapter 4.1.2.1, 4.1.3). Furthermore, the primarily used modified R2A medium and the GDWR medium are not favoured by pathogenic bacteria. The bacterial test procedures for water analysis generally identify the bacteria based on specific cultivation and physiological approaches. As a consequence, at the end of the procedure the bacterium is for example identified as *P. aeruginosa* or *L. pneumophila* but is not tested for virulence factors or pathogenicity. In the case of DIN 12780 for detection of *P. aeruginosa*, false negative results were assessed to be the most serious risk to human because there may be *P. aeruginosa* which lack the blue-green pigment production or are of size smaller than the concentration filter pores (chapter 4.1.3).

The failure of cultivation techniques to detect nongrowing bacteria resulted in application of biofilm samples to DNA extraction with the aim of subsequent molecular biological analysis. In microbiological research, different DNA extraction methods have been successfully applied to many habitats, but show their limitation in a habitat with high inorganic impact combined with oligotrophic conditions like the biofilms of this study. Because of these drawbacks it is of particular importance to check DNA extraction methods and subsequent analysis procedures for recovery efficiency and reproducibility. (chapter 4.2.2) The molecular techniques are promising in detecting pathogens more sensitive and specific and may overcome the problem of nonculturable bacteria in many cases. Despite this, it has also to be taken into account that amplification by PCR is possible but may also be problematic for nonculturable bac-

teria. Additionally, it was reported that PCR can be less sensitive than cultivation. (chapter 4.2.1).

The working groups of the cooperation dealing with bacteria, viruses, fungi, and protoza with a described health risk in drinking water have been outlined in the introduction. In general, their results confirm the assessment of the present study. Regarding the investigated bacteria (*P. aeruginosa*, *Legionella* sp., *E. coli*, coliform bacteria, *Campylobacter* sp. and *Yersinia* sp., *H. pylori*, Mycobacteria, *Aeromonas* sp.) the conclusion has been made that the investigated biofilms do not inhabit these bacteria in concentrations of relevance in drinking water practice. Furthermore it has been remarked that, depending on the situation (e. g. rubber coated valves), biofilms can be a reservoir in defined areas. This assessment is the same for fungi, viruses, and the investigated parasites *Cryptosporidium* and *Giardia* (cysts). Only the group investigating the Amoeba critiacally remarked the isolation of thermophilic Acanthamoeba with pathogenic potential. Moreover, they isolated some further Amoeba which have been described to inhabit bacteria. (Flemming 2003)

It can be summarized that it is confirmed by the results of this study that heterotrophic plate count bacteria are considered a useful parameter to assess drinking water treatment efficiency and changes in bacterial water quality during storage and distribution as described by others (Allen et al. 2004; Bartram et al. 2004a). To the author´s opinion it is not a question of bacteria with pathogenic potential being in drinking water systems or not – they are in the systems at least in small numbers or a nongrowing physiological state. The question is if conditions in the system support growth of these organisms. Consequently, it has to be kept in mind that drinking water quality is determined by multiple parameters. It starts with wastewater treatment, followed by drinking water resource management and appropriate treatment procedure. In addition, choice of microbiologically appropriate material of pipes and other plumbing material as well as maintenance and hydraulic management of distribution pipes. Furthermore, as important as the distribution system which should supply microbiologically safe drinking water is the houseinstallation system with main focus on appropriate materials and hydraulic regime (prevention of stagnation).

5 Outlook

The present study gives an insight in the habitat “old drinking water biofilm”. The cognitions and problems with investigations in this habitat as described here make clear why old biofilms have been rarely investigated in the past, particularly with molecular methods. The results of this study with main focus on the culturable bacterial population allow the conclusion regarding consumer`s health that there is a pathogenic potential depending on the environmental parameters but no acute risk.

Regarding further investigations it can be infered from the results of this study that in the problematic habitat middle aged and old drinking water biofilm, from the scientific and health risk point of view, it is of particular importance to apply a mixture of methods that will provide the broadest insight into the habitat.

Therefore, it would be a future challenge to optimize DNA extraction for such problematic habitats and subsequent get a better inside in drinking water ecology and pathogenic potential of these habitats. Despite the author is aware of the fact that scientists are often limited by economical and personnel constraints, from the scientific point of view, it is necessary to check the DNA extraction methods and subsequent analysis for their recovery efficiency and reproducibility. In this context, it would also be promising to test mutants of Taq DNA polymerases that were reported to be resistant to PCR inhibitors (Kermekchiev et al. 2009). A combination of an optimized DNA extraction technique with fingerprinting methods like DGGE, RFLP, and ARDRA would be helpful for a first insight. A deeper understanding would be able by the application of high throughput techniques like sequencing and cloning with focus on functional markers detected by their mRNA like enzymes or virulence factors (Cenciarini-Borde et al. 2009). Furthermore, a focus on virulence factors will allow a more precise assessment of the pathogenic potential of mature drinking water biofilms. The detection of the spatial distribution of active bacteria could be examined by an adapted FISH procedure for this habitat, e. g. CARD-FISH or direct viable counts combined with FISH, and detection of the results by confocal laserscanning microscopy. To get an insight in the physiological potential of the bacterial population and regarding the limitations of molecular techniques, cultivation approaches are as important as the molecular techniques. Depending on the subject of interest media ap-

propriate for the autochthonous population, pathogens or indicator bacteria should be selected. Regarding the pathogenic bacteria there should not only be taken focus on the known but also on the potential of so far not known pathogens. And as proposed by Theron et al. it is important to clarify correlations between detection of pathogens and their viability or infectivity (Theron et al. 2010). Furthermore, analysis has to be based on a statistically valid basis to get a more reliable assessment of microbial community composition and ecology.

6 Summary

In the present study, mature drinking water biofilms were investigated in reactor systems and distribution pipes. The aim of the study was to characterize the microbial community with emphasis on bacteria of hygienic relevance. In the reactor in Berlin and the Ruhrgebiet middle aged biofilms (6 to 24 months exposed) were grown on coupons of the materials glass, copper, PE, stainless steel, and PVC. The experimental setup takes place in between the actual distribution system and inhouse installations. In addition, biofilms of another reactor system were investigated by cooperation with the DTU in Lyngby, Denmark. Furthermore, pipe biofilms were obtained from pipes cut out of the actual drinking water distribution system (8 to 99 years exposed). The investigations of the pipes focussed mainly on materials of relevance in Germany like cast iron, grey cast iron, and cement lined cast iron as well as PE and PVC.

Investigation of the culturable population in biofilm samples revealed 454 isolates in total and were phylogenetically analysed by sequencing or a combined RFLP-sequencing approach. The culturable bacteria from the biofilms of the German and Danish reactor systems clustered in the phyla *Firmicutes*, *Bacteroidetes*, and predominately *Proteobacteria* (79 %). Only two gram-positive *Bacillus* and four *Flavobacteriaceae* were identified. In contrast to this, in the old biofilm samples nearly as much gram-positive (49 %) as gram-negative (51 %) bacteria were found. The gram-negative bacteria were dominated by *Beta*- and *Alphaproteobacteria* (19 % and 18 %, respectively) and a relatively high fraction of *Gammaproteobacteria* (12 %). Gram-positive bacteria were separated in 28 % *Actinobacteria* and 21 % *Firmicutes*.

Some genera with pathogenic potential were identified but not tested for virulence factors or infectivity. In the bulk water phase of the reactor systems the genera *Acinetobacter*, *Aeromonas* and *P. aeruginosa* were found. Additionally, in the biofilms of the Berlin reactor system *Aeromonas* and in the pipes *Mycobacterium* was detected.

Further investigations in the bulk water phase of the reactor system in Berlin regarding *P. aeruginosa* and HPC bacteria suggest that during the initial phase of operation the reactor system is highly influenced by an exhaustive nutrient source and flow regime. The persistence of *P. aeruginosa* in the bulk water phase of the reactor is as-

sumed to be linked to the protection in biofilms and the ability to produce catalase. It has to be elucidated in further experiments if missing culturable *P. aeruginosa* in the biofilm after six months is caused by maturation of the biofilm and/or transition into viable but nonculturable state.

Detection of total cell counts in the middle aged biofilms on glass, PE, PVC, copper, and steel showed an approximation with increased exposition times. This effect is related to less impact of the materials to growth of the middle aged biofilms. In contrast to this is the long lasting effect of the alkalinity of cement to total cell counts of the old biofilms. Moreover, it was shown that scraping and homogenization of coupon biofilms reduced total cell counts. The statistical analysis of heterotrophic plate counts of old biofilms resulted in a ranking of colony forming units on the used media and incubation conditions: R2A+starch > R2A+Tween > GDWR 20°C and 36°C. Furthermore, standard R2A and modified R2A are able to detect quantitative differences in colony forming units between the metallic materials, PVC, and cement of the old biofilms. A significant correlation was found between standard R2A and modified R2A.

The results of this study showed limitations of selected molecular techniques in mature drinking water biofilms. Detection of bacterial DNA was hindered by adsorption of DNA and/or inhibition of PCR by the biofilm extracts. Moreover, neither the different commercially available column systems nor the traditional phenol/chloroform extraction were able to supply bacterial DNA detectable by PCR out of selected biofilm samples. In conclusion, extraction procedures should not only be assessed for their DNA recovery efficiency but also for their reproducibility.

In the problematic habitat mature drinking water biofilm, from the scientific and health risk point of view, it is of particular importance to apply a mixture of methods that will provide the broadest insight into the appearance and fate of microorganisms with hygienic relevance.

Regarding the health risk of consumers the results of this study allow the conclusion that there is a pathogenic potential depending on the environmental parameters but no acute risk.

7 Zusammenfassung

In der vorliegenden Arbeit wurden reife Biofilme in Reaktorsystemen und Rohren aus dem Trinkwasserverteilungssystem untersucht. Das Ziel der Arbeit war die Beschreibung der mikrobiellen Gemeinschaft mit dem Schwerpunkt auf Bakterien mit hygienischer Bedeutung. In den Reaktorsystemen in Berlin und dem Ruhrgebiet wuchsen Biofilme 6 bis 24 Monate (mittleres Alter) auf Glas, Kupfer, PE, Edelstahl und PVC. Der experimentelle Aufbau und Betrieb des Systems zeigte Bedingungen wie sie im Trinkwasserverteilungssystem und in der Hausinstallation herrschen. Zusätzlich wurde in einem Kooperationsprojekt mit der DTU, Lyngby in Dänemark, ein weiteres Reaktorsystem untersucht. Die Untersuchung in 8 bis 99 Jahre alten Trinkwasserrohren beschränkten sich hauptsächlich auf Materialien mit Bedeutung in Deutschland, wie Gusseisen, Grauguss, mit Zement ausgekleidete gusseiserne Rohre sowie Rohre aus den Materialien PE und PVC.

Es wurden insgesamt 454 Bakterien aus den Biofilmproben isoliert. Diese wurden durch Sequenzierung oder eine Kombination aus RFLP und Sequenzierung phylogenetisch zugeordnet. Die aus den Biofilmen des deutschen und dänischen Reaktors gewonnenen kultivierbaren Bakterien wurden den Phyla *Firmicutes*, *Bacteroidetes* und zu einem großen Anteil den Proteobakterien (79 %) zugeordnet. Es wurden nur zwei gram-positive Bakterien der Gattung *Bacillus* und vier *Flavobacteriaceae* identifiziert. Im Gegensatz dazu wurden in den alten Biofilmen der Rohre annähernd so viele gram-positive (49 %) wie gram-negative (51 %) Bakterien gefunden. Bei den gram-negativen Bakterien herrschten *Beta*- and *Alphaproteobacteria* (19 % bzw. 18 %) vor. Außerdem wurde eine relativ große Zahl an Gammaproteobakterien (12 %) identifiziert. Die gram-positiven Bakterien konnten in 28 % *Actinobacteria* und 21 % *Firmicutes* unterteilt werden.

Es wurden einige Gattungen mit pathogenem Potential identifiziert. Diese wurden jedoch nicht auf das Vorhandensein von Virulenzfaktoren oder ihr Infektionspotential überprüft. In der Freiwasserphase der Reaktorsysteme wurden die Gattungen *Acinetobacter*, *Aeromonas* sowie *P. aeruginosa* gefunden. Zusätzlich wurden im Berliner Reaktorsystem die Gattung *Aeromonas* und in den Rohrproben Mykobakterien nachgewiesen.

Ergänzend wurde die Freiwasserphase des Berliner Reaktorsystems auf *P. aeruginosa* und HPC Bakterien untersucht. Die Ergebnisse deuten darauf hin, dass in der Anfangszeit der Reaktor stark durch eine sich erschöpfende Nährstoffquelle und den Durchfluss bestimmt wurde. Es wird angenommen, dass die Überdauerung von *P. aeruginosa* in der Freiwasserphase des Berliner Reaktorsystems durch ein Überleben im Biofilm und die Fähigkeit Katalase zu produzieren unterstützt wurde. Es muss in weiteren Untersuchungen geklärt werden, ob das Fehlen von *P. aeruginosa* in kultivierbarem Zustand in den sechs Monate alten Biofilmen durch die Reifung des Biofilms und/oder den Übergang in den „viable but nonculturable“ Zustand bedingt war.

Die Gesamtzellzahlen in den Biofilmen mittleren Alters auf Glas, PE, PVC, Kupfer und Stahl zeigten eine Annäherung über die Zeit. Dieser Effekt wurde auf einen geringen Einfluss des Materials auf das Wachstum der Biofilme zurückgeführt. Im Gegensatz dazu zeigte der alkalische pH-Wert der Zementproben einen lang anhaltenden Effekt auf die Gesamtzellzahlen in den alten Biofilmen. Das Abschaben und Homogenisieren der Biofilme (mittleres Alter) führte zu einer Reduzierung der Gesamtzellzahl. Die statistische Analyse erlaubte es eine Rangfolge der Anzahl der koloniebildenden Einheiten von den eingesetzten Medien aufzustellen: R2A+Stärke > R2A+Tween > GDWR 20°C and 36°C. Weiter konnte gezeigt werden, dass standard R2A und modifiziertes R2A in der Lage sind quantitative Unterschiede in der Anzahl der koloniebildenden Einheiten in den alten Biofilmen unterschiedlicher Materialien aufzuzeigen (metallische Materialien, PVC, Zement). Es konnte eine signifikante Korrelation zwischen standard R2A und modifiziertem R2A festgestellt werden.

In der vorliegenden Arbeit wurde eine Einschränkung der Anwendbarkeit ausgewählter molekularbiologische Methoden auf die reifen Trinkwasserbiofilme gezeigt. Der Nachweis der bakteriellen DNA wurde durch Adsorption und/oder Hemmung der PCR durch die Biofilmextrakte verhindert. Darüber hinaus konnte weder durch verschiedene kommerziell vertriebene Säulenaufreinigungssysteme noch durch die klassische Phenol/Chloroform Extraktion bakterielle DNA in amplifizierbarer Form erhalten werden. Zusammenfassend kann gesagt werden, dass DNA Extraktionsmethoden nicht nur auf Wiederfindung, sondern auch auf Reproduzierbarkeit getestet werden sollten.

In dem problematischen Habitat reifer Trinkwasserbiofilm ist es sowohl aus wissenschaftlicher Sicht als auch zur Abschätzung des Gesundheitsrisikos wichtig, durch Anwendung verschiedener Methoden einen möglichst differenzierten Einblick in das Vorkommen und den Verbleib hygienisch relevanter Mikroorganismen zu gewinnen.

Bezogen auf das Gesundheitsrisiko für den Verbraucher kann zusammenfassend gesagt werden, dass je nach Umweltbedingungen ein pathogenes Potential vorliegt. Es besteht jedoch kein akutes Risiko.

Danksagung

An erster Stelle Danke ich Prof. Dr. Ulrich Szewzyk für die Möglichkeit an dem spannenden, anwendungsbezogenen Thema zu arbeiten, für die vielen wissenschaftlichen und kreativen Besprechungen sowie seine Unterstützung und Geduld auf meinem langen Weg.

Frau Prof. Dr. Islode Röske danke ich für die Bereitschaft, meine Arbeit zu begutachten.

Prof. Dr. Werner Manz möchte ich für die wissenschaftliche Betreuung in den ersten beiden Jahren und die Einführung in die Technik der Hybridisierung danken.

Ein besonderer Dank gilt Doris Willoweit-Ohl für Ihre hervorragende Arbeit als technische Assistentin und die wertvolle Freundschaft, die aus der Zusammenarbeit entstanden ist.

Hans-Henno Dörries danke ich für die Unterstützung dieser Arbeit während seiner Zeit als studentische Hilfskraft.

Stefan Kalbhen für die sorgfältige Arbeit in seiner Diplomarbeit, deren Ergebnisse in meine Arbeit eingeflossen sind.

Der Arbeitsgruppe von Prof. Dr. H.-C. Flemming, insbesondere Dr. Jost Wingender und Astrid Dannehl, danke ich für die Unterstützung in der Anfangsphase des Projekts und die sehr angenehme Zusammenarbeit im weiteren Verlauf.

Den Berliner Wasserbetrieben danke ich für Ihre Unterstützung bei den Rohrentnahmen sowie beim Aufbau und Betrieb des Reaktorsystems. Darüber hinaus Herrn H. Ehbrecht und Frau Ambellan für ihr offenes Ohr für die Wissenschaft.

Dem Bundesministerium für Bildung und Forschung (BMBF) möchte ich für die finanzielle Unterstützung im Rahmen des Projektes „Erfassung des Wachstums und des Kontaminationspotenzials von Biofilmen in der Verteilung von Trinkwasser“ danken, Förderkennzeichen Teilprojekt 2: 02WT9861.

Prof. Dr. Soeren Molin for the possibility to stay in his lab at the DTU Lyngby, Denmark in 2001 and Dr. Adam C. Martiny for coopertion in the RFLP-sequencing analysis of the reactor isolates.

Dr. Ute Römisch für die geduldige Diskussion der statistischen Auswertung und die Möglichkeit, das Programm STATISTIKA zu nutzen.

Ebenfalls ein besonderer Dank gilt Dr. Christiane Baschien für das Lesen des Mauskripts, dessen sehr konstruktive Diskussion und ihre energische, aber trotzdem freundschaftliche Art mich anzutreiben.

Dr. Karen Bade für die Einführung ins anaerobe Arbeiten und die Beantwortung der vielen Fragen in meiner Anfangszeit im Labor 519.

Ines Kaesler, Gaby Meuser, Dr. Maria Alexandrino und Dr. Inge Graeber ein freundschaftliches Danke für die schöne gemeinsame Zeit an der TU, die vielen anregenden wissenschaftlichen und privaten Gespräche bei einer Tasse Tee und die Bestärkung weiter zu machen.

Gaby Meuser und Anja Höschel für ihre frischen Augen beim Layout dieser Arbeit und ihre freundschlaftliche Unterstützung.

Bei Karin Trojan und Susan Mattke bedanke ich mich für die Hilfe bei der Bewältigung der administrativen Aufgaben.

Allen nicht namentlich erwähnten alten und neuen Kollegen des Fachgebietes Umweltmikrobiolgie danke ich für das angenehme Arbeitsklima und die vielfältige Unterstützung.

Meinen Freunden Anne und Björn Bremer sowie Bernhard Mehner danke ich für Ihre Bestärkung und Unterstützung den Weg zu Ende zu gehen. Meinem Patenkind Carla für das Geschenk der vielen schönen Ablenkungsmomente in ihrem ersten Lebensjahr.

Ein besonders liebevoller Dank gilt meiner Famillie Renate, Hermanus und Constanze Conradi für Ihren Rückhalt und Ihre Geduld, die sehr lange auf die Probe gestellt wurde.

References

Aardema, B. W., M. G. Lorenz, and W. E. Krumbein. 1983. Protection of Sediment-Adsorbed Transforming DNA Against Enzymatic Inactivation. Applied and Environmental Microbiology **46**:417-420.

Abu Al-Soud, W. and P. Radström. 1998. Capacity of Nine Thermostable DNA Polymerases To Mediate DNA Amplification in the Presence of PCR-Inhibiting Samples. Applied and Environmental Microbiology **64**:3748-3753.

Allen, M. J., S. C. Edberg, and D. J. Reasoner. 2004. Heterotrophic plate count bacteria - what is their significance in drinking water? International Journal of Food Microbiology **92**:265-274.

Amann, R. and M. Kühl. 1998. *In situ* methods for assessment of microorganisms and their activities. Current Opinion in Microbiology **1**:352-358.

Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology **56**:1919-1925.

Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews **59**:143-169.

Anderl, J. N., M. J. Franklin, and P. S. Stewart. 2000. Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. Antimicrobial Agents and Chemotherapy **44**:1818-1824.

Anonymous. 2008. Guidelines for drinking-water quality: in corporating 1st and 2nd addenda, Recommendations. World Health Organization, Geneva, Switzerland.

Aramaki, H. and M. Fujita. 1999. In vitro transcription analysis of rpoD in Pseudomonas aeruginosa PAO1. FEMS Microbiology Letters **180**:311-316.

Bartram, J., J. Cotruvo, A. Dufour, S. Hazan, and B. Tanner. 2004a. Heterotrophic Plate Count - IJFM introduction. International Journal of Food Microbiology **92**:239-240.

Bartram, J., J. Cotruvo, M. Exner, C. Fricker, and A. Glasmacher. 2004b. Heterotrophic plate count measurement in drinking water safety management - Report of an Expert Meeting Geneva, 24-25 April 2002. International Journal of Food Microbiology **92**:241-247.

Battin, T. J., W. T. Sloan, S. Kjelleberg, H. Daims, I. M. Head, T. P. Curtis, and L. Eberl. 2007. Microbial landscapes: new paths to biofilm research. Nature Reviews Microbiology **5**:76-81.

Becker, S., P. Boger, R. Oehlmann, and A. Ernst. 2000. PCR Bias in Ecological Analysis: a Case Study for Quantitative Taq Nuclease Assays in Analyses of Microbial Communities. Applied and Environmental Microbiology **66**:4945-4953.

Bennett, P. V., R. W. Gange, H. Hacham, V. S. Hejmadi, M. Moran, S. Ray, and B. M. Sutherland. 1996. Simultaneous Detection of Microorganisms in Soil Suspension Based on PCR Amplification of Bacterial 16S rRNA Fragments. Biotechniques **21**:463-471.

**Bergey`s Manual Trust**. 2001. Bergey`s taxonomic outline, April 2001. http://www. bergeys. org/outlines. html.

Binnerup, S. J. and J. Sorensen. 1993. Long-term oxidant deficiency in Pseudomonas aeruginosa PAO303 results in cells which are non-culturable under aerobic conditions. FEMS Micobiol. Ecol. **13**:79-84.

Bishop, P. L. and B. E. Rittmann. 1995. Modelling heterogeneity in biofilms: Report of the discussion session. Water Science and Technology **32**:263-265.

Bjergbaek, L. A. and P. Roslev. 2005. Formation of nonculturable Escherichia coli in drinking water. Journal of Applied Microbiology **99**:1090-1098.

Block, J. C., K. Haudidier, J. L. Paquin, J. Miazga, and Y. Levi. 1993. Biofilm accumulation in drinking water distribution systems. Biofouling **6**:333-343.

Boe-Hansen, R., H. J. Albrechtsen, E. Arvin, and C. Jorgensen. 2002. Bulk water phase and biofilm growth in drinking water at low nutrient conditions. Water Research **36**:4477-4486.

Botzenhart, K. and R. Kufferath. 1976. Über die Vermehrung verschiedener Enterobacteriaceae sowie *Pseudomonas aeruginosa* und *Alcaligenes spec*. in destilliertem Wasser, entionisiertem Wasser, Leitungswasser und Mineralsalzlösung. Zbl. Bakt. Hyg. , I. Abt. Orig. B **163**:470-485.

Brauns, L. A., M. C. Hudson, and J. D. Oliver. 1991. Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. Applied and Environmental Microbiology **57**:2651-2655.

Brown, M. R. W. and J. Barker. 1999. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. Trends in Microbiology **7**:46-50.

Brown, M. R. W. and P. Gilbert. 1993. Sensitivity of Biofilms to Antimicrobial Agents. Journal of Applied Bacteriology **74**:S87-S97.

Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. McGuiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1998. Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and - rRNA staining. Appl Environ Microbiol **64**:733-741.

Byrd, J. J., H.-S. Xu, and R. R. Colwell. 1991. Viable but Nonculturable Bacteria in Drinking Water. Applied and Environmental Microbiology **57**:875-878.

Camper, A. K., K. Brastrup, A. Sandvig, J. Clement, C. Spencer, and A. J. Capuzzi. 2003. Effect of distribution system materials on bacterial regrowth. Journal American Water Works Association **95**:107-121.

Carter, J. T., E. W. Rice, S. G. Buchberger, and Y. Lee. 2000. Relationships between Levels of Heterotrophic Bacteria and Water Quality Paprameters in a Drinking Water Distribution System. Wat. Res. **34**:1495-1502.

Cenciarini-Borde, C., S. Courtois, and B. La Scola. 2009. Nucleic acids as viability markers for bacteria detection using molecular tools. Future Microbiology **4**:45-64.

Centers for Disease Control and Prevention. 2002. Surveillance for Waterborne-Disease, Outbreaks-United States, 1999-2000. MMWR, Morbidity and Mortality Weekly Report **51**:1-2.

Ceri, H., M. E. Olson, C. Stremick, R. R. Read, D. Morck, and A. Buret. 1999. The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol. **37**:1771-1776.

Chambless, J. D., S. M. Hunt, and P. S. Stewart. 2006. A three-dimensional computer model of four hypothetical mechanisms protecting biofilms from antimicrobials. Applied and Environmental Microbiology **72**:2005-2013.

Cloete, T. E., D. Westaard, and S. J. van Vuuren. 2003. Dynamic response of biofilm to pipe surface and fluid velocity. Water Science and Technology **47**:57-59.

Cochran, W. L., G. A. McFeters, and P. S. Stewart. 2000. Reduced susceptibility of thin Pseudomonas aeruginosa biofilms to hydrogen peroxide and monochloramine. Journal of Applied Microbiology **88**:22-30.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280**:295-298.

De Beer, D., R. Srinivasan, and P. S. Stewart. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Applied and Environmental Microbiology **60**:4339-4344.

Deines, P., R. Sekar, P. S. Husband, J. B. Boxall, A. M. Osborn, and C. A. Biggs. 2010. A new coupon design for simultaneous analysis of in situ microbial biofilm formation and community structure in drinking water distribution systems. Applied Microbiology and Biotechnology **87**:749-756.

DeQueiroz, G. A. and D. F. Day. 2007. Antimicrobial activity and effectiveness of a combination of sodium hypochlorite and hydrogen peroxide in killing and removing Pseudomonas aeruginosa biofilms from surfaces. Journal of Applied Microbiology **103**:794-802.

DIN EN 12780. 2002. DIN EN 12780 Nachweis und Zählung von Pseudomonas aeruginosa durch Membranfiltration. Beuth Verlag GmbH.

DIN EN 38 411 K 6. 1991. DIN EN 38 411 Teil 6 Nachweis von Eschericia coli und coliformen Keimen (K6). Beuth Verlag GmbH.

DIN EN ISO 6222. 1999. DIN EN ISO 6222 Bestimmung der Koloniezahl durch Einimpfen in ein Nähragarmedium. Beuth Verlag GmbH.

Donlan, R. M., W. O. Pipes, and T. L. Yohe. 1994. Biofilm formation on cast iron substrata in water distribution systems. Water Res. **28**:1497-1503.

Dutkiewicz, C. and H. Fallowfield. 1998. Assessment of microbial involvement in the elevation of copper levels in drinking water. Journal of Applied Microbiology **85**:597-602.

Edberg, S. C. and M. J. Allen. 2004. Virulence and risk from drinking water of heterotrophic plate count bacteria in human population groups. International Journal of Food Microbiology **92**:255-263.

Eichler, S., R. Christen, C. Holtje, P. Westphal, J. Botel, I. Brettar, A. Mehling, and M. G. Hofle. 2006. Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. Applied and Environmental Microbiology **72**:1858-1872.

Elkins, J. G., D. J. Hassett, P. S. Stewart, H. P. Schweizer, and T. R. McDermott. 1999. Protective Role of Catalase in Pseudomonas aeruginosa Biofilm Resistance to Hydrogen Peroxide. Applied and Environmental Microbiology **65**:4594-4600.

Evans, D. J., D. G. Allison, M. R. W. Brown, and P. Gilbert. 1991. Susceptibility of Pseudomonas-Aeruginosa and Escherichia-Coli Biofilms Towards Ciprofloxacin - Effect of Specific Growth-Rate. J. Antimicrob. Chemother. **27**:177-184.

Fass, S., M. L. Dincher, D. J. Reasoner, D. Gatel, and J. C. Block. 1996. Fate of *Escherichia coli* experimentally injected in a drinking water distribution pilot system. Wat. Res. **30**:2215-2221.

Flemming, H. C. 1995. Sorption sites in biofilms. Water Science and Technology **32**:27-33.

Flemming, H.-C. 2003. Erfassung des Wachstums und des Kontaminationspotentials von Biofilmen in der Verteilung von Trinkwasser. Berichte aus dem IWW Rheinisch-Westfälisches Institut für Wasserforschung gemeinnützige GmbH, Mülheim Ruhr.

Fletcher, M. and K. C. Marshall. 1982. Are solid surfaces of ecological significance to aquatic bacteria? Adv. Microb. Ecol. (Ed. : K. C. Marshall), Plenum Press, New York **6**:199-236.

Frederick, J. R., J. G. Elkins, N. Bollinger, D. J. Hassett, and T. R. McDermott. 2001. Factors Affecting Catalase Expression in Pseudomonas aeruginosa Biofilms and Planktonic Cells. Applied and Environmental Microbiology **67**:1375-1379.

Frias, J., F. Ribas, and F. Lucena. 2001. Effects of different nutrients on bacterial growth in a pilot distribution system. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology **80**:129-138.

Frostegard, A., S. Courtois, V. Ramisse, S. Clerc, D. Bernillon, F. Le Gall, P. Jeannin, X. Nesme, and P. Simonet. 1999. Quantification of Bias Related to the Extraction of DNA Directly from Soils. Applied and Environmental Microbiology **65**:5409-5420.

German Drinking Water Regulations. 1990. Verordnung zur Änderung der Trinkwasserverordung und der Mineral- und Tafelwasser-Verordnung vom 5. Dezember 1990. BGBl. **I**:2600.

Grohmann, A., U. Hässelbarth, and W. Schwerdtfeger. 2003. Die Trinkwasserverordnung - Einführung und Erläuterung für Wasserversorgungsunternehmen und Überwachungsbehörden. Erich Schmidt Verlag, Berlin.

Hahn, M. W., H. Lunsdorf, Q. Wu, M. Schauer, M. G. Hofle, J. Boenigk, and P. Stadler. 2003. Isolation of Novel Ultramicrobacteria Classified as Actinobacteria from Five Freshwater Habitats in Europe and Asia. Applied and Environmental Microbiology **69**:1442-1451.

Hallam, N. B., J. R. West, C. F. Forster, and J. Simms. 2001. The Potential for Biofilm Growth in Water Distribution Systems. Wat. Res. **35**:4063-4071.

Harry, M., B. Gambier, Y. Bourezgui, and E. Garnier-Sillam. 1999. Evaluation of purification procedures for DNA extracted from organic rich samples: Interference with humic substances. Analusis **27**:439-442.

Hassett, D. J., J. F. Ma, J. G. Elkins, T. R. McDermott, U. A. Ochsner, S. E. H. West, C. T. Huang, J. Fredericks, S. Burnett, P. S. Stewart, G. McFeters, L. Passador, and B. H. Iglewski. 1999. Quorum sensing in Pseudomonas aeruginosa controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Molecular Microbiology **34**:1082-1093.

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters **131**:5-17.

Hogardt, M., K. Trebesius, A. M. Geiger, M. Hornef, J. Rosenecker, and J. Heesemann. 2000. Specific and Rapid Detection by Fluorescent In Situ Hybridization of Bacteria in Clinical Samples Obtained from Cystic Fibrosis Patients. J. Clin. Microbiol. **38**:818-825.

Holden, B., M. Greetham, B. T. Croll, and J. Scutt. 1995. The effect of changing inter process and final disinfection reagents on corrosion and biofilm growth in distribution pipes. Water Science and Technology **32**:213-220.

Hopf, S. Biofilme des Auengebietes "Unteres Odertal" und deren Beeinflussung druch Arzneimittel. 2004. Technische Universität Berlin, Fakultät III, Fachgebiet Umweltmikrobiologie.

Horn, H., H. Reiff, and E. Morgenroth. 2003. Simulation of growth and detachment in biofilm systems under defined hydrodynamic conditions. Biotechnology And Bioengineering **81**:607-617.

Johnson, D. W., N. J. Pieniazek, D. W. Griffin, L. Misener, and J. B. Rose. 1995. Development of A Pcr Protocol for Sensitive Detection of Cryptosporidium Oocysts in Water Samples. Applied and Environmental Microbiology **61**:3849-3855.

Juhna, T., D. Birzniece, S. Larsson, D. Zulenkovs, A. Sharipo, N. F. Azevedo, F. Menard-Szczebara, S. Castagnet, C. Feliers, and C. W. Keevil. 2007. Detection of Escherichia coli in biofilms from pipe samples and coupons in drinking water distribution networks. Applied and Environmental Microbiology **73**:7456-7464.

Kaeberlein, T., K. Lewis, and S. S. Epstein. 2002. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science **296**:1127-1129.

Kalmbach, S. Polyphasic characterization of the microbial population of drinking water biofilms. 1998.

Kalmbach, S., W. Manz, B. Bendinger, and U. Szewzyk. 2000. In situ probing reveals *Aquabacterium commune* as a widespread and highly abundant bacterial species in drinking water biofilms. Wat. Res. **34**:575-581.

Kalmbach, S., W. Manz, and U. Szewzyk. 1997a. Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and in situ hybridization. FEMS Microbiol. Ecol. **22**:265-279.

Kalmbach, S., W. Manz, and U. Szewzyk. 1997b. Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes. Appl. Environ. Microbiol. **63**:4164-4170.

Kalmbach, S., W. Manz, J. Wecke, and U. Szewzyk. 1999. *Aquabacterium* gen. nov., with description of *Aquabacterium citratiphilum* sp. nov., *Aquabacterium parvum* sp. nov. and *Aquabacterium commune* sp. nov.,three in situ dominant bacterial species from the Berlin drinking water system. Int. J. Syst. Bacteriol. **49**:769-777.

Kepner, Jr. R. L. and J. R. Pratt. 1994. Use of Fluorochromes for Direct Enumeration of Total Bacteria in Environmental Samples: Past and Present. Microbiological Reviews **58**:603-615.

Kermekchiev, M. B., L. I. Kirilova, E. E. Vail, and W. M. Barnes. 2009. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. Nucleic Acids Research **37**.

Kerr, C. J., K. S. Osborn, G. D. Roboson, and P. S. Handley. 1999. The relationship between pipe material and biofilm formation in a laboratory model system. Journal of Applied Microbiology **85**:29S-38S.

Klausen, M., M. Gjermansen, J. U. Kreft, and T. Tolker-Nielsen. 2006. Dynamics of development and dispersal in sessile microbial communities: examples from Pseudomonas aeruginosa and Pseudomonas putida model biofilms. FEMS Microbiology Letters **261**:1-11.

Klotz, M. G. and S. W. Hutcheson. 1992. Multiple periplasmic catalases in phytopathogenic strains of Pseudomonas syringae. Applied and Environmental Microbiology **58**:2468-2473.

Knight, I. T. 2000. Molecular Genetic Methods for Detection and Identification of Viable but Nonculturable Microorganisms, p. 77-85. *In* R. K. Colwell and D. J. Grimes (eds.), Nonculturable Microorganisms in the Environment. ASM Press, American Society for Microbiology, Washington, DC.

Kreader, C. A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Applied and Environmental Microbiology **62**:1102-1106.

Lane, D. J. 1991. 16S/23S rRNA sequencing. *In* E. Stackebrandt and M. Goodfellow (eds.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.

LeChevallier, M. W., T. M. Babcock, and R. G. Lee. 1987. Examination and characterization of distribution system biofilms. Appl. Environ. Microbiol. **53**:2714-2724.

Leclerc, H., L. Schwartzbrod, and E. Dei-Cas. 2002. Microbial agents associated with waterborne diseases. Critical Reviews in Microbiology **28**:371-409.

Lee, D. G. and S. J. Kim. 2003. Bacterial species in biofilm cultivated from the end of the Seoul water distribution system. Journal of Applied Microbiology **95**:317-324.

Lee, S. Y., J. Bollinger, D. Bezdicek, and A. Ogram. 1996. Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. Applied and Environmental Microbiology **62**:3787-3793.

Legnani, P., E. Leoni, S. Rapuano, D. Turin, and C. Valenti. 1999. Survival and growth of *Pseudomonas aeruginosa* in natural mineral water: a 5-year study. International Journal of Food Microbiology **53**:153-158.

Lehtola, M. J., M. Laxander, I. T. Miettinen, A. Hirvonen, T. Vartiainen, and P. J. Martikainen. 2006. The effects of changing water flow velocity on the formation of biofilms and water quality in pilot distribution system consisting of copper or polyethylene pipes. Water Research **40**:2151-2160.

Lehtola, M. J., I. T. Miettinen, A. Hirvonen, T. Vartiainen, and P. J. Martikainen. 2007a. Estimates of microbial quality and concentration of copper in distributed drinking water are highly dependent on sampling strategy. International Journal of Hygiene and Environmental Health **210**:725-732.

Lehtola, M. J., K. T. Miettinen, M. M. Keinanen, T. K. Kekki, O. Laine, A. Hirvonen, T. Vartiainen, and P. J. Martikainen. 2004. Microbiology, chemistry and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. Water Research **38**:3769-3779.

Lehtola, M. J., E. Torvinen, J. Kusnetsov, T. Pitkanen, L. Maunula, C. H. von Bonsdorff, P. J. Martikainen, S. A. Wilks, C. W. Keevil, and I. T. Miettinen. 2007b. Survival of Mycobacterium avium, Legionella pneumophila, Escherichia coli, and Caliciviruses in Drinking Water-Associated Biofilms Grown under High-Shear Turbulent Flow. Applied and Environmental Microbiology **73**:2854-2859.

Linos, A., R. Reichelt, U. Keller, and A. Steinbüchel. 2000. A gam-negative bacterium, identified as *Pseudomonas aeruginosa* AL98, is a potent degrader of natural rubber and synthetic cis-1,4-polyisoprene. FEMS Microbiology Letters **182**:155-161.

Lorenz, M. G. and W. Wackernagel. 1987. Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. Applied and Environmental Microbiology **53**:2948-2952.

Lye, D. J. and A. P. Dufour. 1993. Virulence Characteristics of Heterotrophic Bacteria Commonly Isolated from Potable Water. Environmental Toxicology and Water Quality **8**:13-23.

Mah, T. F. C. and G. A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology **9**:34-39.

Manz, W., R. Amann, W. Ludwig, M. Wagner, and K. H. Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. System. Appl. Microbiol. **15**:593-600.

Manz, W., M. Eisenbrecher, T. R. Neu, and U. Szewzyk. 1998. Abundance and spatial organization of gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiology Ecology **25**:43-61.

Manz, W., U. Szewzyk, P. Ericsson, R. Amann, K. H. Schleifer, and T.-A. Stenström. 1993. In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23SrRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. **59**:2293-2298.

Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, and W. G. Wade. 1998. Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify Genes Coding for Bacterial 16S rRNA. Applied and Environmental Microbiology **64**:795-799.

Martiny, A. C., H. J. Albrechtsen, E. Arvin, and S. Molin. 2005. Identification of Bacteria in Biofilm and Bulk Water Samples from a Nonchlorinated Model Drinking Water Distribution System: Detection of a Large Nitrite-Oxidizing Population Associated with Nitrospira spp. Applied and Environmental Microbiology **71**:8611-8617.

Martiny, A. C., T. M. Jorgensen, H. J. Albrechtsen, E. Arvin, and S. Molin. 2003. Long-term succession of structure and diversity of a biofilm formed in a model drinking water distribution system. Applied and Environmental Microbiology **69**:6899-6907.

Matz, C., A. M. Moreno, M. Alhede, M. Manefield, A. R. Hauser, M. Givskov, and S. Kjelleberg. 2008. Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. Isme Journal **2**:843-852.

McFeters, G. A., F. P. Yu, B. H. Pyle, and P. S. Stewart. 1995. Physiological assessment of bacteria using fluorochromes. Journal of Microbiological Methods **21**:1-13.

Melo, L. F. and M. J. Vieira. 1999. Physical stability and biological activity of biofilms under turbulent flow and low substrate concentration. Bioprocess Engineering **20**:363-368.

Miettinen, I. T., T. Vartiainen, and P. J. Martikainen. 1997. Phosphorus and bacterial growth in drinking water. Appl Environ. Microbiol. **63**:3242-3245.

More, M. I., J. B. Herrick, M. C. Silva, W. C. Ghiorse, and E. L. Madsen. 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Applied and Environmental Microbiology **60**:1572-1580.

Morita, R. Y. 1997. Bacteria in oligotrophic environments, Starvation-survival Lifestyle. Chapman & Hall; Fifth Avenue, New York.

Moritz, M. M., H. C. Flemming, and J. Wingender. 2010. Integration of Pseudomonas aeruginosa and Legionella pneumophila in drinking water biofilms grown on domestic plumbing materials. International Journal of Hygiene and Environmental Health **213**:190-197.

Niquette, P., P. Servais, and R. Savoir. 2000. Impacts of pipe materials on densities of fixed bacterial biomass in drinking water distribution system. Wat. Res. **34**:1952-1956.

Norton, C. D. and M. W. LeChevallier. 2000. A Pilot Study of Bacteriological Population Changes through Potable Water Treatment and Distribution. Applied and Environmental Microbiology **66**:268-276.

Ogram, A. 1998. Isolation of Nucleic Acids from Environmental Samples, p. 273-288. *In* R. S. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler (eds.), Techniques in Mircorbial Ecology. Oxford University Press, Inc., New York.

Olson, B. H., H. F. Ridgway, and E. G. Means. 1981. Bacterial colonization of mortar lined and galvanized iron water distribution mains. Proc. Am. Water Works Assoc. Conf. St. Louis, Mo,1027-1039.

Pavlov, D., C. M. E. De Wet, W. O. K. Grabow, and M. M. Ehlers. 2004. Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. International Journal of Food Microbiology **92**:275-287.

Pedersen, K. 1990. Biofilm development on stainless steel and PVC surfaces in drinking water. Water Res. **24**:239-243.

Pepper, I. L., P. Rusin, D. R. Quintanar, C. Haney, K. L. Josephson, and C. P. Gerba. 2004. Tracking the concentration of heterotrophic plate count bacteria from the source to the consumer's tap. International Journal of Food Microbiology **92**:289-295.

Percival, S., R. Chalmers, M. Embrey, P. Hunter, J. Sellwood, and P. Wyn-Jones. 2004. Mikrobiology of Waterborne Diseases. Elsevier Academic Press, San Diego, California, USA.

Percival, S. L., J. S. Knapp, R. Edyvean, and D. S. Wales. 1998a. Biofilm development on stainless steel in mains water. Water Research **32**:243-253.

Percival, S. L., J. S. Knapp, R. G. J. Edyvean, and D. S. Wales. 1998b. Biofilms, mains water and stainless steel. Water Research **32**:2187-2201.

Percival, S. L., J. S. Knapp, D. S. Wales, and R. G. J. Edyvean. 1999. Biofilm development in potable quality water. Biofouling **13**:259-277.

Piriou, P., S. Dukan, and L. Kiene. 1998. Modelling bacteriological water quality in drinking water distribution systems. Water Science and Technology **38**:299-307.

Reasoner, D. J. 1990. Monitoring heterotrophic bacteria in potable water, p. 452-477. *In* Th. D. Brock, U. Sommer, and G. A. McFeters (eds.), Drinking Water Microbiology, Progress and Recent Developments. Springer-Verlag, New York, Berlin.

Reasoner, D. J. and E. E. Geldreich. 1985. A New Medium for the Enumeration and Subculture of Bacteria from Potable Water. Applied and Environmental Microbiology **49**:1-7.

Riffard, S., S. Douglass, T. Brooks, S. Springthorpe, L. G. Filion, and S. A. Sattar. 2001. Occurrence of Legionella in groundwater: an ecological study. Water Science and Technology **43**:99-102.

Ringen, L. M. and C. H. Drake. 1952. A study of the incidence of *Pseudomonas aeruginosa* from various natural sources. J. Bacteriol. **64**:841-845.

Rodriguez-Martinez, J. M. and A. Pascual. 2006. Antimicrobial resistance in bacterial biofilms. Reviews in Medical Microbiology **17**:65-75.

Roeder, R. S., J. Lenz, P. Tarne, J. Gebel, M. Exner, and U. Szewzyk. 2010. Long-term effects of disinfectants on the community composition of drinking water biofilms. International Journal of Hygiene and Environmental Health **213**:183-189.

Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil. 1994. Influence of Plumbing Materials on Biofilm Formation and Growth of Legionella pneumophila in Potable Water Systems. Applied and Environmental Microbiology **60**:1842-1851.

Roose-Amsaleg, C. L., E. Garnies-Sillam, and M. Harry. 2001. Extraction and purification of microbial DNA from soil and sediment samples. Applied Soil Ecology **18**:47-60.

Röske, I. and D. Uhlmann. 2005. Biologie der Wasser- und Abwasserbehandlung. Eugen Ulmer KG, Stuttgart.

Roszak, D. B. and R. R. Colwell. 1987. Survival strategies of bacteria in natural environment. Microbiological Reviews **51**:365-379.

Rubulis, J. and T. Juhna. 2007. Evaluating the potential of biofilm control in water supply systems by removal of phosphorus from drinking water. Water Science and Technology **55**:211-217.

Saby, S., I. Sibille, L. Mathieu, J. L. Paquin, and J. C. Block. 1997. Influence of water chlorination on the counting of bacteria with DAPI (4',6-diamidino-2-phenylindole). Applied and Environmental Microbiology **63**:1564-1569.

Saeki, K., M. Morisak, and M. Saka. 2008. The Contribution of Soil Constituents to Adsorption of Extracellular DNA by Soils. Microbes and Environments **23**:353-355.

Sambrook, J. and D. W. Russel. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sathasivan, A., S. Ohgaki, K. Yamamoto, and N. Kamiko. 1997. Role of inorganic phosphorus in controlling regrowth in water distribution system. Water Science and Technology **35**:37-44.

Sauer, K., M. C. Cullen, A. H. Rickard, L. A. H. Zeef, D. G. Davies, and P. Gilbert. 2004. Characterization of nutrient-induced dispersion in Pseudomonas aeruginosa PAO1 biofilm. Journal of Bacteriology **186**:7312-7326.

Schallenberg, M., J. Kalff, and J. B. Rasmussen. 1989. Solutions to Problems in Enumerating Sediment Bacteria by Direct Counts. Applied and Environmental Microbiology **55**:1214-1219.

Schleifer, K.-H., R. Amann, W. Ludwig, C. Rothemund, N. Springer, and S. Dorn. 1992. Nucleic acid probes for the identification and in situ detection of pseudomonads., p. 127-134. *In* E. Galli, S. Silver, and B. Witholt (eds.), *Pseudomonas*:molecular biology and biotecholoy. American Society for Microbiolgy, Washington, D.C.

Schmeisser, C., C. Stockigt, C. Raasch, J. Wingender, K. N. Timmis, D. F. Wenderoth, H. C. Flemming, H. Liesegang, R. A. Schmitz, K. E. Jaeger, and W. R. Streit. 2003. Metagenome survey of biofilms in drinking-water networks. Applied and Environmental Microbiology **69**:7298-7309.

Schulte, S. and H. C. Flemming. 2006. Causes of the increased resistance of microorganisms in biofilms. Chemie Ingenieur Technik **78**:1683-1689.

Schwartz, T., S. Hoffmann, and U. Obst. 1998. Formation and bacterialcomposition of young, natural biofilms obtained from publicbank-filtered drinking water systems. Water Res. **32**:2787-2797.

Schwartz, T., S. Hoffmann, and U. Obst. 1997. The influence of drinking water conditioning on the biofilm development within a drinking water distribution system. Vom Wasser, Wiley-VCH Verlag,Weinheim **89**:125-138.

Shin, D. H., Y. S. Choi, and Y. H. Cho. 2008. Unusual properties of catalase A (KatA) of Pseudomonas aeruginosa PA14 are associated with its biofilm peroxide resistance. Journal of Bacteriology **190**:2663-2670.

Sly, L. I., T. L. Cox, and T. B. Beckenham. 1999. The phylogenetic relationships of Caulobacter, Asticcacaulis and Brevundimonas species and their taxonomic implications. International Journal of Systematic Bacteriology **49**:483-488.

Smith, G. L. F., C. Sansone, and S. S. Socransky. 1989. Comparison of 2 Methods for the Small-Scale Extraction of Dna from Subgingival Microorganisms. Oral Microbiology and Immunology **4**:135-140.

Stackebrandt, E. and J. Ebers. 2006. Taxonomic parameters revisited: tarnished gold standards. microbiology today, online journal of the Society for General Microbiology, UK **nov.**:153-155.

Stein, L. Y., M. T. La Duc, T. J. Grundl, and K. H. Nealson. 2001. Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. Environmental Microbiology **3**:10-18.

Stelma, G. N., D. J. Lye, B. G. Smith, J. W. Messer, and P. Payment. 2004. Rare occurrence of heterotrophic bacteria with pathogenic potential in potable water. International Journal of Food Microbiology **92**:249-254.

Stewart, P. S. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. International Journal of Medical Microbiology **292**:107-113.

Stewart, P. S. and M. J. Franklin. 2008. Physiological heterogeneity in biofilms. Nature Reviews Microbiology **6**:199-210.

Stewart, P. S., L. Grab, and J. A. Diemer. 1998. Analysis of biocide transport limitation in an artificial biofilm system. Journal of Applied Microbiology **85**:495-500.

Stewart, P. S., J. Rayner, F. Roe, and W. M. Rees. 2001. Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. Journal of Applied Microbiology **91**:525-532.

Stewart, P. S., F. Roe, J. Rayner, J. G. Elkins, Z. Lewandowski, U. A. Ochsner, and D. J. Hassett. 2000. Effect of Catalase on Hydrogen Peroxide Penetration into Pseudomonas aeruginosa Biofilms. Applied and Environmental Microbiology **66**:836-838.

Stoodley, P., S. Wilson, L. Hall-Stoodley, J. D. Boyle, H. M. Lappin-Scott, and J. W. Costerton. 2001. Growth and Detachment of Cell Clusters from Mature Mixed-Species Biofilms. Applied and Environmental Microbiology **67**:5608-5613.

Strunk, O., O. Gross, B. Reichel, M. May, S. Hermann, N. Stuckman, B. Nonhoff, M. Lenke, T. Ginhart, A. Vilbig, T. Ludwig, A. Bode, K.-H. Schleifer, and W. Ludwig. 1999. ARB: a software environment for sequence data. Department of Microbiolgy, Technische Universität München, Munich, Germany, http://www. arb-home. de.

Szewzyk, U., W. Manz, R. Amann, K. H. Schleifer, and Stenstr. 1994. Growth and in situ detection of a pathogenic *Escherichia coli* in biofilms of a heterotrophic water-bacterium by use of 16S- and 23S-rRNA-directed fluorescent oligonucleotide probes. FEMS Microbiol. Ecol. **13**:169-176.

Szewzyk, U., R. Szewzyk, W. Manz, and Schleifer K.-H. 2000. Microbiological Safety of Drinking Water. Annu. Rev. Microbiol. **54**:81-127.

Tebbe, C. C. and W. Vahjen. 1993. Interference of Humic Acids and DNA Extracted Directly from Soil in Detection and Transformation of Recombinant-Dna from Bacteria and A Yeast. Applied and Environmental Microbiology **59**:2657-2665.

Theron, J., T. E. Cloete, and M. de Kwaadsteniet. 2010. Current molecular and emerging nanobiotechnology approaches for the detection of microbial pathogens. Critical Reviews in Microbiology **36**:318-339.

Thomas, V., G. McDonnell, S. P. Denyer, and J. Y. Maillard. 2010. Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. FEMS Microbiology Reviews **34**:231-259.

Tsai, Y. L. and B. H. Olson. 1991. Rapid method for direct extraction of DNA from soil and sediments. Applied and Environmental Microbiology **57**:1070-1074.

Uhl, W. and G. Schaule. 2004. Establishment of HPC(R2A) for regrowth control in non-chlorinated distribution systems. International Journal of Food Microbiology **92**:317-325.

Van der Kooij, D., J. P. Oranje, and W. A. M. Hijnen. 1982. Growth of *Pseudomonas aeruginosa* in tap water in relation to utilization of substrates at concentrations of a few micrograms per liter. Applied and Environmental Microbiology **44**:1086-1095.

van der Wende, E. and W. G. Characklis. 1990. Biofilms in Potable Water Distribution Systems, p. 249-268. *In* G. A. Mc Feters (ed.), Drinking Water Microbiology - Progress and Recent Development. Springer Verlag, New York, Berlin, Heidelberg.

van der Wende, E., W. G. Characklis, and D. B. Smith. 1989. Biofilms and Bacterial Drinking-Water Quality. Water Research **23**:1313-1322.

Vaneechoutte, M. and M. Heyndrickx. 2001. Application and analysis of ARDRA patterns in bacterial identification, taxonomy, and phylogeny, p. 211-247. *In* L. Dijkshoorn, K. J. Towner, and M. Struelens (eds.), New approaches for the generation and analysis of microbial typing data. Elsevier, Amsterdam.

Ward, D. M., R. Weller, and M. M. Bateson. 1990. 16S Ribosomal-Rna Sequences Reveal Numerous Uncultured Microorganisms in A Natural Community. Nature **345**:63-65.

WHO. 2000. World Health Organisation. www. who. int/csr/don/2000_05_30/en/index. html.

Wilson, I. G. 1997. Inhibition and Facilitation of Nucleic Acid Amplification. Applied and Environmental Microbiology **63**:3741-3751.

Wimpenny, J., W. Manz, and U. Szewzyk. 2000. Heterogeneity in biofilms. FEMS Microbiology Reviews **24**:661-671.

Wingender, J. and H. C. Flemming. 2004. Contamination potential of drinking water distribution network biofilms. Water Science and Technology **49**:277-286.

Yeates, C., M. R. Gillings, A. D. Davison, N. Altavilla, and D. A. Veal. 1997. PCR amplification of crude microbial DNA extracted from soil. Letters in Applied Microbiology **25**:303-307.

Zacheus, O. M., E. K. Iivanainen, T. K. Nissinen, M. J. Lehtola, and P. J. Martikainen. 2000. Bacterial Biofilm Formation on Polyvinyl Chloride, Polyethylene and Stainless Steel Exposed to Ozonated Water. Wat. Res. **34**:63-70.

Zacheus, O. M., M. J. Lehtola, L. K. Korhonen, and P. J. Martikainen. 2001. Soft deposits, the key site for microbial growth in drinking water distribution networks. Water Research **35**:1757-1765.

Zhou, J., M. A. Bruns, and J. M. Tiedje. 1996. DNA recovery from soils of diverse composition. Applied and Environmental Microbiology **62**:316-322.

Zwart, G., B. C. Crump, M. P. K. V. Agterveld, F. Hagen, and S. K. Han. 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquatic Microbial Ecology **28**:141-155.

Abbreviations

AOC	assimilable organic carbon
APHA	American Public Health Asssociation
ARDRA	amplified ribosomal DNA restriction analysis
CFU/cm²	colony forming units per square centimeter
CFU/mL	colony forming units per millilitre
CLSM	confocal laserscanning microscopy
CTAB	cetyltrimethylammonium bromide
Cy3	indocarbocyanine
d	day (s)
DAPI	4`,6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DNA	desoxynucleic acid
DVC	direct viable counts
EDTA	ethylenediaminetetraacetic acid
EPS	extracellular polymeric substances
FA	formamide
FISH	fluorescent *in situ* hybridization
GDWR	German drinking water regulation
h	hour (s)
HPC	heterotrophic plate count
NH_4Ac	ammonium acetate
OTU	operational taxonomic unit
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PE HD, MD, LD	polyethylene (high HD, medium MD, and low LD density)
PVC	polyvinyl chloride

R2A	low nutrient agar
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TCC	total cell counts
TE	Tris EDTA
Tween 80	polyoxyethylenesorbitane monooleate
V2A	stainless steel grade V2A
VBNC	viable but nonculturable
w/v	weight/volume