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Bacterial Communities in Drinking Water Biofilms

Katharina Lührig

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DOCTORAL DISSERTATION
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Abstract

Drinking water is not sterile, and serves as a habitat for diverse microorganisms. The majority of the bacteria in drinking water are found in biofilms on the walls of the distribution system. Next-generation sequencing (NGS) was applied to study the bacterial communities in biofilms from drinking water distribution systems (DWDSs). Biofilms from pipes, water meters and a clearwell from two DWDSs in southern Sweden were analyzed. Samples were taken from sites receiving water from the *Ringsjöverket* and *Vombverket* treatment plants. The feasibility and reproducibility of the NGS approach was demonstrated by analyzing biofilms from parallel-installed water meters in dwellings as biological replicates. Analysis of biofilms from both water meters and pipes indicated that the two DWDSs harbored distinct microbial communities. Pipe biofilms represent diverse environments for microbial growth, and the bacterial communities differed in their composition depending on a combination of factors such as surface material, source water, temperature and season. There was almost no overlap in the operational taxonomic units (OTUs) and shared sequences between the bacterial communities from the two DWDSs, suggesting that there is no universal drinking water core community. When water meter biofilms were analyzed, a system-specific core community was observed for one of the two distribution systems. Biofilm samples from an area receiving mixed water from the two treatment plants harbored a community similar to communities from one of the two DWDSs. No OTUs specific to the communities receiving mixed water were observed. This suggests seeding of the biofilm community from the flowing water, and an influence of the source water or the treatment process on the composition of the microbial community in the drinking water. The most abundant OTUs found in the biofilm samples from the two DWDSs studied were the *Sphingomonadaceae* family and the genus *Nitrospira.*

Water meter biofilms were used to study the occurrence of red water after the source water for drinking water production was changed. However, no correlation was found between water meter biofilms and the occurrence of red water. It is believed that changes in the chemistry, for example, the sulfate concentration, caused the red water by disrupting the stability of the corrosion scale in pipes. The work presented in this thesis contributes to a better understanding of bacterial communities in drinking water biofilms, which is important in ensuring the distribution of safe, high-quality drinking water in the future.

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Bacterial Communities in Drinking Water Biofilms

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Coverphoto by Dr. Peter Menzel

The cover picture illustrates the abundance of the OTUs from the complete dataset of the study presented in Paper IV and was created using packCircles (https://github.com/pmenzel/packCircles).

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Popular Scientific Summary

The delivery of safe, clean drinking water is important for public health, and can become challenging in the light of climate change. Climate change could affect the availability of freshwater resources and the presence of pathogens through an increase in temperature and heavy rainfall events. Elevated temperatures can lead to an increase in nutrients in the water, promoting microbial growth and thus a higher risk of contamination by pathogens. Increased concentrations of dissolved organic carbon have been observed in northern Europe since the 1980s, and could affect drinking water production. An example of a health problem related to drinking water is the outbreak of the protozoan parasite *Cryptosporidium* in Östersund, Sweden, in 2010, when 27,000 people were affected.

In Sweden, both surface water and groundwater are used as source water for drinking water production. The quality of the final drinking water is influenced by the chemical and microbial composition of the source water and by the different processes applied in drinking water purification, such as flocculation and sand filtration. One aim of this treatment is the reliable production of microbiologically safe drinking water. Microbial growth in the distribution system can cause problems such as corrosion, taste and odor formation and, in the worst case, a health risk for consumers. The control of microbial growth in the distribution system is therefore important, and can be achieved through disinfection, e.g. chlorination, chloramination or UV irradiation, or the reduction of nutrients in the final water. Multiple barriers and treatment steps are used to ensure the production of safe drinking water without pathogens.

Although drinking water is purified and contains disinfectants, it is not sterile and serves as a habitat for diverse microorganisms. More than 95 % of the bacteria in drinking water is found in biofilms on the walls of the distribution system. Bacteria in biofilms live in a community surrounded by a matrix of biopolymers. This matrix protects the bacteria, aids adhesion to surfaces, and allows a lifestyle entirely different from free-living planktonic cells. However, biofilms can also harbor pathogens, and the biopolymer matrix protects them from disinfectants.

Bacterial communities in biofilms can be influenced by many different factors, such as temperature and season, disinfection, surface material, and the quality of the source water used for drinking water production. Changes in water quality can influence the growth of microorganisms and the community composition within drinking water biofilms. There are often differences in the water quality between surface water from different lakes or rivers, and relatively pure groundwater, which in some cases can be distributed without disinfection. Traditionally, microbiological water quality has been assessed by growth-based cultivation

methods. However, not all microorganisms are culturable, and DNA-based methods can be applied to study biofilms in the distribution system.

In this work, biofilm communities from pipes and domestic water meters were analyzed using DNA-based next-generation sequencing. Biofilm samples from pipes are the most interesting as pipes represent most of the surface area in a DWDS. However, such samples can only be obtained when a pipe in the street is dug up and cut open. Furthermore, a wide variety of pipe materials and sizes are in use, complicating comparisons between different locations. Biofilms were also collected from water meters as these are often made of similar materials, and are more easily accessible. Biofilm communities from two DWDSs receiving source water from two lakes were compared, and a surprisingly high diversity of bacteria was detected. Different bacterial communities were found in the two distribution systems, suggesting that the source water used for drinking water production has considerable impact on the composition of the bacterial community in the biofilm. This raises the question of whether a universal core community exists in drinking water, i.e., whether all drinking water distribution systems harbor the same groups of microorganisms. It is important to elucidate whether there is a universal core community in DWDSs to allow for comparisons between different studies, and for our understanding of the processes that take place in a DWDS over time.

The effect of a change in water quality after switching the source water used for drinking water production on biofilm communities was also studied. Between April 2009 and March 2011, water from Lake Ringsjön was used as source water at the *Ringsjöverket* treatment plant instead of water from Lake Bolmen. During this period, complaints about water quality increased, and red water was observed in some parts of the distribution system. Red water contains elevated levels of iron and manganese causing the red color. Biofilm communities from sites with and without red water were compared, but no correlation was found between the bacteria in the biofilm and the occurrence of red water. A higher sulfate concentration in the water from Lake Ringsjön was identified as the most likely cause of red water after the switch in source water.

The work presented in this thesis contributes to a better understanding of bacterial communities in drinking water biofilms, and is one of the few studies in which microbial communities from real DWDS pipes are described. The study of biofilms is important to improve our understanding of the processes taking place in the distribution system, to ensure the distribution of safe, high-quality drinking water.

Abstract

Drinking water is not sterile, and serves as a habitat for diverse microorganisms. The majority of the bacteria in drinking water are found in biofilms on the walls of the distribution system. Next-generation sequencing (NGS) was applied to study the bacterial communities in biofilms from drinking water distribution systems (DWDSs). Biofilms from pipes, water meters and a clearwell from two DWDSs in southern Sweden were analyzed. Samples were taken from sites receiving water from the *Ringsjöverket* and *Vombverket* treatment plants. The feasibility and reproducibility of the NGS approach was demonstrated by analyzing biofilms from parallel-installed water meters in dwellings as biological replicates. Analysis of biofilms from both water meters and pipes indicated that the two DWDSs harbored distinct microbial communities. Pipe biofilms represent diverse environments for microbial growth, and the bacterial communities differed in their composition depending on a combination of factors such as surface material, source water, temperature and season. There was almost no overlap in the operational taxonomic units (OTUs) and shared sequences between the bacterial communities from the two DWDSs, suggesting that there is no universal drinking water core community. When water meter biofilms were analyzed, a system-specific core community was observed for one of the two distribution systems. Biofilm samples from an area receiving mixed water from the two treatment plants harbored a community similar to communities from one of the two DWDSs. No OTUs specific to the communities receiving mixed water were observed. This suggests seeding of the biofilm community from the flowing water, and an influence of the source water or the treatment process on the composition of the microbial community in the drinking water. The most abundant OTUs found in the biofilm samples from the two DWDSs studied were the *Sphingomonadaceae* family and the genus *Nitrospira*.

Water meter biofilms were used to study the occurrence of red water after the source water for drinking water production was changed. However, no correlation was found between water meter biofilms and the occurrence of red water. It is believed that changes in the chemistry, for example, the sulfate concentration, caused the red water by disrupting the stability of the corrosion scale in pipes. The work presented in this thesis contributes to a better understanding of bacterial communities in drinking water biofilms, which is important in ensuring the distribution of safe, high-quality drinking water in the future.

List of publications

This thesis is based on the following papers:

I. **Bacterial Community Analysis of Drinking Water Biofilms in Southern Sweden.**

Lührig K, Canbäck B, Paul CJ, Johansson T, Persson KM, Rådström P (2015). Microbes Environ 30(1): 99-107.

II. **Bacterial Biofilm Communities from a Drinking Water Clearwell and five Different Drinking Water Pipes from Southern Sweden**

Lührig, K, Paul, CJ, Persson, KM and Rådström, P. *Manuscript.*

III. **Bacterial Community Analysis of Water Meter Biofilms from two Drinking Water Distribution Systems Receiving Different Source Water**

Lührig, K, Chan, S, Paul, CJ, Persson, KM and Rådström, P. *Manuscript.*

IV. **Comparison of Water Meter Biofilm Communities after the Occurrence of Red Water Caused by a Source Water Switch.**

Lührig, K, Paul, CJ, Persson, KM and Rådström, P. *Manuscript.*

My contributions to the studies

Paper I

I participated in the design of the study, performed the experiments, analyzed the data, and drafted the manuscript.

Paper II

I participated in the design of the study, performed the experiments, analyzed the data, and wrote the manuscript.

Paper III

I participated in the design of the study, performed the experiments, analyzed the data, and wrote the manuscript.

Paper IV

I participated in the design of the study, performed the experiments, analyzed the data, and wrote the manuscript.

Abbreviations

AMOVA	analysis of molecular variance
AOC	assimilable organic carbon
DGGE	denaturing gradient gel electrophoresis
DWDS	drinking water distribution system
EPS	extracellular polymeric substances
NGS	next-generation sequencing
NMDS	non-metric multidimensional scaling
OTU	operational taxonomic unit
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
T-RFLP	terminal restriction fragment length polymorphism
UPGMA	unweighted pair group method with arithmetic mean

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1. Introduction

The availability of safe, clean drinking water is important for public health, and will become more challenging in the future as a result of climate change (Delpla et al., 2009). Climate change can affect the availability of freshwater resources (Oki & Kanae, 2006), and can cause an increase in waterborne diseases (Hunter, 2003), for example, through the contamination of drinking water after heavy rainfall events (Rose et al., 2001). Increased temperature can increase the amount of nutrients available in surface water (Delpla et al., 2009), and the occurrence of cyanobacterial blooms reducing water quality (Paerl & Huisman, 2009, Qin et al., 2010). An increase in dissolved organic carbon has been observed in northern Europe and North America since the 1980s, and this can have an impact on drinking water production (Evans et al., 2005, Ledesma et al., 2012). The deterioration of drinking water quality may have deleterious effects on human health (Delpla et al., 2009), but it is not clear how the bacterial communities within the built environment will be affected. This is especially important since an increase in temperature is known to affect microbial communities and growth (Ratkowsky et al., 1982), as well as chemical and biological processes (Delpla et al., 2009).

Drinking water distribution systems (DWDSs) harbor complex microbial communities (Proctor & Hammes, 2015) and are complex ecosystems with a variety of environmental conditions and different habitats for bacteria (Szewzyk et al., 2000). Complex physical, chemical and microbial processes can affect the safety and quality of water in a DWDS, and being able to predict changes may help ensure good water quality (Douterelo et al., 2014a, Rose et al., 2001).

Drinking water is not sterile, and typical cell concentrations range from 10^3 to 10^5 cells mL^{-1} (Proctor & Hammes, 2015). Control of microbial growth is important for the safety of drinking water, and can be achieved through the addition of disinfectants (Berry et al., 2006) or by creating an oligotrophic environment through the reduction of nutrients, such as organic carbon or phosphate, in the distributed water (Szewzyk et al., 2000). However, disinfection byproducts can also be a health concern (Richardson et al., 2007), and ozone disinfection can increase the potential for regrowth by the formation of small organic carbon molecules that are readily available to bacteria (Hammes et al., 2006). The degradation of compounds in the drinking water, or surfaces in contact with it, can also lead to

regrowth in the DWDS (van der Kooij, 1998). In some distribution systems, especially when groundwater is used, biological stability is achieved, and it is possible to distribute the water without disinfectants, for example, in Denmark and the Netherlands (Christensen et al., 2011, Roeselers et al., 2015, van der Kooij, 1998).

According to Flemming (2002), biofilms are the most successful form of life on earth. In natural environments, bacteria are most often found in the form of biofilms (Branda et al., 2005). It has been reported that in drinking water systems more than 95 % of the biomass is located on the walls of the distribution system, and less than 5 % in the bulk water (Flemming, 2002, Liu et al., 2014). Biofilms are populations of microorganisms surrounded by a matrix of extracellular polymeric substances (EPS) (Hall-Stoodley et al., 2004). This EPS matrix protects the bacteria, making them more resistant to chemicals such as disinfectants (Flemming & Wingender, 2010). The EPS matrix consists mainly of polysaccharides, proteins, nucleic acids, and lipids, and is responsible for the mechanical stability of the biofilm and its adhesion to surfaces (Flemming & Wingender, 2010). The lifestyle of bacteria in a biofilm is entirely different from that of planktonic cells (Flemming & Wingender, 2010), and different gene expression patterns have been observed (Sauer, 2003). It has been suggested that hygienically relevant bacteria or parasitic protozoa (e.g. *Cryptosporidium*) could attach to preexisting biofilms and could survive longer while protected in the EPS matrix (Wingender & Flemming, 2011). Another example of the hygienic relevance of biofilms can be found in a study where contamination by coliforms was caused by growth on rubber-coated valves in a DWDS (Kilb et al., 2003). There is a lack of understanding on how biofilms on pipe walls can affect water quality, and how these pipe biofilms are affected by environmental conditions in DWDSs (Douterelo et al., 2014a). A better understanding of the processes and the microbial communities in DWDSs is necessary in order to understand how changes related, for example, to climate change, can affect water quality and safety.

The main goal of the work presented in this thesis was to gain a better understanding of bacterial communities in biofilms in DWDSs. In order to understand what causes problems in a DWDS, it is necessary to know what characterizes a functioning good drinking water biofilm.

The goal of the study described in Paper I was to design a workflow for next-generation sequencing (NGS) of 16S rRNA gene amplicons from drinking water biofilms. The feasibility and reproducibility of the workflow was tested by analyzing parallel-installed water meters to provide biological replicates. The composition of the bacterial community of the biofilms from water meters and pipes was compared, and it was also investigated whether the water qualities

perceived by consumers obtaining their water from the same DWDS could be resolved.

In the second study (Paper II), bacterial biofilm communities from five different pipes and the walls of a drinking water clearwell were analyzed using NGS. The growing conditions of the biofilms varied, for example, season, surface material, and source water. The aim of this study was to investigate the presence of a potential core bacterial community in these different biofilms.

Biofilms from water meters installed in buildings from two DWDSs were analyzed in the third study (Paper III) to investigate the effect of source water on the biofilm communities. The aim was to compare bacterial biofilm communities in water meters growing on the same surface material and under the same flow conditions, and to determine the potential core community from two different DWDSs. The community composition of biofilms from two water meters receiving mixed water from both DWDSs was also analyzed.

The primary goal of the study presented in Paper IV was to understand what caused the problems of red water after a change in the source water used for drinking water production. Biofilm communities from locations with and without problems of red water were compared to determine whether there was a difference in community composition. A second goal was to study the stability of biofilm communities from water meters within the same DWDS.

2. Methods used to analyze bacterial communities in drinking water biofilms

2.1 Culture-based and DNA-based methods for the analysis of bacteria in DWDSs

Water companies routinely use culture-based methods to assess the microbial quality of drinking water (Douterelo et al., 2014a). However, less than 1 % of the bacteria in drinking water is currently culturable (Amann et al., 1995, Kalmbach et al., 1997, Lautenschlager et al., 2010), and cells of usually culturable bacteria can also exist in a viable but non-culturable state, and are therefore not detectable by culture-based methods (Byrd et al., 1991, Oliver, 2005, Szewzyk et al., 2000). If cells are present in a viable but non-culturable state, cultivation methods can lead to underestimation of the amount of bacteria present in the sample and pathogens may go undetected.

The above problems can be overcome by using DNA-based culture-independent molecular biological methods. Different techniques can be used for community analysis, such as clone libraries, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) or NGS of 16S rRNA gene amplicons (Douterelo et al., 2014a, Zinger et al., 2012). The polymerase chain reaction (PCR) has become a powerful tool for the detection of pathogens, for example, in food (Malorny et al., 2003). Although DNA-based methods can be used to analyze non-culturable bacteria, they suffer from the drawback that they do not distinguish between living and dead cells. Active cells can be analyzed using RNA instead of DNA (Henne et al., 2012, Keinanen-Toivola et al., 2006, Revetta et al., 2011), and cells with intact membranes can be amplified through the addition of propidium monoazide in combination with PCR (Nocker et al., 2010). However, methods of detecting active cells are not widely used. Furthermore, the lack of cell activity in a DWDS does not mean that the cells will not become active under more favorable conditions, for example,

when more nutrients are available or when the temperature increases (Oliver, 2010).

2.2 NGS of 16S rRNA gene amplicons

The method of NGS of 16S rRNA gene amplicons provides deeper insight into the community composition than clone libraries or fingerprinting methods. The most widely used method is sequencing of different variable regions of the 16S rRNA gene using 454 pyrosequencing (D'Amore et al., 2016, Douterelo et al., 2014a, Goodrich et al., 2014, Schloss et al., 2011) (Paper I). However, this will be discontinued and replaced by Illumina sequencing in 2016 (Hodkinson & Grice, 2015). In this method, DNA is extracted from biofilm samples, PCR amplified and sequenced, resulting in around 1,000,000 sequences per run (Hodkinson & Grice, 2015). The steps involved in sample processing are illustrated in Figure 1.

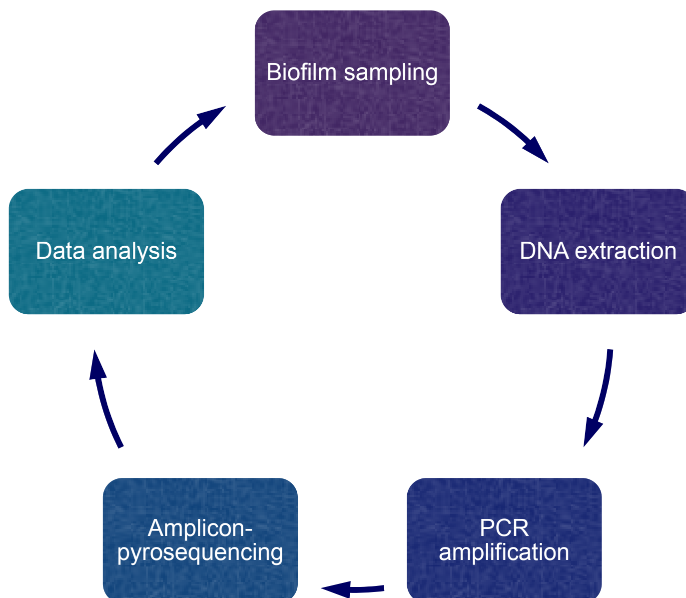

Figure 1. Steps in the analysis of drinking water biofilm communities. The figure illustrates the development of new sampling strategies after the completion of data analysis.

It should be noted that amplicon sequencing of the 16S rRNA gene, as used in the studies described in this thesis, is a method for community analysis, and is not suitable for the identification of individual pathogens in a complex environment such as drinking water biofilms. The resolution of the widely used 16S rRNA gene is not always sufficient for pathogen detection, and more specific target genes are therefore needed for the identification of some organisms (Clarridge, 2004, Janda & Abbott, 2007). Furthermore, due to the short read length, the resolution obtained with NGS methods is currently too low for the identification of bacteria at species level (Douterelo et al., 2014a, Kuczynski et al., 2012). However, the field is evolving rapidly, and longer read lengths may be possible in the near future. Results from studies using NGS have provided valuable insight into bacterial communities in DWDSs. Bacterial communities from model systems (Chao et al., 2015, Kwon et al., 2011), water samples (Pinto et al., 2014, Pinto et al., 2012), and a variety of DWDS biofilms, such as those from water meters (Hong et al., 2010), faucets (Liu et al., 2012), and pipes (Sun et al., 2014a), have been analyzed.

Very few studies using NGS have been performed on eukaryotes in drinking water such as fungi and amoeba or protozoa (Delafont et al., 2013, Douterelo et al., 2016, Liu et al., 2012), although they are an important part of the biofilm community (Buse et al., 2013). The pathogenic bacterium *Legionella pneumophila* can, for example, infect and replicate inside protozoa (Declerck, 2010). It has also been shown that nontuberculous *Mycobacteria* can survive inside amoeba (Delafont et al., 2014). 18S and ITS primers were applied to the samples from the DWDS supplying the town of Landskrona (Paper II and III), but the samples contained too little biomass, and only one sample showed a weak band for 18S, suggesting a low abundance of eukaryotes in these samples.

2.3 Influence of the methodology on microbial communities

When analyzing microbial communities using amplicon sequencing, almost all aspects of the experimental design can introduce bias and therefore influence the observed community composition (D'Amore et al., 2016). In this section the influence of the choice of protocols and analysis methods on the microbial community will be discussed.

2.3.1 Sampling

Different aspects of sampling can influence the results: a) the choice of sample, b) how the samples are collected and processed, and c) how the samples are stored.

When studying DWDSs, there is the choice of sampling from the actual DWDS or using model systems (Berry et al., 2006, Douterelo et al., 2014a, Gomes et al., 2014, Martiny et al., 2003). Samples can also be taken from the water phase or from biofilms. A variety of different surfaces can be used for sampling DWDS biofilms, such as PVC or iron in pipes (Gomez-Smith et al., 2015, Kelly et al., 2014, Sun et al., 2014a), concrete walls of clearwells (Zhang et al., 2012), brass and plastic surfaces of water meters (Hong et al., 2010), or indoor domestic installations, such as rubber gaskets in faucets (Liu et al., 2012).

Water samples are easy to collect, but they do not represent the habitat of the majority of the communities in the DWDS since more than 95 % of the bacteria are found in biofilms on the walls of the distribution system, and not in the water phase (Flemming, 2002, Liu et al., 2014). Several studies have revealed differences between communities in water samples and in biofilms (Henne et al., 2012, Liu et al., 2014, Roeselers et al., 2015). It can take several years for a stable biofilm community to become established (Martiny et al., 2003), which limits the relevance of short-term studies using model systems (Berry et al., 2006). It has also been pointed out that small-scale model systems do not represent the dynamics of diverse communities in real DWDSs (Douterelo et al., 2014a).

The choice of water or biofilm samples depends on the questions to be studied. Water samples are easier to obtain, and it is also easier to obtain replicate samples. In the present work, biofilm samples were collected from the actual DWDSs to ensure that the results reflected the conditions in a real DWDS. Samples were taken from a drinking water clearwell, eight pipes, and 29 water meters. Water meters were chosen as an alternative to pipes due to their easier accessibility. All the samples analyzed in this work were obtained from mature biofilms that had been established in the actual DWDS over several years.

Another aspect of sampling is the collection of the biofilm sample. Biofilms can be collected from pipes by removing a section of the pipe and removing the biofilm using glass beads (Wingender & Flemming, 2004), or brushing (Ren et al., 2015). Biofilm samples can also be collected with sterile cell scrapers (Långmark et al., 2005), spatulas (Gomez-Smith et al., 2015, Kelly et al., 2014), or cotton swabs (Ling et al., 2016). In this work, cotton swabs, cell spatulas and a plastic scraper were used for sampling. However, this did not allow the whole biofilm to be removed. Furthermore, samples of biofilms were collected from pipe sections remaining in the distribution system after cutting to reduce the risk of contamination with soil.

Another aspect that could influence the observed community is the way in which samples are stored (Goodrich et al., 2014). Storage conditions could for example impact DNA quality and yield (Goodrich et al., 2014).

2.3.2 DNA extraction

The DNA extraction method has been shown to influence microbial community composition (Kennedy et al., 2014, Wu et al., 2010a). Since all DNA extraction protocols introduce some sort of bias (Brooks et al., 2015), it is important to compare samples analyzed with the same methods. In this study, the Fast DNA Spin Kit for Soil (MP Biomedicals) was chosen, which has been shown to be suitable for extracting DNA from water meter biofilms (Hwang et al., 2012a). This kit has been widely used to extract DNA for studies applying NGS (Gomez-Smith et al., 2015, Sun et al., 2014a), and has also been used for complex samples such as sediments (Schippers et al., 2005). The protocol includes bead beating, which enables DNA extraction from cells with thick cell walls, for example, bacterial spores (Dineen et al., 2010).

Problems associated with DNA extraction from drinking water biofilms include the presence of PCR-inhibitory substances such as humic acids and iron, and the low biomass in the samples (Brettar & Höfle, 2008, Hwang et al., 2012a). The DNA extraction method must thus be able to remove the PCR inhibitors and must be suitable for samples with low biomass. Another important factor in the choice of DNA extraction method in the present work was the suitability for quantitative PCR (qPCR). DNA extractions should be reproducible, so they can be used to detect specific groups of bacteria using qPCR. The Fast DNA Spin Kit for Soil has been shown to give reproducible results (Hwang et al., 2012a), and has also been used for studies using qPCR (Li et al., 2010, Schippers et al., 2005).

The DNA extraction protocol of Porteous et al. (1997) and the Power Biofilm DNA Isolation kit (MO BIO) were also tested. The protocol devised by Porteous et al. (1997) has been shown to result in high-quality DNA suitable for qPCR (Bonot et al., 2010), but it was not suitable for the biofilm samples in the present work due to the low biomass. The Power Biofilm Isolation kit gave lower DNA yields, and the Fast DNA Spin Kit for Soil was therefore chosen for DNA extraction.

2.3.3 PCR

PCR amplification of the 16S rRNA gene creates millions of copies of the desired amplicons enabling NGS even for samples with a low DNA content. It has been shown that the PCR step can introduce bias into the analysis of bacterial communities (Huse et al., 2010, Pinto & Raskin, 2012).

The choice of polymerase, number of cycles, and template dilution can affect the resulting community composition (Wu et al., 2010b). When handling samples with low biomass it is not always possible to reduce the cycle number due to the low amount of DNA available. Increasing the number of cycles can increase the number of chimeras or artifacts, but has relatively little influence on the overall composition of the community (Brooks et al., 2015, Wu et al., 2010b). The choice of polymerase can influence the taxa richness and community structure (Wu et al., 2010b). Some polymerases are, for example, more tolerant to PCR inhibitors (Hedman et al., 2009), which is important for samples from drinking water biofilms. In the study described in Paper I, the inhibitor-tolerant PicoMaxx polymerase was used. This was changed to ExTaq in the other studies (Papers II-IV), as it was not possible to obtain a batch of PicoMaxx without bacterial contamination. To reduce errors in the sequences, high-fidelity polymerases with the ability for proofreading can be used. However, proofreading enzymes with low processivity can increase the formation of chimeras, and thus inflate diversity (Smyth et al., 2010). Chimeras can form in the PCR step due to incomplete primer extension, and are a combination of two or more sequences (D'Amore et al., 2016, Smyth et al., 2010). Chimeras are a serious problem in community analysis, since they can constitute up to 45 % of the PCR products (Haas et al., 2011), and have been reported to be included as bacterial species in databases (Ashelford et al., 2005). Optimized PCR conditions, including choice of polymerase, number of cycles, primer, and template concentration, can reduce the amount of chimeras formed in PCR (Smyth et al., 2010). Chimeras are difficult to detect and several programs are available for computational chimera detection and removal, such as ChimeraSlayer (Haas et al., 2011), Perseus (Quince et al., 2011), and Uchime (Edgar et al., 2011).

Contamination is an important issue when working with low-biomass samples such as drinking water biofilms and PCR (Goodrich et al., 2014, Salter et al., 2014). Sequencing of negative controls has been suggested, since contamination can originate from the laboratory environment, DNA extraction kits or PCR reagents (Salter et al., 2014). Negative PCR and DNA extraction controls were sequenced in the later studies (Papers II-IV), but no abundant operational taxonomic units (OTUs) were found in the negative controls. However, the number of shared OTUs was influenced by contamination, and this must be considered when defining a drinking water core community (Papers II-IV).

2.3.4 PCR primers

PCR primers can also influence the observed community composition due to mismatches and lack of sensitivity to certain bacterial groups (Kuczynski et al., 2012). Primer choice depends on the sequencing method and the required amplicon length, and a variety of different primer combinations are available (Klindworth et al., 2013, Kuczynski et al., 2012). It is known that "universal" primers do not capture the complete bacterial community (Baker et al., 2003, D'Amore et al., 2016, Hong et al., 2009, Klindworth et al., 2013). Different regions of the 16S rRNA gene evolve at different rates (Ghyselinck et al., 2013, Schloss, 2010) and can give different pictures of the community. The sequence variability can therefore also depend on the region of the 16S rRNA gene sequenced (Goodrich et al., 2014), making it more difficult to compare results between studies.

Samples can be multiplexed using barcodes for simultaneous sequencing of several samples in the same run. There is the choice between longer primers already containing the adaptor for the sequencing platform and the barcodes, or regular primers and ligation of the adaptor and barcodes afterwards. The longer primers have been shown to introduce bias (Berry et al., 2011) and increase the cost of sequencing. However, using long primers containing the adaptors requires less handling of the PCR products and thus reduces the risk of contamination.

In this work, the V1-V3 region of the 16S rRNA gene was chosen because it is suitable for 454 pyrosequencing, and it has been used previously, especially in studies related to drinking water biofilms (Hong et al., 2010, Liu et al., 2012, Zhang et al., 2012). The length of the amplicon is around 600 base pairs including the sequencing adaptors.

2.3.5 Sequencing and data analysis

Errors are introduced into the sequences by the sequencing platforms, and these can be misinterpreted as new species (Kuczynski et al., 2012), thus inflating diversity (Huse et al., 2010, Kunin et al., 2010, Schloss et al., 2011). Typical errors in 454 pyrosequencing are those related to homopolymers, which are stretches of nucleotides with identical base pairs (Hodkinson & Grice, 2015). Furthermore, variation has been reported between sequencing centers and different sequencing runs (Schloss et al., 2011). Quality filtering and data processing can significantly reduce the error rate (Schloss et al., 2011).

A variety of programs are available for the analysis of amplicon sequences (Escobar-Zepeda et al., 2015). The two major programs for data analysis are mothur (Schloss et al., 2009) and QIIME (Caporaso et al., 2010). Mothur was used in the

later studies (Papers II-IV) since it is a well-established, frequently updated program, which has been shown to give similar results to other programs, for example, QIIME (Plummer & Twin, 2015). A combination of different programs was used in the first study (Paper I, Supplemental Figure S1), since at that time mothur did not have as many options for data analysis as is has today. The various steps in the data analysis are described in detail elsewhere (Goodrich et al., 2014, Kuczynski et al., 2012, Schloss et al., 2011), and only a brief description of the analysis is given below, focusing on clustering and classification of the sequences.

In general, the sequences are first "denoised" to reduce sequencing errors, and then quality filtered according to different criteria, such as sequence length, quality scores and mismatches with primers and barcodes (Schloss et al., 2011). Equal numbers of sequences should be used for each sample to allow comparisons between communities, to compensate for differences in sequencing depth (Schloss et al., 2011). For the identification of microbial groups, sequences are clustered into OTUs. OTUs are based on sequence identity, and 97 % identity is commonly adopted as the level corresponding to species level (Goodrich et al., 2014). The 97 % threshold is only an estimate, and it has been shown that more than one species can be present in one OTU (Vetrovsky & Baldrian, 2013). The result is an OTU table containing the different OTUs found in each sample and the number of sequences present in each OTU. These numbers should be regarded as semi-quantitative since different organisms can contain between 1 and 15 copies of the 16S rRNA gene (Klappenbach et al., 2001) and the efficiency of amplification of these in the PCR amplification step may vary (Pinto & Raskin, 2012).

The OTU table is the basis for further analysis such as non-metric multidimensional scaling (NMDS) plots, dendrograms or heat maps (Paliy & Shankar, 2016). Errors in the construction of the OTU table will also influence the interpretation of the results. A variety of clustering algorithms are available, for example, in mothur (Schloss et al., 2009) and QIIME (Caporaso et al., 2010), or programs such as CROP (Hao et al., 2011), Usearch (Edgar, 2010), and CD-HIT (Fu et al., 2012). Clustering algorithms can produce unstable OTUs in which the memberships depend on the number of sequences analyzed (He et al., 2015). A closed-reference approach, where sequences are clustered against a database of known sequences may solve this problem (He et al., 2015). However, this seems not to be suitable for samples from drinking water, which contain many unclassified taxa that are not found in the databases. This instability was observed in the first study (Paper I), where CROP was used for clustering. Some OTUs were observed to split into several OTUs depending on the number of sequences included in the analysis (data not shown).

Classification is another important step in the data analysis. Databases may not accurately link a sequence to a specific bacterium (Clarridge, 2004). The main

databases used for classification of amplicon sequences are SILVA (Pruesse et al., 2007), RDP (Wang et al., 2007), and Greengenes (DeSantis et al., 2006). The RDP database was used in the present work as it is widely used. It has been shown that the choice of database and the training set used for classification can affect the resulting community (Newton & Roeselers, 2012). There may be difficulties associated with the classification, even at phylum level. For example, the three databases mentioned above can be inconsistent in the classification of some candidate phyla (McDonald et al., 2012).

Different classifications for different OTUs depending on the database were also observed in this work. For example, OTU 08 was assigned as unclassified *Betaproteobacteria* using the RDP database, while it was classified as the genus *Methylotenera* using the SILVA database (Paper II). This is important, because in another study on water meters using the SILVA database by Ling et al. (2016) *Methylotenera* was found to be an abundant OTU, while it was not found among the abundant OTUs when the RDP classifier was used in the present work (Paper II). This was not the case for most OTUs, and similar identifications were obtained when the mothur-formatted SILVA and RDP databases were compared (data not shown). This illustrates the need to be aware of the limitations of the data analysis when comparing results from different studies.

2.4 Replicates

The necessity of replication has been recognized in NGS studies (Prosser, 2010), and the need for replicates in drinking water studies has also been stressed (Bautista-de Los Santos et al., 2016). However, it is difficult to obtain replicates when the samples contain little biomass, and the samples are difficult to access, such as drinking water biofilms from water meters and pipes.

2.4.1 Parallel installed water meters (biological replicates)

In order to evaluate the methodology, biofilms from water meters installed in parallel were analyzed in this study (Figure 2). In apartment buildings or factories parallel-installed water meters are used to avoid interrupting the water supply when the water meters are changed. The biofilms in these water meters are true biological replicates, which have developed under the same environmental conditions. The biofilms from parallel-installed water meters at three locations were analyzed (Papers I and III). Similar heat map profiles were obtained in all three cases, and they shared between 75 and 90 % of the sequences.

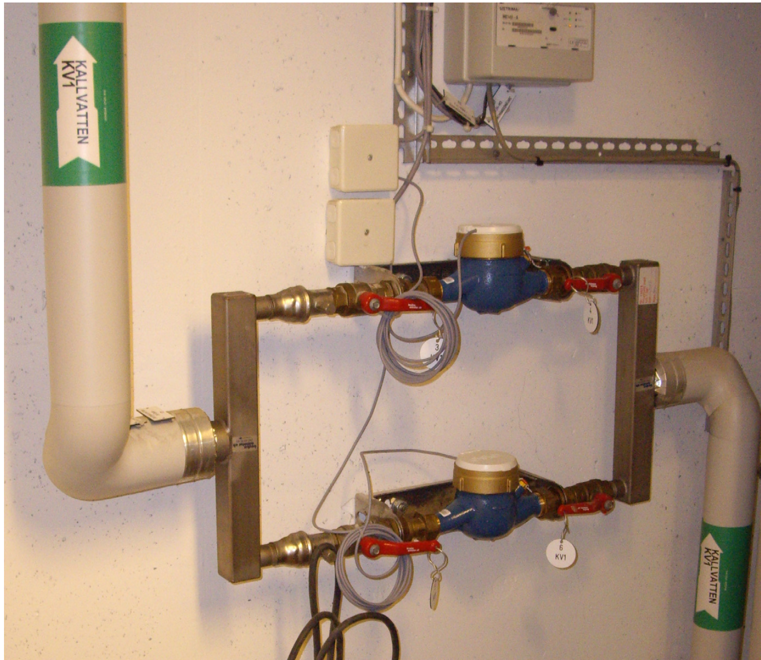

Figure 2. Parallel-installed water meters in an apartment building. Equal amounts of water flow through both water meters.

2.4.2 Template dilution

Process replicates were obtained from the clearwell sample by sequencing the same DNA three times (Paper II). Undiluted DNA was used in two of the replicates, while 10-fold diluted DNA was used in the PCR in one replicate. These three replicates shared 90 % of the sequences. The template DNA concentration seemed to have an effect on the number of singletons, which could consist of erroneous sequences or chimeras. The 10-fold diluted DNA resulted in about 100 fewer OTUs and 100 fewer singletons than the undiluted DNA (Table 4, Paper II). The composition of the community was not affected by dilution regarding the heat map or beta-diversity estimates such as the Bray-Curtis dissimilarity (Figure 2, Paper II, Figure 4, Chapter 3). Template dilution has also been found to have an effect in other studies (Chandler et al., 1997, D'Amore et al., 2016, Wu et al., 2010b). It has been suggested that different DNA dilutions should be used to achieve maximum diversity (Chandler et al., 1997), or compensate for the template concentration added to the PCR (D'Amore et al., 2016). However, this might not be feasible when working with low-biomass samples where it is difficult to measure the DNA concentration. The results from the clearwell samples indicated that

further studies of the effect of the initial concentration of the DNA may be interesting.

2.4.3 Run-to-run variation

Run-to-run variations have been reported to influence the resulting communities (Schloss et al., 2011). In this work, the sample from the clearwell was included in two sequencing runs (Papers II and III). Similar results were obtained, which were clustered together in the NMDS ordination plot (Figure 4, Chapter 3), suggesting only small variations between sequencing runs in the present work.

2.4.4 Storage of DNA

The biofilm samples from the water meters used in the first study (Paper I) were stored as frozen DNA at -80 °C for four years, and were sequenced together with samples in a later study (Paper IV). Storage of the DNA at -80 °C and the different sequencing platform seemed to have no significant influence on the community composition, as samples from the same DNA isolation clustered together in the NMDS ordination plot (Figure 3).

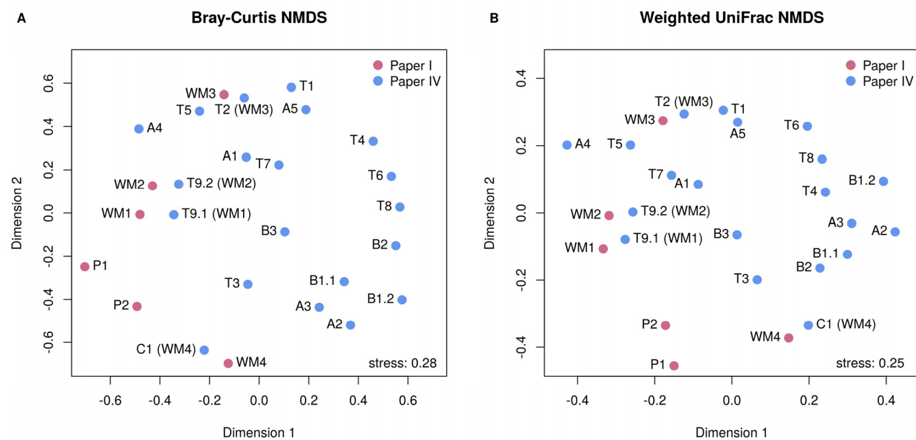

Figure 3. NMDS ordination plots based on Bray-Curtis dissimilarity and UniFrac distance for water meter biofilm communities from the DWDS of Landskrona (Papers I and IV). The samples WM1-4 were sequenced in both studies. The same DNA was sequenced on different pyrosequencing platforms after storage for four years.

2.5 Conclusions

When analyzing bacterial communities the picture obtained is not complete, but represents a window depending on the methods of sample preparation and analysis. In order to compare samples, it is very important to treat all the samples in the same way. Care must be taken when comparing results obtained in different studies, especially when different types of samples are compared, different methods have been used for sample treatment, DNA extraction or data analysis, or when different regions of the 16S rRNA gene are analyzed.

A workflow for the analysis of drinking water biofilms is presented in Paper I. The feasibility and reproducibility of the approach has been demonstrated by the analysis of communities from parallel-installed water meters, which provide biological replicates for DWDS biofilms.

3. Biofilms in DWDSs

Biofilms can grow in DWDS in a diversity of unique habitats with different physicochemical and nutritional conditions (Szewzyk et al., 2000). In order to understand the processes taking place in DWDSs, it is necessary to understand what is happening in the biofilm. Examples of processes in DWDSs are corrosion, nitrification, taste and odor formation, microbial regrowth, the occurrence of red water, and the persistence of pathogens (Berry et al., 2006, Li et al., 2010, Skjevrak et al., 2004). In this work, biofilm samples from distribution system pipes, a clearwell and water meters were analyzed. The samples originated from two DWDSs employing different treatment processes for the source water originating from different lakes.

3.1 DWDSs and water treatment plants

Different types of source water can be used for drinking water production, i.e. surface water from lakes and rivers, or groundwater. In the DWDSs studied in this work, both surface water and artificial groundwater were used as the source of raw water.

The DWDS supplying the city of Malmö in southern Sweden distributes water from the *Vombverket* treatment plant in which drinking water is produced from artificial groundwater. Water from Lake Vomb is filtered through infiltration ponds, with a retention time of approximately three months, to obtain artificial groundwater. The treatment includes aeration, softening, adjustment of pH, coagulation using iron chloride ($FeCl_3$) and rapid sand filtration. Monochloramine is used as disinfectant, formed through the addition of ammonium sulfate and sodium hypochlorite.

The *Ringsjöverket* treatment plant receives its raw water from Lake Bolmen, and the water is distributed to several towns including Landskrona, also in southern Sweden. Water from Lake Bolmen is transported through the 82 kilometer long Bolmen tunnel to the treatment plant. The Bolmen tunnel had to be repaired between April 2009 and March 2011, and during this period water from Lake Ringsjön was used instead. The steps included in the treatment are flocculation,

adjustment of pH, sedimentation, rapid sand filtration, and slow sand filtration. Chlorination is used for disinfection.

Water quality data representative of the different source waters are summarized in Table 2 in Paper II.

3.2 Clearwell biofilm

Biofilm taken from a clearwell used for storage of drinking water from the *Ringsjöverket* treatment plant before distribution was also analyzed. At the time of biofilm sampling, water from Lake Ringsjön was used as the raw water source. The bacterial community in the clearwell was dominated by *Alphaproteobacteria*, with more than 70 % sequence abundance. The family *Sphingomonadaceae* (contributing 18 % to the community) and the genus *Hyphomicrobium* (contributing 11 % to the community), were the dominant members within the *Alphaproteobacteria* (Paper II). Clearwells could be alternative sampling sites for DWDS biofilms due to their easier accessibility and the lower risk of contamination with soil compared to pipe biofilms. However, there are only a few clearwells in the studied DWDSs, limiting the number of sites available for sampling, and the flow conditions are not the same as in pipes.

3.3 Pipe biofilms

Pipe biofilms provide the most representative samples of the conditions in different parts of the distribution system, but are the most difficult samples to obtain, since the sample can only be collected when a pipe in the street is dug up and cut. This type of sample also has the highest risk of contamination with soil. Pipes in the DWDS can have a variety of surfaces such as concrete, asbestos cement, steel, cast iron, ductile iron and PVC/PE (Ren et al., 2015, Vreeburg, 2007). An even larger variety of materials is used in household installations, for example, copper, synthetic polymers, stainless steel and elastomers (Flemming et al., 2013).

For the work described in this thesis, biofilm samples were taken from eight DWDS pipes representing eight unique bacterial communities (Papers I and II). It was not possible to obtain samples from two pipes with the same surface material, during the same season, with the same source water. Therefore, it was not possible to elucidate the causes of differences in community composition between the different samples, or to estimate the variation within biological replicates from pipes. Abundant members found in the different pipe biofilms were members of

the *Sphingomonadaceae* family, the orders *Acidimicrobiales* and *Rhizobiales,* and the genera *Hyphomicrobium*, *Desulfovibrio*, *Mycobacterium*, *Nocardia, Methylibium,* and *Sulfuricurvum*.

3.4 Water meter biofilms

Water meter biofilms were analyzed, since the environmental conditions in water meters are more similar due to comparable surface materials and flows, although the bacterial communities in water meters were different from those found in pipes when comparing samples from the same street (Paper I). Water meter biofilms shared only 21-23 % of the sequences found in a pipe from the same street, despite the fact that the majority of the abundant OTUs were shared. The composition of the communities varied between water meters and pipes. For example, the family *Sphingomonadaceae* was the most abundant OTU in the two parallel-installed water meters, contributing 22-36 % to the communities, while it showed a very low abundance in the pipe, contributing only 2 % to the community (Table 4, Paper I). This difference in community structure may be due to several factors such as temperature, surface material or hydraulic conditions.

Biofilms from water meters from the two DWDSs supplying Malmö (DWDS A in Paper III) and Landskrona (DWDS B in Paper III) with drinking water were compared. The two DWDSs harbored different bacterial communities, and almost no overlap in OTUs was seen. Eleven samples included in this study shared 14 OTUs, and none of the shared OTUs had an abundance of at least ten sequences in each sample. Furthermore, it could be shown for the Landskrona DWDS that a system-specific core community existed in biofilms from water meters (Paper IV). Six of the ten most abundant OTUs were present in all samples analyzed in this study. The abundant OTUs of the core community specific to the DWDS of Landskrona were the *Sphingomonadaceae* family, genus *Nitrospira*, the *Hyphomicrobiaceae* family, the *Candidatus Pelagibacter* family, unclassified *Betaproteobacteria* and the genus *Sphingomonas* (belonging to the *Sphingomonadaceae* family) (Paper IV).

For seventeen of the eighteen sites sampled in the DWDS of Landskrona, either a high abundance (above 10 %) of the genus *Nitrospira* (5 sites) or the *Sphingomonadaceae* family (10 sites), or both (2 sites), was observed (Paper IV). It was not possible to determine the environmental conditions selecting for *Nitrospira* or *Sphingomonadaceae.*

Figure 4 shows the results of analyzing the data from water meters and pipes and the clearwell together (Papers II and III) to produce a combined NMDS plot based

on the Bray-Curtis dissimilarity. The same clearwell DNA was sequenced in both runs showing good reproducibility. The bacterial communities of the two distribution systems were statistically significantly different when data from both water meters and pipes were included in the analysis (AMOVA (analysis of molecular variance), p< 0.001).

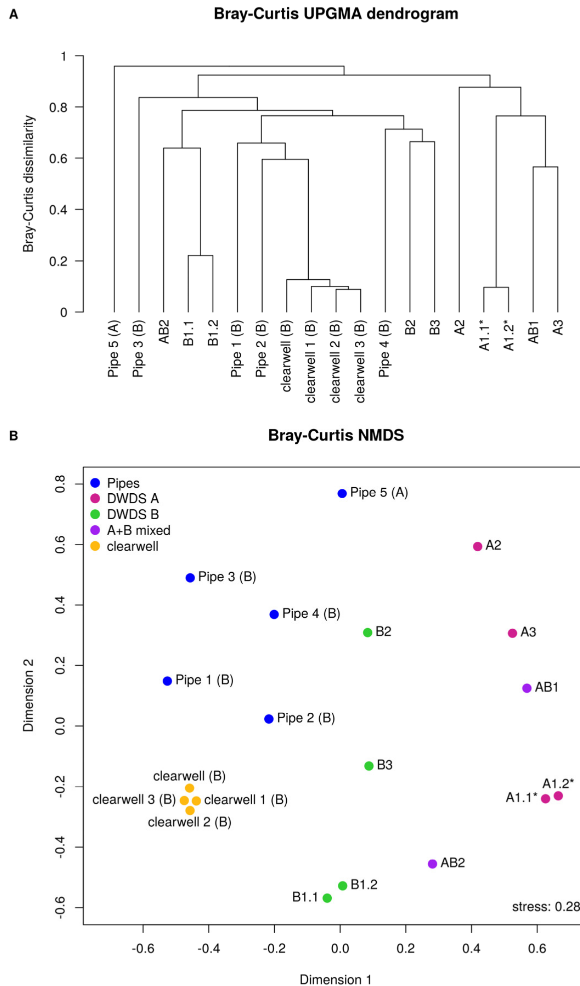

Figure 4. Dendrogram and NMDS ordination plot based on the Bray-Curtis dissimilarity for clearwell, pipe and water meter biofilm communities from the two DWDSs supplying Malmö (A) and Landskrona (B). Samples from Lund (AB) received a mixture of water from both DWDSs. DNA samples from the clearwell 1-3 (B) were sequenced in the study described in Paper II, and clearwell (B) was sequenced in the study described in Paper III. *Indicates samples with additional groundwater. (Papers II and III).

3.5 Environmental factors influencing microbial communities in DWDSs

A variety of factors influence bacterial biofilm communities, such as surface material (Yu et al., 2010), source water (Eichler et al., 2006) (Paper III), temperature and season (Henne et al., 2013, Ling et al., 2016), nutrients (Escobar et al., 2001, Szewzyk et al., 2000, van der Kooij, 1998), disinfectants (Eichler et al., 2006, Hwang et al., 2012b), hydraulic regimes (Douterelo et al., 2016, Douterelo et al., 2013), and stagnation time (Lautenschlager et al., 2010). These could explain the differences in community composition found at the different sites in different drinking water biofilms analyzed in this work, and will be discussed in more detail in the following sections.

3.5.1 Source water

To compare the results from different studies and different DWDSs it is necessary to understand the influence of the source water on bacterial communities. The effects of source water on the community composition have been reported in several studies (Eichler et al., 2006, Gomez-Alvarez et al., 2015, Roeselers et al., 2015) (Papers II and III). Different bacterial communities were found in water samples from 32 DWDSs in the Netherlands, suggesting that DWDSs could be distinguished by their microbial community profiles (Roeselers et al., 2015). It has also been found to be possible to distinguish bacterial communities from two areas in a DWDS using ground- and surface water by multivariate analysis (Gomez-Alvarez et al., 2015). In the present work, it was found that only 2-10 % of the sequences were shared between the bacterial communities from pipes receiving water from the *Vombverket* treatment plant and from the *Ringsjöverket* plant (Paper II). This was further confirmed in Paper III by analyzing biofilms from water meters with comparable conditions, such as surface material and flow. Almost no overlap in OTUs and shared sequences was found between biofilms from the two DWDSs.

Several studies have shown that the bacterial communities in the distribution system are seeded with bacteria from the flowing water (Douterelo et al., 2016, Lautenschlager et al., 2014, Pinto et al., 2012). The seeding of biofilms from flowing water was also observed in this work (Paper III). Interestingly, the communities in the biofilms from the part of the distribution system receiving mixed water were similar to communities from one of the two DWDSs (Paper III). No OTUs specific to the mixed communities were observed (Paper III). The specific environmental conditions at the sites receiving mixed water could have

selected the local biofilm community. For example, hydraulic regimes or the local temperature at the sampling site could play an important role in shaping the biofilm community (Douterelo et al., 2016) (Paper IV). The seeding of the biofilm from biological filters could be investigated for the two distribution systems in the future, since the two treatment plants apply different filtration steps. At *Vombverket* artificial groundwater is produced by filtration through infiltration ponds, while at *Ringsjöverket* slow sand filtration is employed at the end of the treatment process before the disinfection step. It was not possible to determine from the studies carried out here whether the source water or filtration influenced the community composition in the biofilms.

3.5.2 Surface material

Another important aspect affecting biofilms is the surface material, which has been shown in several studies to influence the community composition (Sun et al., 2014a, Yu et al., 2010). In the present work, biofilms were sampled from pipes with different surface materials (Papers I and II) and from water meters with surfaces of both brass and plastic (Papers I, III and IV). Hwang et al. (2012a) found different communities on brass and plastic surfaces of water meters, however, not all DNA extraction methods could resolve this difference.

Biofilms growing on different surface materials such as concrete, PVC, cast iron and ceramic lined iron, were studied (Paper II), showing differences in community composition. However, other factors such as source water, pipe diameter, and season also differed between the samples. Biofilm samples were collected from three different surface materials (the clearwell, a PVC pipe and a cast iron pipe) with the same source water, during the same season. The two pipes shared 32 % of the sequences, while the clearwell shared 34 % of the sequences with the PVC pipe and 41 % of the sequences with the cast iron pipe.

An attempt was made to collect samples from a ceramic-lined iron pipe, but no visible biofilm was observed (Paper II). When processing this sample it was not possible to obtain enough sequences for analysis. This could be an indication that the biofilm formation on ceramic-lined pipes is low, but further studies are needed to confirm this.

3.5.3 Disinfectant

Another important factor is the kind of disinfection used, which is usually chlorination, chloramination, ozonation, or UV irradiation (Richardson et al., 2007). The use of disinfectants can influence microbial communities (Eichler et al.,

2006). Differences in community composition have been observed depending on whether chlorination or chloramination was used (Gomez-Alvarez et al., 2012, Hwang et al., 2012b). Shifts in the abundance of classes within *Proteobacteria* can, for example, depend on chlorine concentration (Mathieu et al., 2009). *Alphaproteobacteria* were more sensitive to the disinfectant, while *Beta-* and *Gammaproteobacteria* tolerate higher concentrations of free chlorine residuals (Mathieu et al., 2009). Monochloramine is used for disinfection at the *Vombverket* treatment plant, while chlorination is used at *Ringsjöverket*.

3.5.4 Temperature and season

Temperature can affect the growth of microorganisms and plays a role in the different habitats in the DWDS. For example, the environment in pipes buried underground is colder than in water meters installed indoors, and the temperature of the distributed drinking water varies with the season. There is conflicting evidence regarding the effects of season on the bacterial community in the DWDSs. Season has been reported to have an effect on the bacterial community in a DWDS (Henne et al., 2013, Pinto et al., 2014), and Ling et al. (2016) found seasonal variation to be the key factor in the variation of biofilm communities from water meters. However, in other studies, the community composition of drinking water was found to be stable over several months (Eichler et al., 2006, Roeselers et al., 2015).

3.5.5 Nutrients and organic carbon

The availability of nutrients, for example, organic carbon and phosphate, can influence the regrowth of microorganisms in a DWDS and therefore influence biological stability (Szewzyk et al., 2000, van der Kooij, 1998). Assimilable organic carbon (AOC) can indicate the potential for bacterial regrowth in a DWDS (Escobar et al., 2001). The AOC content is dependent on the kind of disinfection. Ozonation, for example, can increase the AOC content by the formation of smaller, more easily degradable organic carbon molecules, leading to an increase in regrowth in the DWDS (Escobar et al., 2001).

3.5.6 Spatial variability

Henne et al. (2012) observed a stable bacterial community in water samples throughout the studied DWDS, however, spatial variability of the bacterial core communities was observed in the biofilm. Similar observations were made in the

present work, where water meter biofilms had different community compositions, despite the fact that the conditions in the DWDS seemed similar (Paper IV, samples A1-A3).

3.6 Conclusions

A variety of factors can influence microbial communities in DWDSs. One problem associated with the DWDS biofilms analyzed in this work is that there are many variables, and it is thus not possible to differentiate the effect of a single variable on the biofilm community, or to predict changes in community composition. These variables can be controlled in model systems, but such studies are often not conducted over a sufficiently long time period for a mature biofilm to become established. There is a need for more studies on biofilms in real DWDSs, since the conditions in model systems are not representative of those in real DWDSs (Douterelo et al., 2014a). When sampling biofilms in DWDSs, the factors mentioned above must be considered in order to obtain results depending on fewer variables whose effects are easier to interpret.

4. Bacterial community composition of DWDS biofilms

4.1 Bacterial diversity in DWDSs

Drinking water is far from sterile, and it has been reported that one mL of distributed drinking water can contain between 10^3 and 10^5 cells (Proctor & Hammes, 2015). The number of OTUs observed in drinking water studies can vary from hundreds to thousands (Hong et al., 2010, Kwon et al., 2011, Ling et al., 2016, Pinto et al., 2012, Roeselers et al., 2015). In the present work, diverse bacterial communities were found in biofilms from two DWDSs, with between 216 and 1,315 OTUs being detected in the analyzed samples. The majority of these OTUs consisted of singletons, and it is debatable whether they correspond to bacteria in the sample, or are sequencing or PCR errors. The observed number of OTUs was significantly reduced when rare sequences were excluded (Paper I). When comparing OTUs obtained in different studies, several factors must be considered, for example, sequencing depth, quality filtering of the sequences, clustering method, singletons, polymerase, and number of cycles in the PCR. All these factors could influence the number of OTUs, and it is therefore difficult to estimate the number of species present in a DWDS.

4.2 Core communities in drinking water

Although the number of studies on the microbial communities in drinking water is increasing, the question of whether there is a general drinking water core community is still open (Proctor & Hammes, 2015). The difference in community composition between biofilms and bulk water, and the changes in abundance and community structure resulting from drinking water treatment and distribution pose challenges in defining a drinking water core community (Proctor & Hammes, 2015). Holinger et al. (2013) found very similar communities in drinking water sampled over a large geographic distance, while Roeselers et al. (2015) suggested

that each DWDS might have its own community profile. However, water samples were analyzed in these studies. In a study where both water and biofilm samples were analyzed, a stable bacterial community composition was observed in the water samples, but not in the biofilm (Henne et al., 2012). It should be noted that the biofilm samples in that study were growing on different materials such as glass, stainless steel, PVC, copper, and Teflon (Henne et al., 2012).

In the present work, a system-specific core community was observed in water meter biofilm samples from the DWDS of Landskrona (Paper IV), but no core community was observed when samples from two different DWDSs in southern Sweden were compared (Papers II and III). The different community compositions in the two DWDSs were observed for pipe (Paper II) and water meter biofilms (Paper III). The two DWDSs varied in many respects: the source water came from two different lakes, different treatment steps were used in the plants, and the disinfection strategies were different.

4.3 Bacteria present in the DWDSs

Although no core community was found when comparing biofilms from two DWDSs in the same region (Paper III), many bacterial families or genera have been found in drinking water studies worldwide. *Proteobacteria* is the most abundant phylum in the drinking water environment, regardless of whether biofilm or water samples are analyzed, and independent of geographic area or treatment processes (Douterelo et al., 2013, Liu et al., 2012, Pinto et al., 2014, Proctor & Hammes, 2015). *Proteobacteria* was also the most abundant phylum detected in all four studies described in this thesis. However, *Proteobacteria* are very diverse, and can carry out a variety of metabolic processes (Kersters et al., 2006). The abundance of the different classes within *Proteobacteria* varies in different studies on different DWDSs (Proctor & Hammes, 2015). One explanation of this could be the influence of the source water used for drinking water production (Paper III). In the Landskrona DWDS, *Alphaproteobacteria* was the most abundant class, while this was not the case for most samples from the DWDS of Malmö (Paper III).

The three most abundant OTUs found in the two DWDSs analyzed in this work were the *Sphingomonadaceae* and *Hyphomicrobiaceae* families, and the genus *Nitrospira*; and these will be discussed in the following sections. These have also been found in other drinking water studies. Due to the diversity within the groups, more detailed analysis such as metagenomics or transcriptomics must be performed, to determine their function in drinking water biofilms. This diversity can be illustrated by the variety of habitats in which the groups can occur. All three

groups have also been isolated from marine environments (Eguchi et al., 1996, Koops & Pommerening-Röser, 2001, Moore, 1981).

4.3.1 The *Sphingomonadaceae* family

Members of the *Sphingomonadaceae* family are commonly observed in the drinking water environment (Hong et al., 2010, Ren et al., 2015, Vaz-Moreira et al., 2011). A high amount of *Sphingomonadaceae* was observed in the biofilm from a clearwell analyzed in this work (Paper II), and also in a drinking water clearwell in China (Zhang et al., 2012). The *Sphingomonadaceae* family consists of eleven genera, and they are found in different environments such as soil, corals, plant surfaces and clinical environments (Vaz-Moreira et al., 2011). The genera *Sphingomonas, Novosphingobium, Sphingopyxis* and *Sphingobium* are commonly called sphingomonads, and have the ability to grow in man-made environments such as DWDSs (Vaz-Moreira et al., 2011). The genus *Sphingomonas* alone consists of more than 20 species with diverse phylogenetic, ecological and physiological properties (Sun et al., 2013).

Sphingomonas spp. may be related to drinking water quality, as it is believed they may be responsible for initial biofilm formation (Bereschenko et al., 2010), and several strains with a high resistance to chlorine and chloramine have been isolated from drinking water (Sun et al., 2013). *Sphingomonas* spp. are widely distributed in nature (Sun et al., 2013), and are therefore adapted to a wide range of temperatures. A *Sphingomonas* strain isolated from the seawater of Alaska can, for example, grow at 5 °C, but had an optimal growth temperature between 35 and 40 °C (Eguchi et al., 1996). Another example is *Sphingomonas chlorophenolica*, with an optimal growth temperature of around 30 °C (Yang et al., 2006). The optimum growth temperature could explain the higher abundance of *Sphingomonadaceae* observed in water meters compared to pipes (Paper I). The temperature could also explain variations in biofilms from water meters at different sites if the water meter is installed at different locations in the house subjected to different temperatures, for example, the kitchen compared to the basement (Paper IV).

4.3.2 The *Hyphomicrobiaceae* family

Hyphomicrobiaceae were found in all samples from the Landskrona DWDS. The main groups identified within the *Hyphomicrobiaceae*, at genus level, were *Hyphomicrobium* and *Pedomicrobium.* A difference in classification between different variable regions of the 16S rRNA gene was observed (Paper I). The genus *Pedomicrobium* was detected when the V1-V2 region was used. Classification did not reach genus level in the V3 region, and the observed OTU was the family

Hyphomicrobiaceae, suggesting differences in resolution in the classification of *Pedomicrobium* depending on the variable region (Tables 4 and 5 in Paper I).

Hyphomicrobium and *Pedomicrobium* are budding bacteria with the ability to deposit iron and manganese oxides (Moore, 1981). *Hyphomicrobium* has been detected in pipe biofilms (Ren et al., 2015) and in a model DWDS (Williams et al., 2004). *Pedomicrobium manganicum* has been isolated from drinking water, and growth was observed in the temperature range from 13 to 35 ºC (Sly et al., 1988). The isolates were heterotrophic, required organic nitrogen, were facultative oligotrophic, and oxidized manganese but not iron (Sly et al., 1988). *Pedomicrobium ferrugineum*, however, is able to oxidize iron (Cox & Sly, 1997).

4.3.3 The genus *Nitrospira*

The second most important phylum after *Proteobacteria* found in biofilm samples from the two DWDSs studied was *Nitrospirae*. *Nitrospirae* have been observed in drinking water studies all over the world (Gomez-Alvarez et al., 2012, Martiny et al., 2003, Roeselers et al., 2015). It has recently been discovered that bacteria from the genus *Nitrospira* can carry out complete oxidation from ammonia to nitrate (Daims et al., 2015). This has also been shown for an isolate from biologically active filters used for drinking water treatment (Pinto et al., 2016), and in the groundwater well of a drinking water treatment plant (Daims et al., 2015). This finding could explain the role of *Nitrospira* in drinking water systems, and the low abundance of groups such as *Nitrosomonas* or *Nitrosospira* (Martiny et al., 2005), which were also scarce in the samples analyzed in the present work. The finding of the enzymes for complete nitrification in *Nitrospira* illustrates the need for metagenomic approaches to understand the processes carried out by certain groups of bacteria. Transcriptomics could further elucidate the role of these bacteria in the DWDS if the difficulty associated with obtaining samples could be overcome.

4.4 What is a good biofilm?

A good biofilm is one that is free of pathogens, does not cause deterioration of water quality, or lead to corrosion. Since it was not possible to determine a core community in two DWDSs geographically very close to each other, the question of what constitutes a good drinking water biofilm cannot be answered based on the microbial communities in these DWDSs. Many factors can influence the microbial community in a certain location, and it cannot be said which OTUs are good and which are not, especially as sequence reads of pyrosequencing are too short to

identify bacteria at species level. Neither is the presence of a certain family helpful in describing a good biofilm, because of the diversity within bacterial families. Since even geographically close DWDSs seem to have a system-specific core community rather than a general one, the community structure of a good biofilm must be determined for each system. Furthermore, all problems in a distribution system are not visible in the biofilm at a certain location. For example, problems related to the observation of red water were not detectable in biofilm communities, when samples were analyzed with and without the occurrence of red water (Paper IV).

5. Red water in DWDSs

Discolored or red water is a problem in many DWDSs, and it is one of the most common reasons for consumer complaints (Vreeburg et al., 2008). Discoloration is caused by changes in the distribution system leading to the mobilization of accumulated precipitates by a variety of mechanisms (Vreeburg & Boxall, 2007). Particles can originate from the drinking water itself or enter the DWDS from many sources, for example, the treatment process (Vreeburg et al., 2008). One reason for the mobilization of particles could be changes in water quality after switching the source water for drinking water production (Li et al., 2010). Red water has been observed in several places after a source water switch (Imran et al., 2005, Li et al., 2010) (Paper IV), but has also been observed when no source water switch occurred (Husband et al., 2008, Vreeburg & Boxall, 2007). Discolored water typically contains elevated levels of iron and manganese and has increased turbidity (Ginige et al., 2011, Husband et al., 2008). There is a correlation between the natural organic matter in the water and the mobilization of iron and manganese (Ginige et al., 2011).

5.1 Red water in the DWDS of Landskrona

Between April 2009 and March 2011 water from Lake Ringsjön was used as source water at the *Ringsjöverket* treatment plant instead of water from Lake Bolmen. During this period, complaints about water quality increased and red water was observed in some parts of the distribution system (Figure 5), but not in other parts receiving the same water. Complaints were mainly received from the villages around the town of Landskrona, while almost no problems were experienced in the town itself (Paper IV). After the source water was switched back to water from Lake Bolmen, the problem of red water disappeared.

Figure 5. Photograph of flowing water in a kitchen sink taken during sampling while red water occurred.

Biofilm samples from areas with and without complaints were taken before the source water was switched back to water from Lake Bolmen in an attempt to determine whether the occurrence of red water could be related to bacterial communities in the biofilm (Paper IV). Water meter biofilms were chosen as it was not possible to obtain a sufficient number of pipe samples from both areas. Water samples were deemed unsuitable, as red water did not occur all the time, and sampling took place several weeks after the last red water event at many of the sites. Sampling sites were chosen based on complaints by consumers.

Red water was observed only at one site during sampling (Figure 5). Chemical analyses showed elevated levels of turbidity, iron and manganese (Paper I). A biofilm sample from the water meter at this site had a distinct microbial community composition (Paper I), however, this could not be confirmed when more samples were analyzed (Paper IV). Thus, no correlation was found between the occurrence of red water and the community composition in water meter biofilms (Paper IV).

5.2 Microbiology

Red water events are difficult to study because they occur over short periods of time and for unpredictable reasons (Vreeburg & Boxall, 2007). The role of microorganisms in red water events is not fully understood, and knowledge on the role of biofilms in the DWDS and the process of discoloration is limited (Douterelo et al., 2013). Detachment of pipe biofilms due to cell death and increased flow could release iron and manganese accumulated in the biofilm,

resulting in red water (Ginige et al., 2011). Few studies have been conducted on the correlation between red water and microbial communities, and very few samples, between two and four samples per study, have been analyzed (Li et al., 2010, Li et al., 2016, Wu et al., 2014, Yang et al., 2014). Most of these studies dealt with the event of red water caused by a switch of the source water used for drinking water production in the DWDS of Beijing, China, in 2008 (Li et al., 2010). However, microorganisms observed in red water in China, such as *Gallionella* (Li et al., 2010) and *Limnobacter* (Wu et al., 2014), were not among the abundant OTUs in the DWDS of Landskrona (Paper IV).

A possible correlation between corrosion, biofilm and red water formation has been investigated (Li et al., 2015, Sun et al., 2014b, Zhu et al., 2014). It was found that the formation and transformation of corrosion products is closely related to the composition of the biofilm community (Sun et al., 2014b). The presence of sulfate-reducing, sulfur-oxidizing, or iron-oxidizing bacteria could increase corrosion, while iron-reducing bacteria could inhibit iron corrosion and release (Sun et al., 2014b). However, the formation of red water might not be related to corrosion. The lack of sulfate-reducing bacteria and ferric-iron-reducing bacteria in red water samples suggests that biocorrosion does not play a major role in the occurrence of red water (Li et al., 2010). This is further supported by studies from the Netherlands, where red water is observed despite the fact that the distribution system consists mainly of plastic pipes (Vreeburg & Boxall, 2007).

5.3 Water chemistry and corrosion scales

Since it is not clear whether there is a correlation between red water and biofilms in a DWDS, red water could also be explained by changes in the chemical composition of the drinking water. Imran et al. (2005) found indications that alkalinity, chlorides, sulfates, sodium, and dissolved oxygen in the source water could have an effect on the formation of red water. In the DWDS of Beijing, the new source water contained significantly higher concentrations of sulfate (Li et al., 2010). This increase in sulfate was suggested as the reason for the red water phenomenon after the source water switch, due to disruption of the stable shell of scales on the pipe surfaces (Zhang et al., 2014). In the present work, a higher concentration of sulfate was observed in the treated water from Lake Ringsjön than in treated water from Lake Bolmen (Paper II). It is very likely that the increase in sulfate was also responsible for the occurrence of red water in the DWDS of Landskrona.

It was observed that red water in the DWDS of Beijing occurred only in areas historically supplying drinking water purified from local groundwater, and not in

areas where surface water was used (Yang et al., 2012). Different corrosion scales in pipes were observed depending on the history of water passing through the pipe (Yang et al., 2012). Pipes transporting groundwater had thinner corrosion scales than pipes transporting surface water (Yang et al., 2012). In another study, corrosion scales from pipes from two DWDSs had different physicochemical characteristics (Sarin et al., 2001). The chemical stability of these corrosion scales can affect the release of iron and, therefore, the formation of red water (Yang et al., 2014). Unstable and less protective corrosion scales could lead to red water when the water quality in the DWDS changes (Li et al., 2015). An increase in sulfate concentration can disrupt the equilibrium of the corrosion scales, resulting in the release of iron, and thus red water (Yang et al., 2014). The effect was more pronounced for pipes with unstable corrosion scales and a history of groundwater, and less pronounced for pipes with more stable corrosion scales after transporting surface water (Yang et al., 2014). The biofilm could play a role in the formation of corrosion scales and influence whether the scales are thin and loose, or thick and dense (Li et al., 2015, Yang et al., 2014).

It is not known how the occurrence of red water in the DWDS of Landskrona was affected by the corrosion scales in the pipes. Both Landskrona and the surrounding villages received the same water, but only the villages were affected by problems with red water after the source water switch.

Another explanation could be hydraulic pathways and stagnation. Stagnation at the end of the DWDS could be one explanation of the red water. Stagnation can cause the slow depletion of chlorine and oxygen causing the layer on the pipe to dissolve and release red water (Zhang et al., 2014). High sulfate concentration can accelerate the dissolution of the layer on the pipe and therefore increase iron release (Zhang et al., 2014). It has been reported that stagnation has an influence on the release of iron (Sarin et al., 2004), which could explain the occurrence of red water in the villages, but not in the town.

5.4 Control measures

Various measures are available for the control of red water. Flushing of pipes to remove loose material is one means of managing problems with red water in a DWDS (Vreeburg & Boxall, 2007). Blending the water with other water sources could also help to improve water quality and reduce red water formation (Zhang et al., 2014). Another measure could be the addition of phosphate to decrease the amount of iron released (Zhang et al., 2014). This, however, might be problematic if phosphate is a growth-limiting nutrient as the addition of phosphate might increase bacterial growth in the DWDS. High levels of pH and alkalinity have also

been found to reduce the iron release rate (Zhang et al., 2014). It has also been suggested that an increase in the concentration of oxidants in the water and maintaining flow conditions could reduce iron release from pipes (Sarin et al., 2004). Ultrafiltration can also be used to reduce the amount of particles in the water (Vreeburg et al., 2008).

5.5 Conclusions

It was not possible to resolve differences between areas with and without complaints concerning red water using water meter biofilm communities. It might be that red water is caused solely by chemical changes in the DWDS, and that there is no correlation between red water and bacterial communities in biofilms. Red water could also be caused by changes in hydraulic regimes. In this case, it would be more suitable to study water chemistry, or to compare the composition of corrosion scales in pipes. According to the literature, one reason for red water could be the change in sulfate concentration, and this is very likely also the case in the DWDS of Landskrona. Model systems could be useful to recreate source water switches and to study the chemistry combined with the analysis of biofilm communities. For example, tests with experimental pipe loops using excavated DWDS pipes could be helpful in studying the effect of the new source water on the existing pipe corrosion scales (Yang et al., 2014). Since the problem was solved when the water source was switched back, it was not possible to conduct any further tests in the DWDS of Landskrona.

6. Conclusions

The main conclusions drawn from the work presented in this thesis are given below.

- A workflow for the analysis of drinking water biofilms was established. The feasibility and reproducibility of the approach was demonstrated by the analysis of biofilm communities from parallel-installed water meters to provide biological replicates. The results in Paper I show that water meters can be a good alternative for the study of DWDS biofilms. Water meters are relatively easy to access and have the same surface material, enabling comparisons between different sites.
- A surprisingly high diversity of bacterial communities was found in biofilms from water meters and pipes of the distribution systems studied in this thesis. This illustrates that the composition of the microbial community in drinking water biofilms is affected by the diversity of habitats in the DWDS.
- A variety of factors can influence bacterial growth. Bacterial communities in the samples studied differed in their composition depending on a combination of factors such as surface material, temperature, source water, and season. It could not be determined how each factor influenced the community composition in the analyzed biofilms.
- Biofilm samples from domestic water meters and pipes from two DWDSs were compared. No general drinking water core community could be identified in the two distribution systems. A system-specific core community was observed in water meter biofilms for one of the distribution systems, suggesting that the source water had the greatest effect on the biofilm community.
- When analyzing samples from sites where red water occurred, no correlation could be found between the communities in biofilms from water meters and the occurrence of red water.
- The reason for the occurrence of red water in the studied distribution system was most likely a change in the chemical parameters, more

specifically, an increase in the sulfate concentration in the new water source, and was not related to the bacterial communities in the biofilm.

7. Future studies

Pipes constitute most of the surface area in DWDSs, and more studies on pipes are necessary for a better understanding of drinking water biofilms. The influence of different conditions, such as source water, season, temperature, disinfection agents and hydraulic conditions, on pipe biofilm communities should be studied in more detail. However, it is difficult to obtain comparable pipe samples with the same properties, such as surface material, diameter, or age. It would also be interesting to study more pipe samples from different DWDSs with different treatment steps. This could confirm the results presented in Paper III, where different communities were found in water meter biofilms from two DWDSs.

To understand the relationship and the exchange between the bacterial communities in water and biofilms, more studies are required to compare bacterial communities from the water phase and biofilms. This information could be helpful in determining when water samples can be used instead of biofilm samples when studying the effects of changes in the distribution system. So far, only one study has compared the community compositions in DWDS pipe biofilms and water samples (Liu et al., 2014). In all other studies comparing biofilm communities with water samples, pipes from household installations, water meters or model systems were studied (Douterelo et al., 2013, Henne et al., 2012, Ling et al., 2016, Roeselers et al., 2015). The best way to compare water and biofilm communities would be to sample the water over a long period of time (a few weeks or months) and then compare the bacterial community to that in the biofilm from pipe sections through which the sampled water had passed.

The results presented in Paper II indicate less biofilm formation on ceramic-lined pipes. It would be interesting to investigate this further using model systems or sampling from more sites with ceramic-lined pipes.

Another interesting line of investigation would be the study of the material removed by flushing of pipes (Douterelo et al., 2014b). This would provide an easier way of accessing the more easily removable parts of the biofilm without cutting the pipe. However, it remains to be verified how much of the bacterial community originates from the water and how much from the biofilm when applying this method.

In order to obtain a better understanding of DWDS biofilms, the influence of different variables must be studied in real distribution systems, and not in model systems. The most important variables are source water, season and temperature. Understanding the influence of changes in temperature on biofilm communities would be especially important bearing in mind the effects of climate change.

More studies on the influence of source water on bacterial communities would help in the comparison of results between different studies from different geographic regions. The influence of the source water could, for example, explain inconsistent distributions among *Proteobacteria* found in different studies (Proctor & Hammes, 2015). Understanding the influence of source water on bacterial communities would also be important in areas where different source waters are blended at different ratios. The blending of source water could make the study of other variables difficult.

It has been suggested that it is not the source water, but the biological filtration step that shapes the bacterial community (Pinto et al., 2012). In the DWDSs studied in the present work, not only source water varied, but also the biological filtration steps. Therefore, it is difficult to discern the causes of the difference in the systems studied. It is therefore also important to understand how the water treatment process shapes the community in the distribution system, and to ascertain whether it is possible to include, change or remove treatment steps to improve the composition of the bacterial community in the distribution system.

Studies on the occurrence of red water might be more informative if biofilm samples from pipes were studied instead of biofilms from water meters. Since it is not clear whether there is a correlation between biofilm composition and red water, chemical analysis of the water and the corrosion scales on the pipes should be performed. Model systems with pipe sections (Yang et al., 2014) would be useful to test the effect of different parameters, and to study the effect of water quality on the stability of corrosion scales.

The function of the biofilm cannot be understood using 16S rRNA gene amplicons alone. Metagenomics could be used to learn more about the genes present in the biofilm. Metatranscriptomics should be applied to study the activity and function of the biofilm. However, the problem of too little biofilm material must be overcome before this can be done. With the improvement in sequencing technologies, lower amounts of DNA might be sufficient in the near future to apply metagenomics or metatranscriptomics to drinking water biofilms.

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