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Water and biofilm in drinking water distribution systems in the Netherlands

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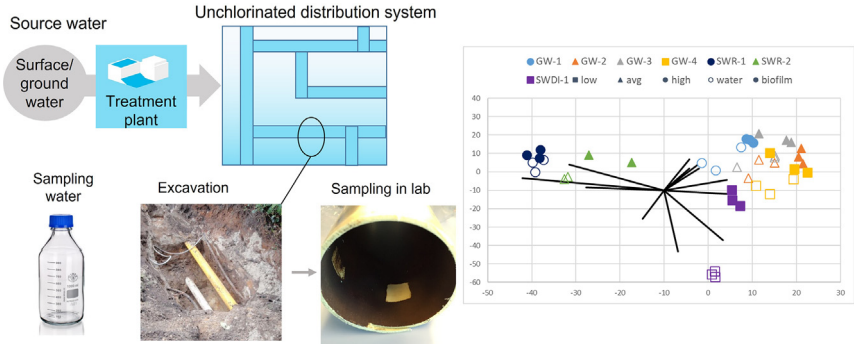
HIGHLIGHTS

- Microbiology in Dutch unchlorinated drinking water distribution systems was studied.
- Growth potential best explains the variation of the bacterial composition.
- Source of drinking water did not significantly affect bacterial composition in biofilm.
- Different pipe materials did not significantly affect bacterial composition in biofilm.

GRAPHICAL ABSTRACT



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ABSTRACT

To keep the high quality of drinking water in the future for non-chlorinated drinking water systems, knowledge about the variables that most strongly affect this quality is necessary in order to know where to focus on and possibly even change aspects of drinking water production and distribution. Therefore, the aim of this study was to investigate which variables (source of drinking water, growth potential and pipe material type) have the biggest influence on bacterial community composition and biomass concentration of drinking water and biofilm in distribution systems. Ten different distribution systems were sampled for water and biofilm, obtained from four different pipe materials, throughout the Netherlands. The distribution systems are supplied either with drinking water produced from groundwater or surface water, and differ in drinking water quality parameters such as the growth potential. We found a significant relationship for growth potential and ATP concentration in water, but for the ATP in the biofilm none of the parameters showed a significant effect. Furthermore, the source of the drinking water and the pipe material did not significantly affect the ATP concentration in water and biofilm. The bacterial composition of in both water and biofilm was significantly different between distribution systems delivering water with low and high growth potential and between drinking water produced from groundwater or surface water. In contrast, the different pipe materials did not significantly affect composition of biofilm-associated communities. We conclude from these results that the growth potential of the treated water best explains the variation in biomass and bacterial composition in water and biofilm of non-chlorinated drinking water distribution systems followed by the drinking water source, whereas pipe materials seem to be of lesser importance.

1. Introduction

Microbial growth in drinking water distribution and/or premises plumbing systems can have a negative impact on water quality. For instance, opportunistic pathogens might be able to multiply in the biofilm attached to the pipe material (van der Kooij et al., 2003), which poses a risk

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for public health. In addition, microbial growth can also result in aesthetic problems, e.g. color (Hambsch et al., 2014), taste/odor (Hoehn, 1988), growth of invertebrates visible by the naked eye (Christensen et al., 2011; van Lieverloo et al., 2012), technical problems, e.g. corrosion of pipe materials (Camper, 2014) and clogging of water meters (van der Kooij and van der Wielen, 2014). Most countries limit growth of microorganisms in drinking water by maintaining a disinfection residual during distribution. In contrast, drinking water companies in several countries, including the Netherlands, Denmark, Switzerland and parts of Germany, prevent growth of microorganisms in the distribution system by reducing biodegradable organic carbon (BDOC) in the drinking water (Rosario-Ortiz et al., 2016; van der Kooij, 2003). BDOC can be present in the treated water leaving the plant, but these biodegradable compounds can also come from pipe materials used in drinking water systems (Escobar et al., 2001). To this end, factors have been identified that could influence the drinking water quality. These include, for instance, the source of the drinking water (ground or surface water) (van der Kooij et al., 2003; van der Kooij and van der Wielen, 2014; van der Wielen and Lut, 2016), the growth potential of the finished drinking water (van der Kooij et al., 2015; Hijnen et al., 2018), hydraulics (Donlan et al., 1994; Kirisits et al., 2007; Boks et al., 2008; McClaine and Ford, 2002; Shen et al., 2015; Lehtola et al., 2006; Ollos et al., 2003; Tsagkari and Sloan, 2018; Douterelo et al., 2013; Prévost et al., 2014), and pipe materials used (Flemming et al., 2014; Prévost et al., 2014; Yu et al., 2010; Jang et al., 2011; Buse et al., 2014; Chao et al., 2015; Learbuch et al., 2021).

In the Netherlands, the source of drinking water is groundwater (oxic or anoxic) or surface water. The microbial growth potential of the finished drinking water derived from these different sources can differ significantly and can be assessed using different parameters (van der Kooij and Veenendaal, 2014). Among these, the assimilable organic carbon (AOC) concentration is probably the best-known growth potential parameter (van der Kooij and van der Wielen, 2014) and has lately been extended with the AOC-A3 test, which determines the concentration of biodegradable polymers (Sack et al., 2011; van der Kooij et al., 2015). More recently, the biomass production potential (BPP) test was developed as an improved AOC-test in which the maximum biomass concentration in a drinking water sample observed within one week of incubation is measured (van der Kooij et al., 2015; Hijnen et al., 2018). Finally, a method including concentration using ultrafiltration has been developed to quantify particulate and/or high-molecular organic carbon (PHMOC) as a measure of slowly biodegradable organic compounds (van der Kooij et al., 2015; Hijnen et al., 2018). These methods have been successfully used to determine the growth potential of drinking water in several studies and showed that a combination of the three methods, i.e. AOC-A3, BPP and PHMOC, describes the biological stability of the drinking water better than each of these parameters alone (van der Kooij et al., 2015, 2017; Hijnen et al., 2018; van der Wielen, 2018).

As outlined above, several studies have shown that different factors can specifically affect the microbial community in the drinking water ecosystem. It was for instance observed that the microbial community structure of drinking water differed between distribution systems of different treatment plants (Roeselers et al., 2015). Furthermore, Pinto et al. (2012) showed that treatment process operations affect the bacterial community composition in drinking water, which was also confirmed in another study that showed that the core microbiota in drinking water was found to be dependent on treatment strategy (El-Chakhtoura et al., 2015). A particular limitation of all these studies is that only one factor (e.g. water quality, treatment processes, pipe material, distance) was specifically addressed, which makes is difficult to determine which of the different factors has the largest influence on the microbial community composition in drinking water distribution systems. Another limitation of some of these studies is that only one distribution system was sampled, which makes it difficult to determine whether the observed results can be extrapolated to other distribution systems as well.

To be able to keep the high microbial quality of non-chlorinated drinking water or to improve this quality in the future, knowledge about which factors have the highest influence on the bacterial community composition in drinking water systems is necessary to manage regrowth problems in drinking water management. Therefore, the aim of this study was to determine which variables (source or growth potential of drinking water, material type) have the biggest influence on the bacterial community composition and biomass concentration of drinking water and biofilm in the distribution system of ten different treatment plants in the Netherlands.

2. Material and methods

2.1. Sample locations

In the summer and early autumn of 2015 (August – October 2015) ten different distribution systems throughout the Netherlands were sampled for water and biofilm. Six of these distribution systems are supplied with drinking water produced from groundwater and four distribution systems with drinking water produced from surface water. The drinking water of these ten different treatment plants differ in drinking water quality and growth potential.

The six groundwater treatment plants treat the source water with aeration and rapid sand filtration. Two surface water treatment plants (SWR-1 and SWR-2) treat the source water with coagulation, sedimentation, rapid sand filtration, UV, activated carbon and chlorine dioxide. The other two surface water locations (SWDI-1 and SWDI-2) treat the source water with rapid sand filtration, infiltration into the dunes, softening, powdered active carbon dosing, aeration, rapid sand filtration and slow sand filtration. Due to these different sources for drinking water production and the different treatment processes, the growth potential of the drinking water differed between the different treatment plants studied. We grouped the drinking water from the ten treatment plants that we sampled in three different growth potential categories; high, average and low (Table 1). These categories are based upon AOC-A3 concentrations, the maximum biomass during the first seven days of the BPP-test (BP_7) and PHMOC values (Supplementary Table A.3). In the past AOC-A3, BP_7 and PHMOC have been determined in the treated water (Hijnen et al., 2018; van der Wielen, 2018). In the distribution system of six treatment plants (three treating ground water: GW-1, GW-2, GW-3, and three treating surface water: SWR-1, SWR-2, SWDI-1) only PVC-U materials were excavated. These PVC-U materials came from three different geographic locations relative to the treatment plant (proximal, middle and distal site). In the distribution systems of the other four plants (three treating groundwater: GW-4, GW-5, GW-6, and one treating surface water: SWDI-2), different pipe materials were taken out (PVC-U, PE, cast iron or asbestos cement). These pipe materials were all sampled at different locations situated near the middle of the distribution system.

2.2. Sampling procedure drinking water and biofilm

The treated water at the plant was sampled as well as drinking water and pipe materials at three different geographic locations in the distribution system (two different locations for distribution system SWR-2). First, drinking water (1 l) at the tap was sampled after flushing drinking water from the tap until the water temperature was constant for 30 s, so that the drinking water came from the distribution system instead of the premises plumbing system. Next, a pipe segment in the same street was excavated (± 0.50 m), wrapped in sterile bags and put on ice. In total, one treated water sample, three drinking water samples from the distribution system and three pipe segments from the distribution system were obtained per treatment plant. In the laboratory, four areas (±25 cm^2 each) of biofilm on the inside of the pipe were removed using sterile swabs, as recommended by Liu et al. (2020). The swabs were added to 10 ml drinking water that was sterilized by autoclaving for 15 min at 121 °C and subsequently treated four times for 2 min of Low-Energy Sonication (LES, at 40 KHz) to release the biofilm from the swabs into the water. After sonication, the water samples with the biofilm from four areas of one pipe were pooled and further treated as one sample. At location GW-4 two different materials

Table 1
Detailed information on the treatment plants and sampling locations analyzed. GW = groundwater, SWR = surface water reservoir, SWDI = surface water dune infiltration.

Treatment Plant	Source of water	Growth potential	Materials analyzed	Sampling period
GW-1	Anoxic groundwater	High	PVC-U	August 2015
GW-2	Anoxic groundwater	Average	PVC-U	August 2015
GW-3	Anoxic groundwater	Average	PVC-U	September 2015
GW-4	Anoxic groundwater	Low	PVC-U & AC	October 2015
GW-5	Oxic groundwater	Low	PVC-U, AC & CI	October 2015
GW-6	Anoxic groundwater	High	PVC-U, PE & CI	October 2015
SWR-1	Surface water after reservoir	High	PVC-U	August 2015
SWR-2	Surface water after reservoir	Average	PVC-U	September 2015
SWDI-1	Surface water after dune infiltration	Low	PVC-U	October 2015
SWDI-2	Surface water after dune infiltration	Low	PVC-U, AC & CI	October 2015

(PVC-U and AC) were sampled in the same street, and as a result there is only one tap water sample for this specific situation.

2.3. Analytical methods

2.3.1. ATP

The ATP concentration was determined in all drinking water and biofilm samples by measuring the amount of light produced in a luciferin-luciferase assay. Briefly, a nucleotide-releasing buffer (NRB, Celsis International B.V., Maastricht-Airport, The Netherlands) was added to the sample to release ATP from the cells. The generated light signal was measured as Relative Light Units (RLU), after a 2 s delay time and a 10 s integration time with a luminometer (Celsis Advance II, Celsis). The concentration of ATP was calculated from the RLU values using a conversion factor determined in calibration measurements. The detection limit of the luminometer is 1 ng ATP l^{-1}. A more detailed description of the method to measure ATP in drinking water associated environments (including calibration curves and the use of standards) is given in CEN-EN16421. ATP concentrations in drinking water are expressed as ng l^{-1}, whereas those from the biofilm are expressed in pg cm^{-2}. In addition, ATP concentrations from drinking water and biofilm were also converted per meter of pipe using a pipe diameter of 110 mm. The statistical test Kruskal-Wallis with Bonferroni post-hoc was performed on the ATP data to determine statistical differences between samples at a p-level of 0.05.

2.3.2. DNA isolation

Water (350–750 ml) and biofilm (25–100 ml) samples were vacuum filtrated through polycarbonate track-etch membrane filters with a diameter of 50 mm and a pore size of 0.22 μm (Sartorius; Goettingen, Germany). Filters were then transferred to a bead tube of the PowerBiofilm™ DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, USA), containing 350 μl of Solution BF1 of the kit. The samples were stored at −20 °C. Samples were processed, at a different time, to isolate DNA and remove PCR inhibitors according to the manufacturer' protocol and purified DNA was finally eluted in 200 μl with elution buffer BF7.

2.3.3. Illumina sequencing of 16S rRNA gene amplicons

16S ribosomal RNA (rRNA) gene amplicons were generated using previously described 515F and 806R primers (containing Illumina adapter overhangs as described by Illumina) targeting the V4 hyper variable region of the 16S rRNA gene (Caporaso et al., 2011). Amplicons were generated in duplicate PCR reactions with 5 μl DNA extract in a reaction volume of 25 μl. Duplicate reactions were pooled and 25 μl of this mixture was cleaned, indexed and sequenced as described in the Illumina MiSeq 16S Metagenomic sequencing library preparation protocol (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf, June 2016). The final amplicon concentration loaded on the MiSeq system was 4 pM supplemented with 10% PhiX (Illumina; San Diego USA) for control and to add diversity. Negative controls were included in every experiment to monitor the presence of contaminating DNA.

Version 1.37.0 of the MOTHUR software package (Schloss et al., 2009) was used to process all MiSeq datasets using the procedure previously described (Kozich et al., 2013) and summarized in the MiSeq standard operating procedure (http://www.mothur.org/wiki/MiSeq_SOP, June 2016). The sequences were filtered by removing sequences containing ambiguous bases and sequences with an average quality score below 35. Paired-end reads were first assembled into contigs. The quality of the filtered reads and the assembled reads was further improved by (i) removing sequences containing homopolymers of minimally eight nucleotides, (ii) removing sequences with ambiguous bases and (iii) by using a 1% precluster error. The remaining sequences were aligned to the SILVAv128 16S RNA gene sequence database (Pruesse et al., 2007), and chimeric sequences were removed using UCHIME (Edgar et al., 2011). The retained high-quality reads were clustered into operational taxonomic units (OTUs), with an identity cut-off of 97%. The R package ampvis2 was used to generate abundance heatmaps at class and genus level (Andersen et al., 2018). The program PRIMER-e V7 (www.primer-e.com) was used to determine differences in bacterial community composition between samples by calculating Bray-Curtis dissimilarities, which were subsequently used as input for principle coordinates analysis (PCoA). A permutational multivariate analysis of variance (PERMANOVA) was done on the constrained axes used in ordination, using a *p*-value of 0.05 as cut-off value for statistical significance.

3. Results

3.1. Influence of drinking water source and growth potential

We analyzed whether the drinking water source and/or growth potential had an impact on the active biomass (ATP) and the bacterial community composition. The average ATP concentrations in the treated water were all lower than 6 ng l^{-1} (Table 2). These values are lower than 10 ng l^{-1} ATP, which has been suggested as an attention value for drinking water in the distribution systems in the Netherlands and above which possible regrowth problems can be expected (van der Kooij and Veenendaal, 2014). The average ATP concentration of the drinking water in the distribution system varied between 1.5 and 69.1 ng l^{-1}, with four of the six distribution systems showing an average ATP concentration higher than 10 ng l^{-1}. There was no significant difference found between ATP concentrations in drinking

Table 2
Average ATP concentrations (± standard deviation, SD) in drinking water and biofilm on PVC-U material per distribution system and the different samples at the three different locations within each distribution system. TP = treatment plant; DS = distribution system.

Distribution system	Drinking water (ng/L)		biofilm
	TP	DS	pg/cm^2
GW-1	5.9 ± 0.4	44.4 ± 65.5	265.4 ± 46.2
GW-2	2.4 ± 0.1	14.9 ± 17.2	326.2 ± 55.5
GW-3	5.6 ± 0.3	14.2 ± 2.4	1623.5 ± 2296.8
SWR-1	0.6 ± 1.2	69.1 ± 114.6	490.6 ± 422.7
SWR-2	2.7 ± 1.3	4.5 ± 1.1	1255.8 ± 1002.5
SWDI-1	1.2 ± 0.0	1.5 ± 0.8	83.5 ± 42.1

water produced from ground water or surface water (Kruskal-Wallis, $p > 0.05$). The ATP concentrations in drinking water that has a low growth potential were significantly lower than in drinking water with an average or high growth potential ($p < 0.05$). The ATP concentrations in drinking water with average or high growth potentials did not differ significantly from each other ($p > 0.05$).

The average ATP concentration in the biofilm on PVC-U materials ranged between 83.5 and 1623.5 pg cm^{-2} (Table 2). Similar to the ATP concentrations in drinking water, the ATP concentrations in the biofilm were not significantly different between the different sources used for drinking water production (Kruskal-Wallis, $p > 0.05$). The ATP concentrations in the biofilms were also not significantly different between the distribution systems fed with drinking water having a different growth potential (Kruskal-Wallis, $p > 0.05$) This was in contrast to the ATP concentrations in the drinking water. The results, thus, showed that the growth potential of the drinking water significantly correlates with the ATP concentration in water but not in the biofilm.

Next to the biomass determination, the bacterial community composition was determined using 16S rRNA gene sequencing. Differences in bacterial communities were examined by analyzing the alpha and beta diversity. The results showed that the alpha diversity (expressed as the Shannon index) did not differ between the distribution systems investigated (Supplementary Table A.1). The beta diversity between the bacterial communities of the water and biofilm samples was visualized with a PCoA analysis and results showed that the drinking water samples from plants using groundwater as source (GW-1-4) clustered together, but apart from treatment plants using surface water as source (Fig. 1). In addition, drinking water samples from the distribution systems of plants that used surface water without dune infiltration (SWR-1 & 2) clustered together as well, but apart from the distribution system fed with drinking water produced from surface water treated with dune infiltration (SWDI-1) that clustered separate from all other treatment plants (Fig. 1). PERMANOVA showed that the bacterial community in drinking water differed between treatment plants ($p < 0.05$), with pair-wise comparisons showing that SWDI-1 differed significantly from SWR-1 and from SWR-2. PERMANOVA also showed that the source of the drinking water (groundwater vs. surface water) had a significant effect ($p < 0.05$) on the bacterial community composition. Finally, it was also observed that the communities were significantly different in drinking water with a low and high growth potential ($p < 0.05$), but did not differ significantly between low and average growth potential or between average and high growth potential.

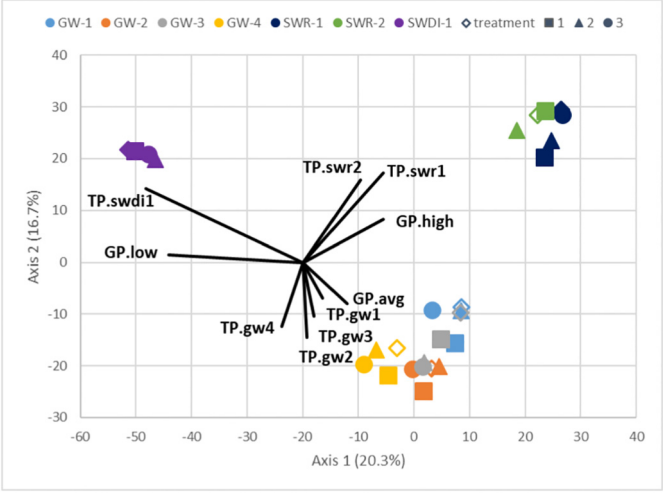

Fig. 1. Principal Coordinates Analysis (PCoA) plot of the OTU composition in drinking water samples from different treatment plants, based on pairwise Bray Curtis dissimilarities. The environmental variables are displayed as vectors and vector abbreviations are as follows: GP = growth potential; TP = treatment plant. Percentages given for both axes indicate the relative amount of variation explained. Samples were taken at the treatment plant (treatment) and at three locations in the distribution system (different symbols).

PCoA of the water samples thus showed three significantly different clusters that relate to the source and the growth potential of the drinking water. Additional analyses showed that different bacterial genera could be observed for these three clusters as well (Fig. 2). For instance, the bacterial group *Acidobacteria,* subgroup 7 and *Proteobacteria*, TRA3–20 (candidate division) had a higher relative abundance in samples from treatment plant SWDI-1 than for the other plants. Likewise, *Sediminibacterium* and *Pedomicrobium* were most predominant in samples from treatment plant SWR-1. Furthermore, *Acidobacteria*, subgroup 6 was less abundant in the samples of treatment plants SWR-1 and SWR-2 compared to the samples of the other treatment plants. Another observation was that some genera had much higher relative abundance in the treated water of treatment plant GW-3 than in the distributed drinking water. This was mainly due to higher relative abundance of *Limnobacter* and *Nitrospirae*, 0319-6A21 (candidate division) in the treated water, but not in the distributed drinking water. Such clear differences between treated and distributed water were not observed for the other plants.

The comparison of the bacterial community composition in the biofilm on PVC-U materials showed a similar clustering as was observed for the water samples with the four groundwater treatment plants clustering together, but separate from SWR-1 and SWR-2 (that clustered together), and SWDI-1 (which clustered separately form the other treatment plants) (Fig. 3). The bacterial communities differed significantly between the different treatment plants, the source of the drinking water and between distribution systems fed with drinking water that had a low or high growth potential (PERMANOVA, $p < 0.05$). The different biofilm samples within the distribution system from each of the plants GW-1, SWDI-1 or SWR-1 had bacterial communities that clustered closely together. SWR-2, GW-3 or GW-4 had each one biofilm sample from the distribution system showing a community composition that differed from the other two biofilm samples of the same distribution system.

An analysis of the 30 most abundant genera in the PVC-U biofilm samples showed that some specific bacterial genera could be observed for certain clusters of treatment plants (Fig. 4). The genus *Nitrospira* seemed to have a slightly more relative abundance in the biofilm samples of SWR-1 compared to the rest, whereas *Chloroflexi,* KD4–96 (candidate division) was only observed among the top 30 genera in the two biofilm samples from the distribution system of treatment plant SWR-2. It was observed that the relative abundance of *Gemmatimonadetes*, BD2–11 (candidate division) was higher for the biofilm samples from the distribution system of the four groundwater treatment plants compared to treatment plants using surface water as a source.

We also analyzed the water and biofilm samples together to determine whether the bacterial community composition differed between these two different matrices. PCoA of the OTU distribution showed that the drinking water samples of all treatment plants clustered separately from the biofilm samples (Fig. 5) and these differences were statistically significant (PERMANOVA, $p < 0.05$). The largest distance between drinking water and biofilm samples was observed for distribution system SWDI-1 and the lowest distance for distribution system SWR-1.

The relative abundance of the genera *Legionella*, *Pseudomonas* and *Mycobacterium*, all known to contain opportunistic pathogenic species, was examined to determine whether these genera were more water- or biofilm-associated (Supplementary Table A.2). The relative abundance of the three genera were in general lower in the biofilm than in the drinking water samples, indicating that these genera might be more water than biofilm related. However, absolute numbers should be determined as well, before such a conclusion can be made. Furthermore, not all three genera were observed in all drinking water samples. The genus *Mycobacterium* was in general characterized by higher relative abundance in drinking water than *Pseudomonas* and *Legionella*. In the PVC-U biofilm samples, the relative abundances of all three genera were comparable to each other.

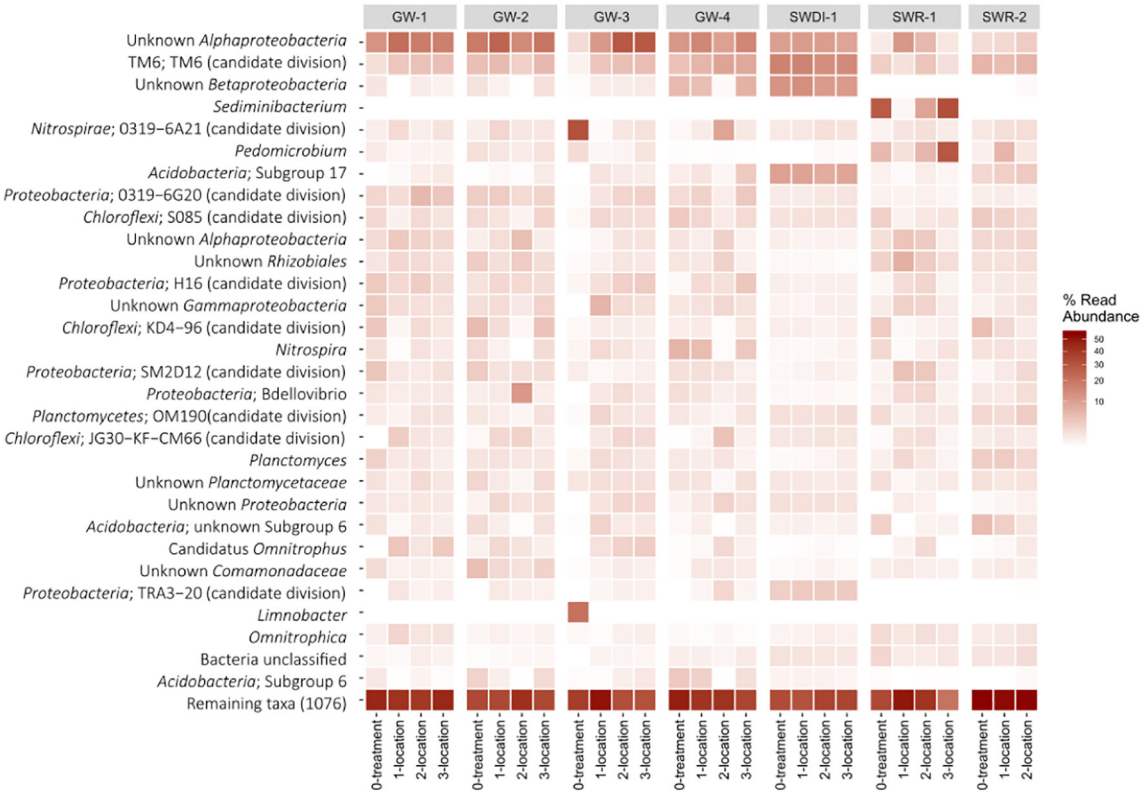

Fig. 2. Relative abundance of the top 30 bacterial genera in drinking water sampled at the treatment plant and three locations in the distribution system of seven different treatment plants in the Netherlands.

3.2. Influence of materials

To determine the effect of materials on bacterial biomass and community composition, only biofilm samples were analyzed. The mean ATP concentrations in the biofilm of different materials ranged between 31 and 3091 pg cm^{-2} (Table 3). The mean ATP concentration in the biofilm of AC materials was 1224 ± 1153 pg cm^{-2} (range: 98–3091 pg cm^{-2}; n = 5). The mean ATP concentration in the biofilm of CI materials was 1103 ± 1375 pg cm^{-2} (range: 221–2687 pg cm^{-2}; n = 3). The mean ATP concentration in the biofilm of PVC-U materials was 390 ± 456 pg cm^{-2} (range: 31–1150 pg cm^{-2}; n = 6).

PVC-U had often the lowest biofilm concentration compared to the other pipe materials sampled within one distribution system, whereas, cast iron had usually the highest biofilm concentration of the materials sampled within one distribution system. However, ATP concentrations in the biofilm did not significantly differ between the different pipe materials (Kruskal-Wallis, $p > 0.05$). This lack of statistical significance, is probably because other factors that differ between the distribution systems, have a bigger influence on the ATP-concentration in the biofilm (e.g. source of the drinking water or growth potential).

The results of the bacterial community analyses showed that the alpha diversity (expressed as the Shannon index) did not differ between the different material types (Supplementary Table A.1). PCoA and PERMANOVA based on the OTU distribution showed no significant difference between the bacterial community composition in the biofilm from the different materials ($p > 0.05$). Furthermore, biofilm samples from the distribution system of the treatment plant GW-5 or SWDI-2 clustered separately from the biofilm samples from the distribution system of the other treatment plants (Fig. 6). PERMANOVA showed that these differences between distribution systems were significant ($p < 0.05$). In addition, the community composition of the biofilm samples from the distribution system where drinking with a low growth potential was distributed, differed significantly from those where drinking water with a high growth potential was distributed ($p < 0.05$, PERMANOVA). These significance findings support the findings described in paragraph 3.1, i.e. the significant influence of treatment plant and growth potential on the bacterial community composition in the biofilm.

An additional taxonomic analysis at genus level demonstrated that *Acidobacteria*, subgroup 6 was specific for the biofilm on the pipe material cast iron in the distribution system of treatment plant GW-5 and GW-6 (Fig. 7). For each of the other pipe materials (PVC-U, PE, asbestos cement) no specific bacterial genera were observed in the biofilm samples. However, candidate division OYR10d3 from phylum *Proteobacteria* was more abundant in the biofilm on the materials PVC, PE and asbestos cement than in the biofilm on cast iron. The observations that there are not many specific bacterial genera in the biofilms of each material type is consistent with PCoA and

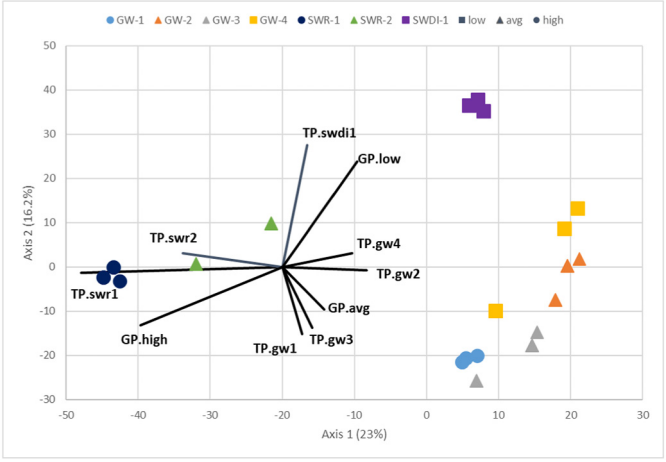

Fig. 3. Principal Coordinates Analysis (PCoA) plot of the OTU composition in biofilm samples on PVC-U from different treatment plants, based on pairwise Bray Curtis dissimilarities. The environmental variables are displayed as vectors and vector abbreviations are as follows: GP = growth potential and TP = treatment plant.

Fig. 4. The relative abundance of the top 30 bacterial genera in PVC-U biofilms sampled at three locations in the distribution system of seven different treatment plants in the Netherlands.

PERMANOVA, which showed that pipe material did no significantly affect the community composition in the biofilm.

4. Discussion

4.1. Influence of drinking water quality

Previous studies have shown that drinking water quality influences the associated microbial communities (Chao et al., 2015; Yu et al., 2010; Liu et al., 2014; Henne et al., 2012; Roeder et al., 2010; Learbuch et al., 2019, 2021). Studies on unchlorinated drinking water systems in the Netherlands showed for instance that the bacterial community composition of unchlorinated drinking water, loose deposits and biofilm samples differed between three drinking water distribution system that were fed with drinking water having a different growth potential (Vavourakis et al., 2020). Especially the distribution system fed with drinking water having a low growth potential differed most strikingly from the other two distribution systems by the exclusive presence of *Pseudonocardia* in the biofilm and the absence of *Limnobacter* in the water and loose deposits during summer. Another study showed, however, that the bacterial community composition of the pipe wall biofilm and of the loose deposits largely determined the bacterial community composition in the drinking water sampled at the tap (El-Chakhtoura et al., 2018). Based on their results, these authors suggested that the impact of water source and treatment strategy on the bacterial community composition is minimal/less important. Furthermore, it was observed that pipe materials have an influence on the bacterial community when in contact with unchlorinated drinking water (Learbuch et al., 2021).

A limitation of these previous studies is that they did not include factors (e.g. growth potential, source or pipe material) that directly affect the

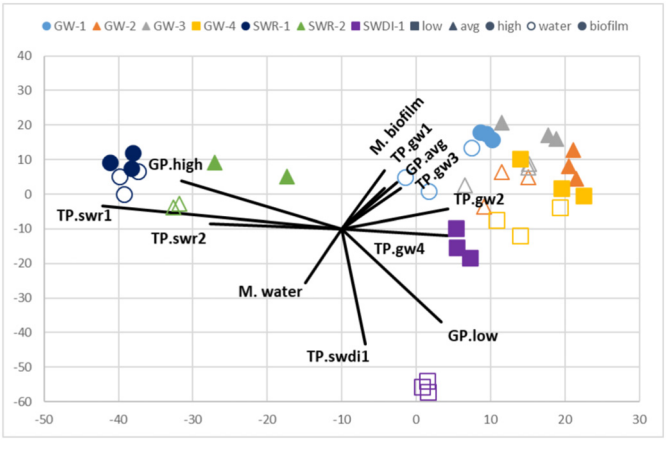

Fig. 5. Principal Coordinates Analysis (PCoA) plot of the OTU composition in drinking water and PVC-U biofilm samples from different treatment plants, based on Bray Curtis dissimilarities. The environmental variables are displayed as vectors and vector abbreviations are as follows: GP = growth potential and TP = treatment plant.

Table 3
Mean ATP concentrations (± standard deviation; SD) in the biofilm of each excavated pipe material for the distribution systems where different pipe materials were sampled.

Distribution system	Material	Biofilm (± SD) pg cm^{-2}
GW-4	PVC-U	1150.1 ± 5.3
	AC	3091.3 ± 164.2
	PVC-U	174.2 ± 2.9
	AC	475.7 ± 28.5
	PVC-U	34.3 ± 0.7
	AC	1162.0 ± 18.5
GW-5	PVC-U	30.8 ± 4.1
	AC	98.4 ± 8.4
	CI	220.9 ± 28.4
GW-6	PE	508.7 ± 0.4
	CI	2687.3 ± 160.0
	PVC-U	152.9 ± 4.6
SWDI-2	CI	401.0 ± 13.8
	PVC-U	209.4 ± 5.1
	AC	1294.3 ± 67.1

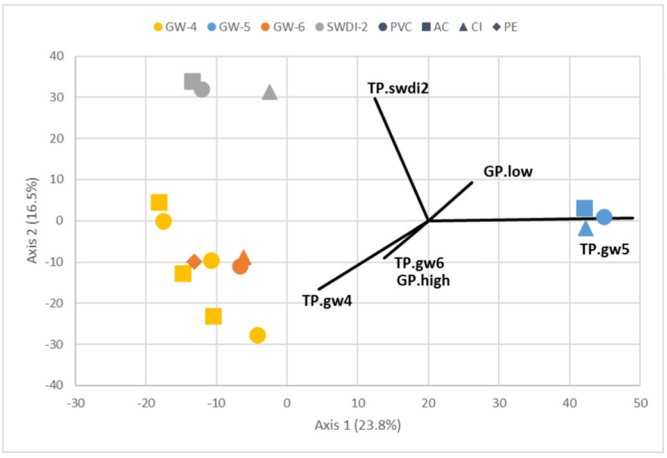

Fig. 6. Principal Coordinates Analysis (PCoA) plot of the OTU composition of biofilm samples from PVC-U (PVC), PE, cast iron (CI) and asbestos cement (AC) materials taken from the distribution system of different treatment plants, based on the Bray Curtis dissimilarities. The environmental variables are displayed as vectors and vector abbreviations are as follows: GP = growth potential and TP = treatment plant.

nutrient concentration used by bacteria to grow in drinking water distribution systems. Consequently, it remains unclear which of those factor(s) play (s) the dominant role in determining the bacterial community structure in distributed drinking water and biofilm. The results of our study showed that the source of the drinking water (surface water versus groundwater) and the growth potential of the treated water, but not the pipe material, had a significant effect on the bacterial community composition of both drinking water and biofilm. In addition, the biomass concentration in the drinking water was only significantly affected by the growth potential of the treated water, but not by the source or pipe materials. In addition, the biofilm concentrations we observed on PVC-U pipes were higher than those found with the BPP-test for materials (Hambsch et al., 2014), which underlines that biofilm biomass in the distribution systems studied was related to nutrients from the drinking water and not from the pipe material.

We conclude from these results that of the three factors investigated, the growth potential of the treated water explains most of the variation in biomass concentration and bacterial community composition, whereas pipe materials had the lowest impact.

4.2. Influence of pipe materials

In our study the biofilms developed on four different pipe materials, namely PVC-U (n = 6), AC (n = 5), CI (n = 3) and PE (n = 1), were studied. Because PE is not often used as a distribution pipe material in the Netherlands (approximately 7% of the distribution system), we were only able to sample one PE pipe. Our result that pipe material had no significant effect on the bacterial community composition and biomass between different pipe materials was in contrast with the results from a previous study from our group (Learbuch et al., 2021). The apparent differences between these two studies might have been caused by several experimental differences between our previous and current study. An important difference is that in our current study drinking water and biofilm samples were taken from full scale distribution systems. In the other study, the effect of pipe materials on bacterial community composition was investigated with new and thoroughly cleaned materials under laboratory conditions. Another aspect is that in the previous study the experiments were performed with the same drinking water quality taken from one tap. In our field study, we took samples from different taps in different buildings within and from different distribution systems. Hence, when pipe materials are studied among other factors under field conditions, different conclusions can be drawn than when studied as single factor under laboratory conditions. This is an important finding to consider when studying the influence of a given variable on the microbiological drinking water quality. Furthermore, as our current study has shown that the growth potential of the drinking water has a significant effect in shaping the bacterial community in drinking water systems, research determining the influence of different factors on drinking water microbiology should not be done in a single distribution system, because then only a single growth potential and water quality is included. Up till now, numerous studies were performed in a single distribution system and authors often draw generalized conclusions extrapolated to all drinking water distribution systems (Eichler et al., 2006; El-Chakhtoura et al., 2015; Lautenschlager et al., 2013; Liu et al., 2016;

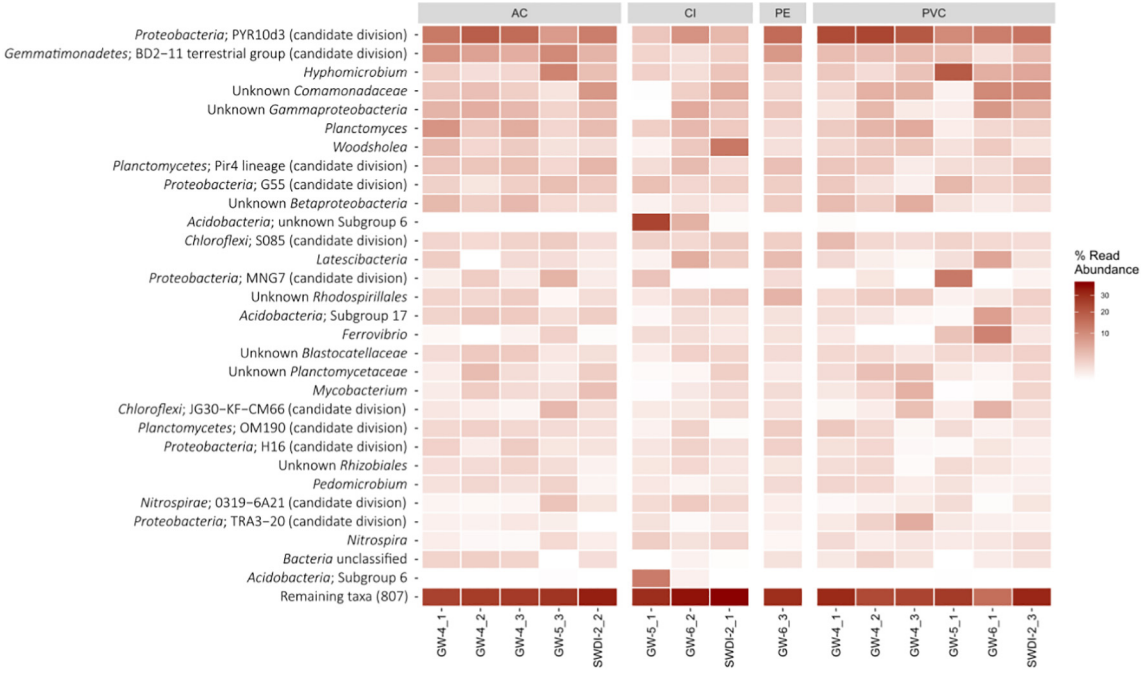

Fig. 7. The relative abundance of the top 30 bacterial genera in PVC-U (PVC), PE, cast iron (CI) and asbestos cement (AC) biofilms sampled in the distribution system of four different treatment plants in the Netherlands.

Lührig et al., 2015; Pinto et al., 2012; Revetta et al., 2016). Consequently, another important finding from our study is that phenomena observed in a single distribution system do not necessarily occur in other distribution systems where drinking water with for instance a different growth potential or quality is distributed.

Several studies have determined which phase (water versus biofilm) contains most of the biomass in drinking water systems. Flemming (2002) found that more than 95% of the entire biomass is in the biofilm and less than 5% is in the water phase. Liu et al., 2014 demonstrated that the bulk water bacteria (including suspended solids) contributed less than 2% of the total bacteria, whereas bacteria associated with loose deposits and biofilm accounted for over 98% of the total bacteria. Also van der Wielen and Lut (2016) demonstrated in their study, where they sampled three PVC-U pipes from full-scale distribution systems, that most of the biomass was found in the biofilm and sediment.

We also observed that the ATP concentration was always higher in biofilm than in water and that the bacterial communities from the different matrices (water and biofilm) at the same location were different from each other. From these comparisons between water and biofilm we conclude that analysis of the bacterial community of drinking water does not hold much information on the bacterial communities dominantly present in the distribution system (i.e. biofilm). As a result, it remains difficult to draw conclusions about the bacterial biomass and composition in drinking water distribution systems based on drinking water analysis only, without taken the biofilm into account. The latter was also observed by Vavourakis et al. (2020) and Roeselers et al. (2015), who both showed that biofilm communities where different from drinking water communities. These findings in our study and previous studies are in contrast to El-Chakhtoura et al. (2015) who observed that the bacterial community in drinking water at one location in the distribution system differed from the community measured at the treated water at the plant. Although, these authors have not determined the biofilm or particle-associated bacterial community, they hypothesize that the bacterial community in the distributed drinking water samples was most likely influenced by the biofilm and particle associated communities. Considering the results from our study as well as the other two studies mentioned above, it is highly unlikely that this hypothesis is correct. The difference between the study of El-Chakhtoura et al. (2015) and our and both other studies is that El-Chakhtoura et al. (2015) only sampled one location from one distribution system, whereas the other studies sampled multiple locations from different distribution systems. Moreover, the distribution system studied by El-Chakhtoura et al. (2015) was also studied by Vavourakis et al. (2020) and our study, and both more elaborated studies could not confirm the results found by El-Chakhtoura et al. (2015). From the results of our study and previous studies (Wullings and Van Der Kooij, 2006; van der Wielen et al., 2013; van der Wielen et al., 2013; Van der Wielen et al., 2016) we can conclude that only analyzing drinking water is not sufficient to describe the microbiology in drinking water distribution systems, because the biofilm microbiology (including possible regrowth problems related to biofilm) is missed. An indicator organism for biofilm microbiology that is also present in drinking water is, therefore, needed. Furthermore, our results show again that it is important that drinking water field studies should sample multiple locations from different drinking water distribution systems to be able to draw reliable and scientifically sound conclusions on drinking water quality. Locations should also be chosen where hydraulics are different, and the locations should be sampled at different periods during the year.

4.3. Comparison of drinking water with or without disinfectant residual

Several studies also determined the microbial community of drinking water with or without a disinfectant residual (e.g. El-Chakhtoura, 2015 & 2018; Lautenschlager et al., 2013; Roeselers et al., 2015; Vavourakis et al., 2020; Eichler et al., 2006; Henne et al., 2012; Ji et al., 2015; Lührig et al., 2015; Pinto et al., 2012; Potgieter et al., 2021; Revetta et al., 2016). These studies showed that the microbial community in chlorinated, chloraminated and non-chlorinated drinking water is overall rather similar at the phylum level. The phyla reported by these previous studies were also observed in our study. This indicates that at low taxonomic resolution microbial communities in the drinking water distribution system comprise the same bacterial phyla, regardless of the presence or absence of a disinfectant residual. However, when focusing on a higher taxonomic resolution, some genera are present in the communities in drinking water with or without a disinfectant residual, whereas others are only present in either chlorinated or non-chlorinated drinking water. For instance, the following genera were found to be present in both non-chlorinated and chlorinated drinking water: *Flavobacterium*, *Fluviicola*, *Legionella*, *Limnobacter*, *Nitrospira*, *Pedomicrobium*, *Planctomyces*, *Polaromonas* and *Pseudomonas*. Besides different genera in drinking water with or without a disinfectant residual, these studies also showed that samples from a distribution system fed with drinking water without a disinfection residual are more diverse than from those fed with drinking water with a disinfection residual.

When we compare our results with results from studies that also sampled non-chlorinated drinking water, we observed both similarities as differences in the observed genera. For instance, *Nitrospira*, *Pseudonocardia*, candidate division OM190 from phylum Planctomycetes and candidate divisions Subgroup 6 &17 from phylum *Acidobacteria* that were observed in our study, were also found by Vavourakis et al. (2020). Likewise, *Nitrospira* and *Planctomyces* were both found in our study and the study by Liu et al. (2016). Other genera (e.g. candidate divisions G55 and H16 both from family *Desulfurellaceae*, *Pedomicrobium*) found in our study were, however, not observed in the other studies that investigated the microbial communities in drinking water distribution systems fed with drinking water without a disinfectant residual (El-Chakhtoura, 2015 & 2018; Lautenschlager et al., 2013; Roeselers et al., 2015). This indicates that those genera might be specific for the distribution systems we sampled or for the time period in which we sampled in our study.

Specifically looking at several genera known to comprise opportunistic pathogens showed no noticeable effect of the source of drinking water production or the growth potential of the drinking water. However, we did not specifically determine whether pathogens were present in these unchlorinated drinking water distribution systems. Such studies have been done in the past and although low numbers of *Pseudomonas aeruginosa*, *Aspergillus fumigatus* and *Stenotrophomonas maltophilia* were sporadically observed (van der Wielen and van der Kooij, 2013), most of the *Legionella* and *Mycobacterium* species identified in these earlier studies were not-yet cultivated species for which there are no indications that they are involved in human infections (Wullings and Van Der Kooij, 2006; van der Wielen et al., 2013).

The observation that bacterial communities differ between distribution systems fed with unchlorinated drinking water produced from different sources or with a different growth potential doesn't necessarily mean that this is a problem. Studies have shown that not so much community composition but rather the biomass concentration has negative effects in unchlorinated drinking water systems, on for instance the presence of opportunistic pathogens (van der Wielen and Lut, 2016; van der Kooij et al., 2017) and invertebrate animals (van der Kooij et al., 2017). When there are regrowth problems in a distribution system, the most effective strategy might thus be to minimize the growth potential of the water as this lowers the biomass concentration in the drinking water distribution systems (van der Kooij and Veenendaal, 2014). Still, it is important to stress that the exact influence of the bacterial community composition on regrowth problems is still unknown. For instance, it has been shown that biofilm concentrations in drinking water systems relate to numbers of *Legionella pneumophila*, because host protozoans feed on this biofilm (van der Kooij et al., 2017). Some studies, however, have suggested that these host protozoans seem to prefer certain bacterial taxa (e.g. *Betaproteobacteria*) (van der Kooij et al., 2017, 2018; Shaheen and Ashbolt, 2021), indicating that the bacterial community composition can indirectly affect L. *pneumophila* in drinking water distribution systems. Furthermore, Wang et al. (2013) hypothesized that the microbiome of the drinking water can be used to control opportunistic pathogens via their ecological interactions. This observation led these authors to conclude that probiotics approaches, that e.g. take advantage of

competition of ecological niches, encourage growth of antagonists to knock-out keystone species, could control growth of nuisance microorganisms in the distribution system (Wang et al., 2013). These observations and hypotheses thus emphasize the need to better study the relationship between regrowth problems and microbial community compositions in drinking water distribution systems.

5. Conclusions

Our study showed that the growth potential of the treated water best explains the observed variation in bacterial community composition in water and biofilm of non-chlorinated drinking water distribution systems followed by the drinking water source (groundwater vs surface water), whereas pipe materials seem to be of lesser importance. In addition, the biomass concentration in the drinking water was only significantly affected by the growth potential of the treated water, but not by the source or pipe materials. Furthermore, the comparison of our data with previous studies reinforces the importance to sample multiple locations from different drinking water distribution systems in drinking water field studies to be able to draw reliable and scientifically sound general conclusions on factors that affect the bacterial ecology and drinking water quality in drinking water distribution systems.

CRediT authorship contribution statement

K.L.G. Learbuch: Conceptualization, Investigation, Software, Data curation, Formal analysis, Visualization, Writing – original draft, Validation. **H. Smidt:** Conceptualization, Writing – review & editing. **P.W.J.J. van der Wielen:** Funding acquisition, Conceptualization, Formal analysis, Data curation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154940.

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