Long-Term Influences of Pipe Material on Bacterial Communities of Matured Biofilms (>40 Years' Old) in Drinking Water Distribution System

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Abstract: Pipe material is important for biofilm in drinking water distribution system. However, there is controversy in the literature that studies debating if pipe material shaped the composition and diversity of bacterial communities. To study the long-term influences of pipe material on biofilm, the matured biofilm on PVC-U, grey cast iron, asbestos cement (> 40 years' old) was sampled, in triplicates for each material, from three areas of an unchlorinated drinking water distribution system in the Netherlands. The Illumina sequencing results showed that there were 773 OTUs detected (730 OTUs - 814 OTUs) within biofilm on the three pipe materials, which were all dominated by Proteobacteria (36.2 - 46.1%). Both the alpha and beta diversity results showed that the bacterial communities of biofilm formed on different pipe materials were highly similar. The neutral community model revealed that the assembly of biofilm communities is governed by environmental selection rather than neutral process. Among the 142 shared OTUs between water and biofilm, there are 25 OTUs enriched (e.g. OTU7, assigned as *Nitrospira* spp.) which accounted for 62.6% of the sequences, while 16 OTUs disadvantaged (e.g. OTU14 and OTU40, assigned as Hyphomicrobiaceae) which accounted for 2.2% of the sequences. The harmonizing process, which means biofilm with significant differences driven by the pipe material developing towards biofilm with similar quantity and community over time, is proposed and discussed. Our finding offers valuable insights on the long-term biofilm development, which bridged the essential gap of the current conflicted debates regarding the influence of pipe material, highlighted the importance of long-term study, and pointed out the potentially masked harmonizing process during the biofilm development over years/decades.

Keywords: pipe material; biofilm communities; environmental selection; harmonizing effects; long-term influences

1. Introduction

Biofilm formed on the inner surface of drinking water distribution pipe is a complex mixture of microbes, organic and inorganic material accumulated with microbially-produced polymeric matrix (Flemming and Wingender 2010; Menaia and Mesquita 2004). Regardless the maintenance of disinfectant residuals or not, the formation of biofilm is unavoidable and unwanted in drinking water distribution systems (DWDSs) (Liuet al. 2020; Van der Kooij 1999; Van Der Wendeet al. 1989), because it is the reservoir for (opportunistic) pathogens (Wingender and Flemming 2011), may cause microbial corrosion (Beech and Sunner 2004), and continuously releasing microbes into bulk water (Chanet al. 2019), especially during the switching of supply water quality (Chenet al. 2020; Liuet al. 2017b). Therefore, biofilm has attracted increasingly research attention over the last decades. For example, studies have covered both pilot and full-scale distribution systems on the biofilm formation potential (Okabeet al. 2002; Van der Kooij 1999), the quantity and community of biofilm (Liuet al. 2017a; Liuet al. 2016a), the (opportunistic) pathogens in biofilm (Feazelet al. 2009; Septemberet al. 2007; Wingender and Flemming 2011) and the key factors for the development and management of biofilms (Doutereloet al. 2013; Hwanget al. 2012; Proctoret al. 2017; Sunet al. 2014; Tsvetanova and Hoekstra 2009; Yuet al. 2010).

Typically, the pipelines of DWDSs have a length of tens to several hundreds of kilometers, e.g. 0.4 million kilometers in the Netherlands (Vreeburg and Boxall 2007), 1.1 million kilometers in China, while 20k kilometers water pipelines in the city of Beijing (Development 2022). As such, there is no doubt that the material of those distribution pipes contact with drinking water is important regarding its potential contribution to water quality deterioration and energy consumption (Brooet al. 2001). This is especially true when considering its significant influences on planktonic bacterial growth and biofilm formation (Van der Kooij and Veenendaal 2001; Wenet al. 2015). However, there is controversy in the literature regarding

how pipe material can affect microbial communities of biofilm with studies debating if pipe material shaped the composition and diversity of bacterial communities. Some researchers found significant differences in bacterial communities among biofilm formed on different pipe material (Kerret al. 1998; Proctoret al. 2016; Wanget al. 2014a; Yuet al. 2010), while others found similar bacterial communities among biofilm formed on different pipe material (Gomez-Smithet al. 2015; Henneet al. 2012; Inkinenet al. 2014). Though valuable knowledge has been obtained, the critical differences in the scale (pilot vs. full scale), duration (days vs. years) and sampling strategies (flushing vs. swabbing) of reported studies make it impossible to have reasonable cross comparisons to draw a solid conclusion.

Since the opportunities to sample biofilm from field distribution systems are limited, most of the reported studies used model distribution networks and removable coupons for short periods from days to months (Berryet al. 2006; Wanget al. 2014a; Wanget al. 2014b) or taken faucets and water meters as alternatives for sampling field DWDSs biofilm (Honget al. 2010; Liuet al. 2012). The limitations of such studies have been clearly illustrated and well noted by long-term (3 years) studies with model system (Martinyet al. 2003), field studies of mature biofilm (> 20 years) (Gomez-Smithet al. 2015; Henneet al. 2012), and the clear influences of hydraulic regimes (Doutereloet al. 2013). Whereas, the study period of 3 years is still too short comparing to the mature biofilm in field DWDSs. The field studies of mature biofilm in Germany focused mainly in a small distribution zone within the campus (7 out of the 8 samples), which attributed the similarity of biofilm on different pipe material to the fluence of adjacent biofilm communities (Henneet al. 2012).

In this study, to investigate the long-term influences of pipe material, planktonic bacteria and mature biofilms (> 40 years) were sampled from different pipe materials in three distribution areas supplied with same drinking water treatment plant, including PVC-U, asbestos cement (AC) and gray cast iron (GCI). Our finding offers valuable insights on the long-term influence

of pipe material on biofilm in drinking water distribution system, which brought forward the understanding of biofilm development, highlighted the importance of long-term study, and pointed out the potentially masked harmonizing process along the bacterial community succssion over years.

2. Material and Methods

2.1 Description of the drinking water supply system

At Katwijk treatment plant of Dunea, Den Haag, the Netherlands, the source water is transported for 30 km to a dune area of natural lakes for natural infiltration. After an average residence time of 2 months, the infiltrated water is extracted and post treated by softening, powdered activated carbon filtration, aeration, rapid sand filtration, and slow sand filtration before being pumped into the distribution system. Chlorination and usage of disinfectant residuals are avoided in the Netherlands.

2.2 Sampling program

As illustrated in Figure 1, planktonic bacteria were sampled at treatment plant and three distribution sites (TP, L1, L2 and L3, n=4), while biofilm samples were taken from three distribution areas in triplicates (n=9). The pipe material in L1 is unplasticized polyvinyl chloride (PVC-U), in L2 is asbestos cement (AC), in L3 is gray cast iron (GCI), the pipe diameter in all three locations is 110 mm and the pipe age is 42 years, 58 years and 50 years respectively. For planktonic bacteria sampling, 500 mL water was collected at each sampling point. For biofilm sampling, three sections (length = 30 cm) were cut from each distribution point to sample the biofilm. Two sections were swabbed immediately after cutting the distribution pipes, with a swabbing area of approximately 10 cm^2 at least 5 cm from a cut end to minimize the risk of biofilm disturbance or contamination from the chop saw. One section was sealed with pre-disinfected caps and filled with 1L of DNA-free water (Millipore) to keep the inner surface wet during transport. All samples were stored at 0˚C and transported to the laboratory as quickly as

possible (< 4 hours). To detach the bacteria from the biofilm, the pipes were pre-treated by ultrasonication for three periods of two minutes each at 42KHz(Magic-Knezev and van der Kooij 2004). The obtained suspensions were used for further DNA extraction and sequencing.

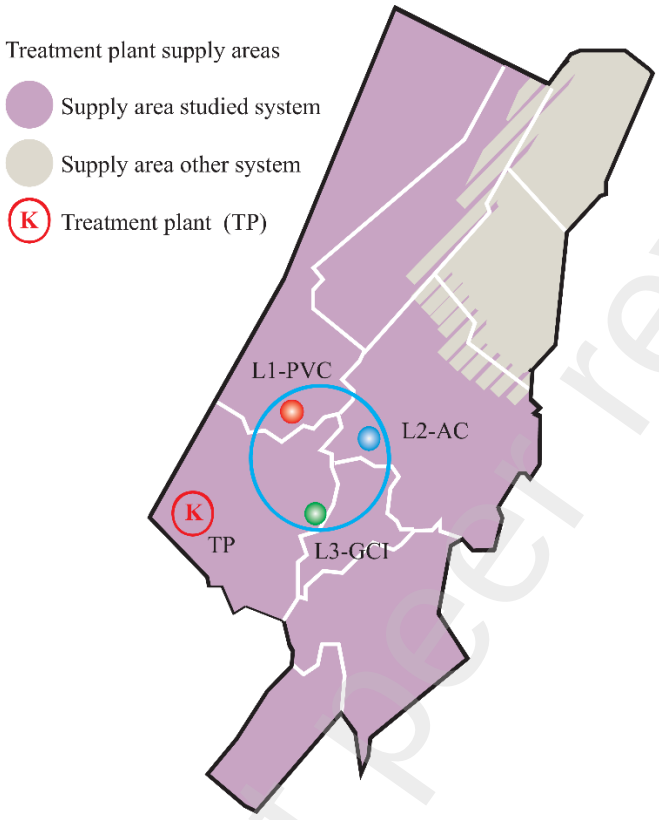

Figure 1. *Layout of the distribution area and sampling locations. L1, PVC-U pipe (1978, 42 years old); L2, AC pipe (1962, 58 years old); L3, GCI pipe (1970, 50 years old).*

2.3 DNA extraction, illumina sequencing, and data processing

The water samples and obtained suspensions biofilm samples were filtered through 0.2 µm polycarbonate membrane filters (Whatman, UK). DNA was recovered from filters or rayon swabs using the FastDNA Spin Kit for Soil (Q-Biogene/MP Biomedicals, Solon, OH, USA), following the manufacturer's instructions (Hwanget al. 2011; Tamakiet al. 2011). V3-V4 region was amplified with the bacterium-specific forward primer 341F (5'-CCTACGGGNGGCWGCAG-3') and the reverse primer 805R (5'-GACTACHVGGGTATCTAATCC-3')(Proctoret al. 2018). The sequencing was performed on

the Illumina Life Sciences GS FLX series genome sequencer (Roche, Switzerland). The obtained DNA sequences were deposited in the DDBJ sequence read archive (Accession Number: PRJNA648471).

The sequences generated from the Illumina Miseq analysis of the 16S rRNA gene amplicons were processed (i.e., filtered, clustered, and taxonomically assigned and aligned) using the Quantitative Insights Into Microbial Ecology (QIIME2, v2018.6) pipeline with the default settings (Bolyenet al. 2018; Caporasoet al. 2010). Raw sequences were first processed using DADA2 (Callahanet al. 2016), including quality filtering, denoising, paired-end sequence merging, and chimera filtering. DADA2 generated unique amplicon sequence variants that were equivalent to 100% similarity operational taxonomic units (OTUs) in the conventional practice. In this publication, we still use the term OTU for the purpose of simplicity (referred as Feature elsewhere). Taxonomy was assigned using q2-feature-classifier (Bokulichet al. 2018), customized for the primer set used in this study with Silva SSU database release 132 (Quastet al. 2012). Multiple sequence alignment and phylogenetic tree construction were performed using the QIIME 2 plugin q2-phylogeny. Alpha and beta diversity analyses were performed using the QIIME 2 plugin q2-diversity.

Weighted and unweighted UniFrac distance matrices were constructed from the phylogenetic tree and used to conduct a principal coordinate analysis (PCoA) (Liuet al. 2014b). The dominant OTUs are defined as the OTUs with a defined cut-off of relative abundance (>1%) within each phase/pipe. The significance of beta diversity differences among different sample categories was determined by the PERMANOVA test in QIIME2. Differences were considered significant when the p-value was lower than 0.05 ($p < 0.05$). Venn diagrams exhibiting the similarity of the microbial populations among distinct sample categories were drawn with VennDiagram package in R (3.5.3).

2.4 Neutral community model (NCM)

To explore the contribution of neutral processes and environmental selection on community assembly of the filter communities, an evolved NCM following null hypothesis was performed (Morriset al. 2013). Specifically, the bulk water samples were considered to be the source community, whereas the biofilm samples were the local target communities. The empirically observed frequency of detection was expressed as the number of biofilm samples, in which a target OTU was detected over the total number of biofilm samples. In the implementation of this model, only shared OTUs between target and source communities were employed. Consequently, the expected frequency of detection in the target communities, which were present via dispersal and ecological drift, was calculated following a beta probability distribution (Sloanet al. 2006). The neutral model was constructed by 95% binomial confidence intervals based on the Wilson method with the Hmisc package in R (Morriset al. 2013). Theoretically, OTUs that fell between the confidence intervals were considered to be a result of neutral dynamics of stochastic births and deaths within the local communities and stochastic immigration from the source communities, according to the neutrality assumption. OTUs falling outside the upper or lower bound of the confidence interval were detected at disproportionately higher or less frequencies in the local communities than predicted by the neutral model, based on their relative abundance in the source communities, which are advantaged or disadvantaged by the local environment (Vignolaet al. 2018).

3. Results

In total, 333,660 sequences were generated from the 13 samples (4 water and 9 biofilm), which were assigned as 10431 OTUs. The rarefaction curves reached a plateau after 4,000 sequence reads were obtained, indicating that enough sample coverage was obtained in this study (Figure S1).

3.1 Number of observed OTUs

Figure 2 shows the number of OTUs observed from water and biofilm samples. On average, 1205 OTUs (n=4) were observed in water samples, which was much higher than that observed in biofilm samples (773 OTUs, on average, n=9). For the biofilm formed on different pipe material among the three locations, 814, 775 and 730 were observed for AC (L2), PVC-U (L1) and GCI (L3), respectively, in descending order. The different observations between water and biofilm were statistically significant, while the differences among biofilms on different material were not significant.

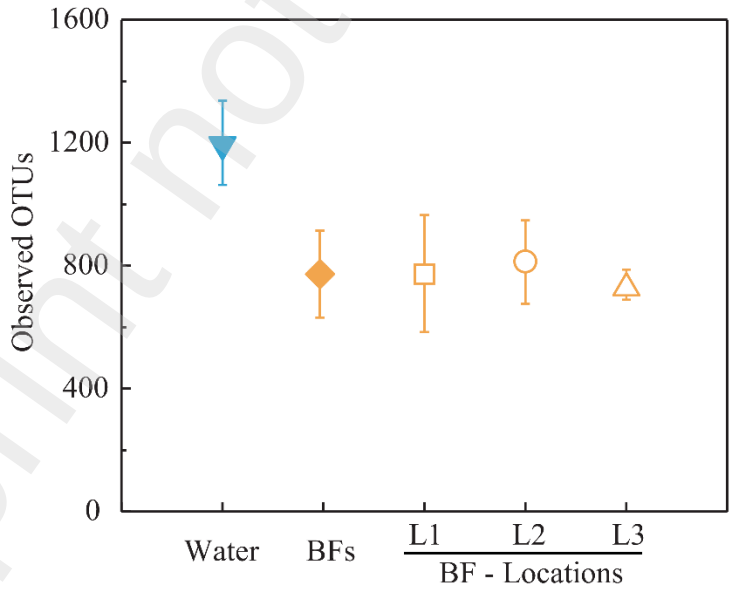

Figure 2. *The number of observed OTUs in water samples from all locations (n=4) and biofilm sampled from pipes of different material (L1 – PVC, n=3; L2 – AC, n=3; L3 – GCI, n=3).*

3.2 Bacterial community composition

At phylum level, both water and biofilm samples were dominated by Proteobacteria, the relative abundance of which was higher in the biofilm (36.2 - 46.1%) than that in the water (15.5 - 25.2%) (Figure S2). The community of water bacteria was dominated by, in descending order, OD1 (16.4 - 20.1%), OP3 (3.0 - 3.6%), Acidobacteria (1.9 - 2.3%), Planctomycetes (1.6 - 2.2%), Nitrospirae (0.9 - 2.8%), Chlamydiae (1.0 - 2.4%), Bacteroidetes (0.8 - 2.3%) and TM6 (0.5 - 1.5%). For biofilm, the bacterial community was dominated by Planctomycetes (5.4 - 11.1%), Acidobacteria (3.2 - 6.3%), Actinobacteria (1.5 - 5.7%), Nitrospirae (2.2 - 4.4%), Chloroflexi (2.0 - 3.7%), OD1 (0.7 - 4.1%) and Gemmatimonadetes (0.9 - 3.0%). It is remarkably to observed that, at phylum level, there were minor differences among the biofilms on different pipe material of PVC-U, AC and GCI.

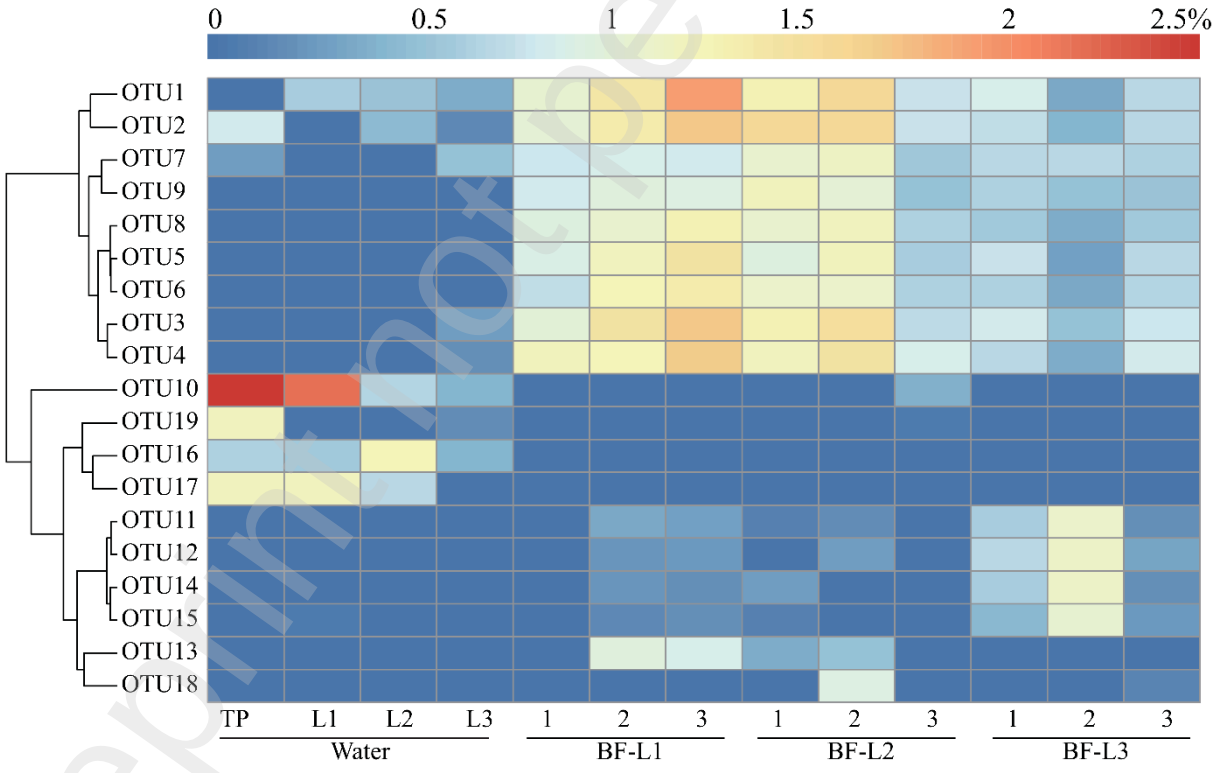

Figure 3. *Heatmap shows the dominant OTUs and their relative abundance in all samples. The complete list of their relative abundances and taxonomy information was shown in Figure S3.*

There were 19 core OTUs detected in the water and biofilm samples (Figure 3, Table S2). In

water, OTU10 (f_Hyphomicrobiaceae) and OTU16 (f_Hyphomicrobiaceae) were the most dominant OTUs (relative abundance, 0.5 - 2.7%; occupancy, 100%). The OTU16 was only detected in water, but not in the biofilms on any pipe materials. For biofilms across all locations and pipe materials, the core OTUs including OTU1, OTU2, OTU3, OTU4, OTU5, OTU6 and OTU8 that assigned to class of Gammaproteobacteria (relative abundance, 0.4 - 2.1%; occupancy, 100%), and OTU7 and OTU9 that assigned to *Nirospira* spp. (relative abundance, 0.6 - 1.2%; occupancy, 100%). The OTU5, OTU6, OTU8 and OTU9 were detected only biofilms, but not in water.

Comparing the dominant OTUs among biofilms formed on different materials, most of the dominant OTUs were shared by all pipe material (13/17 OTUs, Venn gram Figure S4). There were 2 OTUs (OTU10, OTU19) presented only in biofilm formed on AC pipe, but not detected in biofilms on the PVC-U and GCI pipes. Similar with the observation at phylum level, there was minor differences of dominant OTUs among the biofilms formed on PVC-U, AC and GCI pipes, indicating minor effects of pipe material on the biofilm formation.

3.3 Bacterial community similarity

PCoA plot based on UniFrac distance showed clearly the two clusters of water and biofilm (Figure 4, Table 1, $p < 0.05$). The bacterial communities of water samples showed clear distance with each other, suggesting clear variations of bulk water bacteria among the sampling locations. Moreover, it is especially clear that all biofilm samples clustered closely together, indicating the high reproducibility of the obtained results (the triplicate samples from each pipe material), and the little influences of pipe material on bacterial community of biofilm, which agrees with the observations on the above presented bacterial community composition.

The beta diversity results of biofilm samples from different locations and different sampling strategies are represent in a PCoA plot (Figure S3). The bacterial communities of biofilm samples are still having little variations between different locations with very low variation

explanation in the PCoA plot which we can see the differences from the Venn gram (Figure S4) are OTU10, OTU13, OTU18 and OTU19. A comparison of biofilm samples collected by pipe specimen ultrasound and swabs indicate the variation for different sampling strategies in the same section ($p < 0.01$, PERMANOVA test by QIIME2). For the swab samples, the biofilm swap samples are clustered together in different locations showed the reproducibility of swap samples.

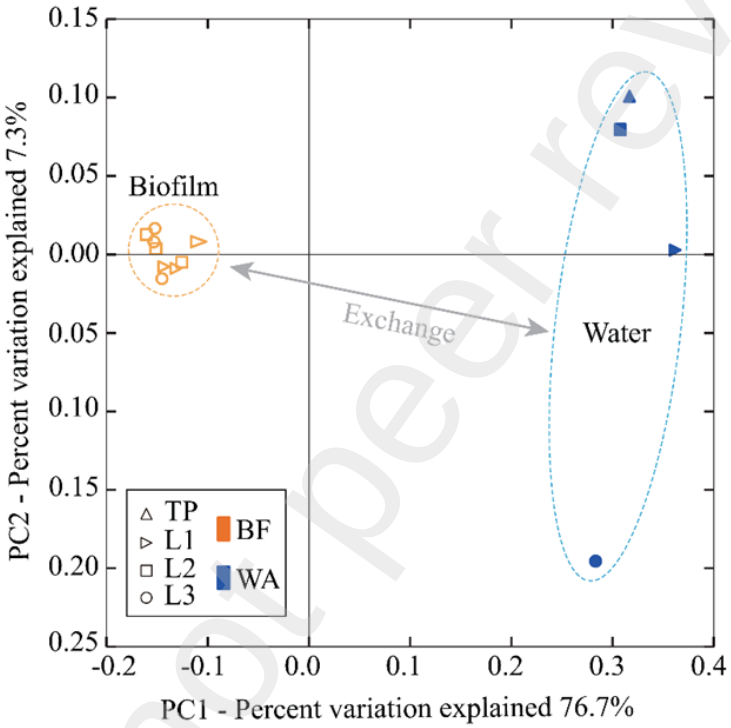

Figure 4. *PCoA plot generated using unweighted UniFrac distance matrix showing the microbial community distribution of different sample categories.*

Table 1. Influences of Pipe Material tested by Two-Way ANOSIM

	Group 1	Group 2	*p* value
Tests	BF	WA	0.04
	L1	L2	0.39
	L1	L3	0.10
	L2	L3	0.10

3.4 Effects of neutral processes on biofilm microbial community assembly

Between the 4694 OTUs and 5879 OTUs detected in water (over three weeks' time) and biofilm samples (> 40 years old), there were only 142 shared OTUs that accounted for 2.4% of number of observed OTUs and 13.2% of the total sequences in the biofilms. To further explore the microbial community assembly within biofilm, those 142 shared OTUs were used to calculate the probability of detecting the OTUs in the biofilm due to the neutral processes, e.g. dispersal and ecological drift (Table 2, Figure 5). Though the number of neutral process droved OTUs accounted for 71.1% of the number of shared OTUs, they accounted for just 35.2% of the shared sequences. The 28.9% of the number of shared OTUs that were environmental selected accounted for 62.6% of the shared sequences, including OTU3, OTU4, OTU7, OTU10, and OTU11 which had a relative abundance >1% and occupancy of 100%. Moreover, the goodness-of-fit (R^2) value is 0.02 (where ≤ 0 is no fit and 1 is perfect fit), further confirmed that taking the water microbes as meta community, the assembly of biofilm bacterial community is governed by environmental selection rather than neutral processes.

Table 2. Spearman rank correlation coefficient

	OTUs	Sequences	Relative Abundance	
Water	4694	73527	11.5% (unique)	
Biofilm	5879	260133	86.8% (unique)	
Share	142	34248	13.2% (biofilm)	
Neutral	101	12069	4.6% (biofilm)	35.2% (share)
Enriched	25	21443	8.3% (biofilm)	62.6% (share)
Disadvantaged	16	736	0.3% (biofilm)	2.2% (share)

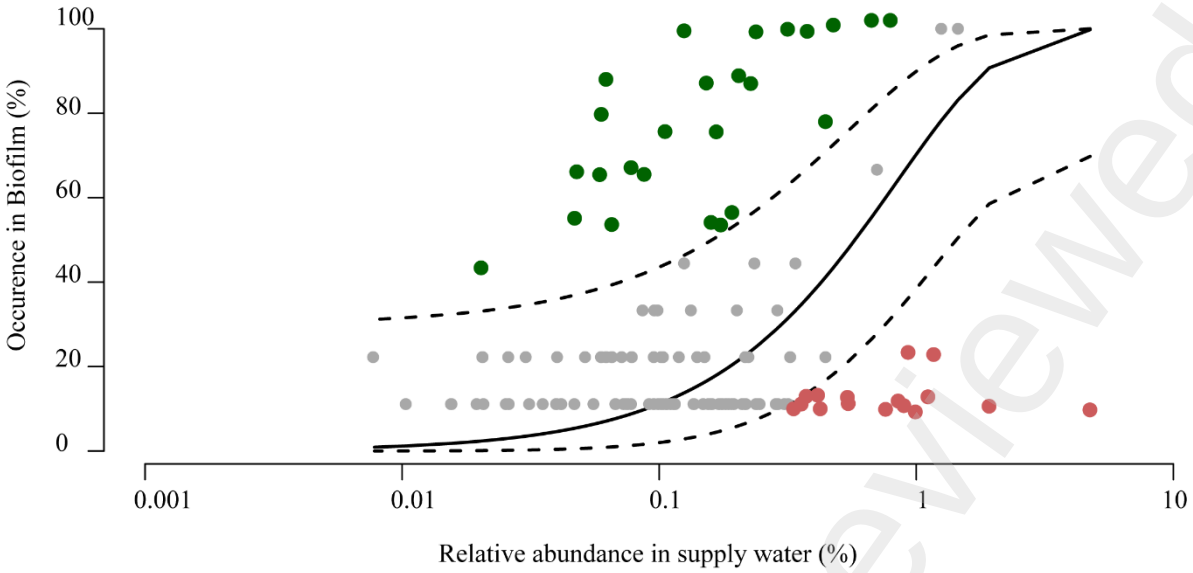

Figure 5. *Neutral community model for the combined biofilm samples (n=9). The solid line is the model prediction and dashed lines represent 95% confidence intervals. Green points represent the OTUs for which the observed frequency is greater than the model prediction (enriched), and red points represent the OTUs for which the observed frequency is less than the prediction (disadvantaged), based on their mean relative abundance in the influent communities.*

4. Discussion

Different from the traditionally used pilot/simulated systems and/or young biofilm sampling, this study investigated matured biofilm on full scale drinking water distribution pipes of different material (i.e. PVC-U, AC and GCI) that installed in different areas supplied by the same water treatment plant. The long-term affects were discussed regarding the influences of pipe material on biofilm formation in drinking water pipes, espeically the possible harmonizing effects on the bacterial community assembly.

4.1 Microbiome assembly in drinking water biofilm revealed by NCM model

The NCM model results based on the shared OTUs between water and biofilm suggested that the assembly of biofilm bacterial community is governed by environmental selection, while water microbes is meta community served as seed bank, which proved the hypothesis proposed by Henne et al. (2012). More specifically, the enriched community member OTU7, assigned as *Nitrospira* spp., has been widely reported to be able to thrive in drinking water biofilm (Henneet al. 2012; Liuet al. 2014a; Martinyet al. 2003). While the disadvantaged community members, such as OTU14 and OTU40 that assigned to the family of Hyphomicrobiaceae, were found to thrive in phosphorus limited environment and form filamentous biofilm in drinking water biofilters (Keithley and Kirisits 2019). They are disadvantaged because the Dutch drinking water supply system are carbon limited, with the pursuit of chlorine free drinking water supply and extremely low AOC (<10 μ g C/L) (Smeetset al. 2009).

However, it should be mentioned that the number of shared OTUs between water and biofilm (142 OTUs) was low in the present study, which is because of the low number of water samples. The biofilm has developed over four decades under the historical water microbiology conditions. Since historical water samples were not available, higher temporal resolution could not be achieved for this study. Moreover, there were significant variations among bacterial communities in bulk water samples, which might be caused by the stagnations and contributions

from plumbing systems (Jiet al. 2015; Lautenschlageret al. 2010; Linget al. 2018). Since the shared members between water and biofilm are picked for NCM analysis, the model results would not be influenced. For future research, the combination of dynamic water and biofilm paired sampling at certain frequency for long-term would offer more valuable insights microbiome assembly over time. Especially, feeding the high resolution data set to NCM model will be able to uncover essential mechanism for biofilm formation and its controlling strategies (Linget al. 2018; Vignolaet al. 2018).

4.2 Influence of pipe material on biofilm formation

As revealed by the compostion (Figure 3) and diversity of bacterial communities (Figure 4), though there are slight differences regarding certain members and their presence and abundance, the > 40 years' old matured biofilm communities formed on different pipe material of PVC-U, AC and GCI were highly similar, suggesting the minor influences of pipe material on the bacterial communities of biofilm. There has been, somehow, a consensus that pipe material is important for both quantity and community of biofilm (Berryet al. 2006; Liuet al. 2016b; Proctoret al. 2016). The observed little influences of pipe material in the present study were different from the commonly found strong inlfuences of pipe mateiral on both composition and diversity of biofilm bacterial community in water supply pipes, such as plumbing system (28 days' old biofilm) (Rogerset al. 1994), shower hoses (8 months' old biofilm) (Proctoret al. 2016), and modeled and field distribution systems (1 month -42 weeks' old bioflm) (Kerret al. 1998; Niquetteet al. 2000; Yuet al. 2010), all of which targeted on young biofilm less than 1 year's old.

On the other hand, there were studies found similar quantity and community of matured biofilm fomred on different pipe materials in an office building in Finland (copper vs. PEX, > 1 year's old months' old biofilm) (Inkinenet al. 2014) and in a distribution main pipe in Germany (steel, copper, PVC, > 20 years' old biofilm) (Henneet al. 2012), both of the study illustrated the

potential importance of vicinity of the biofilm over the support material. Placing different coupon materials in the same reactors, Aggarwal et al. (2018) also found that coupon material did not have a signficant impact on biomass levels or composition of the biofilm communities(Aggarwalet al. 2018). By comparing their results with similar study that using separate reactors for each coupon material (Buseet al. 2014), the authors argued that isolating different material to study their impacts on biofilm could not mimic full-scale systems containing a varity of materials (Aggarwalet al. 2018), which have neglected the mutual influence of biofilms by exchange of bacteria via mitigation and/or diffusion (Henneet al. 2012; McDougaldet al. 2012).

However, in the present study, the biofilm on three pipe materials were taken from different supply areas that are >10 km away from each other. Our finding was complied with an earlier observation in the full scale chloraminated drinking water distribution system of Saint Paul, Minn, USA, which observed surprisingly similar biofilm communities regardless the age, location and pipe material (unlined cast iron vs. cement-lined cast iron, > 53 years' old biofilm) (Gomez-Smithet al. 2015). It is remarkably interesting to notice that the two studies both observed similar bacterial communites on different pipe materials from different locations (spatially distanced), though the the present studied Dutch system was completely different from the system in Saint Paul, espeically considering the disinfection strategies (unchlorinated vs. chloraminated). The key common factor is that both studies investigated matured biofilms >40 years' old. It is rational to hypothesize that years-long (decades-long) acclimatization harmonized the initial signicant differences induced by pipe material. The hypothesis could be proved by the previous observation of less pronounced differences regarding the quantity and community of biofilms formed on four out of six materials after 8 months (Proctoret al. 2016), which might possibly be explained by the diminished nutrient

leaching from the pipe and afterwards the biofilm formation governed by the microbes and nutrients' matrix in the supply water.

4.3 Practical implications

For enssuring the biosafety, considerable attention and efforts have been invested to understand the formation of biofilm and its managing strategis in drinking water distribution network over decades. Pipe material has been considered as one of the key factors, which possibly governing the potential of biofilm formation and its community assembly (Van der Kooij and Veenendaal 2001). However, until now, the critical questions of how and how long the pipe material is influencing the biofilm development remains unanswered. Currently, the available pipe material test for evaluating its potential to promote microbial growth varied from 2 weeks to 16 weeks (Wenet al. 2015). While, as mentioned above, the simulated studies on pipe material's influence on biofilm communities were conducted for periods varied from days to years. Such big variations regarding the scale of study time might be the reason for the conflicted observations and conclusions across the literatures, as well as the reported differences between simulated reactors and the full-scale systems that operated for decades (Aggarwalet al. 2018). Therefore, choices of study time are masking the mechanism of pipe material's influence on biofilm development and its community succssion.

As showed in the present study of unchlorinated Dutch system (> 40 years' old biofilm), in the chlorinated German system (> 20 years' old biofilm) (Henneet al. 2012) and the chlormainated system (> 53 years' old biofilm) (Gomez-Smithet al. 2015), the biofilm was harmonized regardless the pipe material and other environmental circumustances in each full scale distribution system, as long as the different pipe material is supplied with same drinking water. Once the harmonized stable microbial ecology established, there would be potential risks associated with transition effects when the supply water quality subjected to changes, which

may lead to destabilization of biofilm matrix and suddenly release of opportunistic pathogens (Chanet al. 2019; Chenet al. 2020; Liuet al. 2017b).

In addition, from both scientific and practical perspectives, an essential question to be answered is how long the harmonizing process may take before a quantity and community stablized biofilm could be established. Martiny et al. (2003) suggested that the bioilm formation may take 200-300 days to reach stationary density and ~500 days to establish stable population on stainless steel. Proctor et al. (2016) observed less pronouced differences after 8 months than in the early months among the biofilms formed on 4/6 of the tested flexible polymeric pipe materials, suggesting the harmonizing time for different material would be different. To determine the time threshold, the long-term efficacy of pipe material and other essential drinking water biofilm related questions, long-term (years-long) study of biofilm formation dynamics with the latest developed high throughput quantification and sequencing techniques, high resolution water-biofilm paried sampling and microbial ecology model is recommended for the future research.

5. Conclusions

- As showed by the number of observed OTUs, the bacterial community was more diverse in bulk water than that of the biofilms.
- The mature biofilm bacterial communities on PVC-U, AC and GCI pipes are highly similar regarding the alpha and beta diversity, indicating minor influence of pipe material on biofilm.
- As revealed by NCM model, the assembly of biofilm communities is governed by environmental selection rather than neutral process. Members of *Nitrospira* spp. were enriched, while members belong to the family of Hyphomicrobiaceae were disadvantaged.

- The long-term effects and harmonizing process of pipe material's influences on biofilm needs to be explored further.

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