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ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · APRIL 2014

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Pyrosequencing Reveals Bacterial Communities in Unchlorinated Drinking Water Distribution System: An Integral Study of Bulk Water, Suspended Solids, Loose Deposits, and Pipe Wall Biofilm

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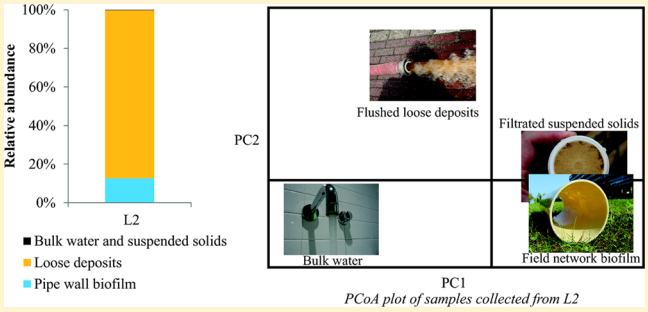
Supporting Information

ABSTRACT: The current understanding of drinking water distribution system (DWDS) microbiology is limited to pipe wall biofilm and bulk water; the contributions of particle-associated bacteria (from suspended solids and loose deposits) have long been neglected. Analyzing the composition and correlation of bacterial communities from different phases helped us to locate where most of the bacteria are and understand the interactions among these phases. In the present study, the bacteria from four critical phases of an unchlorinated DWDS, including bulk water, pipe wall biofilm, suspended solids, and loose deposits, were quantified and identified by adenosine triphosphate analysis and pyrosequencing, respectively. The results showed that the bulk water bacteria (including the contribution of suspended solids) contributed less than 2% of the total bacteria. The bacteria associated with loose deposits and pipe wall biofilm that accumulated in the DWDS accounted for over 98% of the total bacteria, and the contributions of bacteria in loose deposits and pipe wall biofilm were comparable. Depending on the amount of loose deposits, its contribution can be 7-fold higher than the pipe wall biofilm. Pyrosequencing revealed relatively stable bacterial communities in bulk water, pipe wall biofilm, and suspended solids throughout the distribution system; however, the communities present in loose deposits were dependent on the amount of loose deposits locally. Bacteria within the phases of suspended solids, loose deposits, and pipe wall biofilm were similar in phylogenetic composition. The bulk water bacteria (dominated by *Polaromonas* spp.) were clearly different from the bacteria from the other three phases (dominated by *Sphingomonas* spp.). This study highlighted that the integral DWDS ecology should include contributions from all of the four phases, especially the bacteria harbored by loose deposits. The accumulation of loose deposits and the aging process create variable microenvironments inside loose deposits structures for bacteria to grow. Moreover, loose deposits protect the associated bacteria from disinfectants, and due to their mobility, the associated bacteria reach taps easily.



PCoA plot of samples collected from L2

1. INTRODUCTION

Many problems in drinking water distribution systems (DWDSs) are microbial based[1] such as pipe wall biofilm growth,[2] nitrification,[3,4] the biocorrosion of pipe material,[5,6] the deterioration of taste and odor,[7] and the proliferation of opportunistic pathogenic bacteria.[8,9] An understanding of the bacteriology in distribution systems is necessary to design innovative and effective control strategies that will ensure safe and high-quality drinking water;[1] therefore, it is critical to identify the different types of bacteria and their relative abundance in the different phases of DWDSs.[10] According to the characteristics of different phases and the available microenvironments, the phases in DWDSs have been defined and summarized as bulk water (flow through the water main), the pipe wall biofilm (formed on the inner surface of the pipe),

Received: September 5, 2013
Revised: April 25, 2014
Accepted: April 25, 2014
Published: April 25, 2014

Table 1. Detailed Information of Treated Water Quality and Sampling Locations

parameters	AOC (μg c l^{-1})	ATP (ng l^{-1})	TCC (cells ml^{-1})	pipe material	diameter (mm)	distance to treatment plant (km)	flushed length (m)	pipe construction (pipe age, years)
location 1 (L1)						4.5	150	1966 (46)
location 2 (L2)	17.0	4.1 (±0.4)	0.9 (±0.2) × 10^5	PVC	110	9.5	160	1981 (31)
location 3 (L3)						23	180	1984 (28)

suspended solids (particulate matter transported throughout the network) and loose deposits (particulate matter accumulated/settled on the pipe bottom).[11]

Traditionally, it is believed that all bacteria in DWDSs are present in the following two phases: pipe wall biofilm bacteria (95%) and bulk water bacteria (5%).[12−16] Consequently, previous studies have focused only on these two phases. Specifically, reported studies have investigated bacterial abundance, community diversity, and composition at different points (from source to tap),[17] scales (model[18] and field[19] DWDSs), and phases (bulk water, pipe wall biofilm) of DWDSs.[20] Seasonal and diurnal changes;[21] the influence of different pipe materials,[22] sources, and treatment processes governing bacteria in DWDSs;[23] and the interaction between pipe wall biofilm bacteria and bulk water bacteria[20] have been studied.

Several researchers have found that loose deposits are common and abundant in DWDSs, ranging from 30 to 24500 mg m^{-1}.[24−26] Recently, a few studies have investigated loose deposits in distribution systems and discovered that this phase may be a reservoir for organic compounds and bacteria,[27−30] including potentially pathogenic bacteria (1.8−3.9 × 10^5 Mycobacteria/g) as found in these types of loose deposits in Finland.[31] Our previous 10-month pilot study of distribution systems found comparable concentrations of bacteria in loose deposits and pipe wall biofilm;[31] therefore, loose deposits present a very significant fraction of the total microbial population in a DWDS.

However, no research has been conducted regarding the relative abundance of bacteria in loose deposits, bulk water, and pipe wall biofilms. Furthermore, no information is available on the bacterial community composition or structure of loose deposits present in DWDSs. This absence of information is partially attributable to the length of time necessary to form and its dependency on complex factors in field DWDSs, which creates difficulty in studying loose deposits in the short term or using model/pilot systems that have been widely used for pipe wall biofilm studies. Moreover, opportunities to sample loose deposits and biofilms in field distribution systems are limited, especially opportunities to sample different phases together.

Studying the microbial ecology of DWDSs will continue to provide needed insights to help resolve public health concerns associated with bacterial growth in these systems. The considerable contribution of loose deposits to the DWDS environment challenges the current understanding of DWDS microbial ecology, which is primarily limited to pipe wall biofilm and bulk water bacteria. To better understand the DWDS as an integral environment and its microbial ecology, this research describes a sampling program that was conducted in an unchlorinated drinking water distribution system. This program included multiple sample locations in the distribution area. At each location, bulk water, pipe wall biofilm, suspended particles, and loose deposits were sampled simultaneously. Cultivation-independent methods were used to study the quantification and identification of bacteria across sampling locations and phases. This study was undertaken to evaluate the integral DWDSs microbial ecology by involving bacteria in all phases: (I) what fraction of bacteria is present in each phase and where most of the bacteria are located and (II) what the bacterial community structure and composition are within each phase and the correlation of bacterial communities from different phases.

2. MATERIALS AND METHODS

2.1. Drinking Water Treatment Plant and Distribution System. Three sites were selected from an unchlorinated distribution system (L1, L2, and L3) that had not been flushed since their construction (1966−1984, Table 1). The treatment plant belongs to Vitens Water Co. and is located in the northern part of The Netherlands. The treatment plant uses anoxic groundwater as its source water, and after treatment by aeration, rapid sand filtration, softening, rapid sand filtration, and ion exchange (for color removal), the water is pumped to the clean water reservoir and fed to the distribution system. In addition, chlorination is avoided in The Netherlands. PVC pipes with a 110 mm diameter were selected at three locations along the distribution system. Detailed information on treated water quality and sampling location is shown in Table 1.

2.2. Sampling. To sample all four phases together at each site, a water main pipe of at least 150 m was selected (Figure S1, Supporting Information). At the end of the selected water main, a hydrant was present for the sampling of suspended solids and loose deposits. Water samples (WA) were collected from the taps of customers that were connected directly to the main and located close to the hydrants. Flushed pipe specimens (loose deposits were removed) were cut out for biofilm sampling. The order of sampling began by obtaining a water sample, then filtering to collect the suspended solids, followed by carrying out flushing to collect loose deposits, and then cutting out the pipe specimen for biofilm sampling. After removing the loose deposits by flushing, the pipe specimens were cut in duplicate at each sampling location.

The bulk water was sampled at the taps after refreshing the pipe until the water temperature was constant to confirm that the water from the main pipe had reached the tap. The samples were taken during a low water demand period (approximately 11:00am) to sample bulk water and to avoid the possible influence from the resuspension of loose deposits. Suspended solids (SS) were sampled by running a multiple particle filtration system (MuPFiS) at the selected hydrant, as previously described,[32] and the hydrant and customer tap were nearby (at a distance less than 10 m).

Loose deposits (LD) were sampled by flushing the pipelines as previously described at a rate of 1.5 m/s.[26] During flushing, the turbidity and flow of the flushed water were recorded. The first loose deposit samples were taken within 30 s after the flush

started to ensure that the flushed samples of the targeted pipe (over D1) were captured. Every 4 min, 2 L of flushed samples were taken, and extra flushed samples were taken when the measured turbidity changed significantly (an increase or decrease of more than 5 NTU).

Strong linear correlations between turbidity and mass (dry weight, measured as total suspended solids) and between turbidity and biomass (measured as adenosine triphosphate) were found in the flushed water samples from all of the measured locations (Figure S2, Supporting Information). The correlation coefficient was found to be dependent on the location. The correlation between turbidity and biomass was stronger than the correlation between turbidity and mass (suspended solids). The online recorded turbidity was converted to the amount of mass and biomass of loose deposits collected from different locations using the correlation coefficients. Averaged loose deposits mass was calculated over the flushed water main.

Pipe wall biofilm (BF) samples were collected by cutting pipe specimens (30−40 cm) out of the distribution systems, and the two open pipe ends were closed as soon as the specimens were removed. The specimens were filled with one liter of sterilized water to keep the environment wet.

2.3. Adenosine Triphosphate and Flow Cytometry Cell Count. All samples were maintained in an ice box as soon as they were removed from the pipe and then transported to the laboratory within 3 h for pretreatment and analysis, and all analyses were conducted within 24 h. To detach bacteria from the attached surface (suspended solids, loose deposits, and pipe specimens), the samples were pretreated three times for 2 min each by ultrasonication of 42 kHz before further analysis.[33] The recovery rate of the bacteria from the suspended solids, loose deposits and pipe wall biofilm is shown in Figure S3 (Supporting Information). Obtained suspensions were used for further analyses. Cultivation-independent methods, adenosine triphosphate (ATP) measurements, and flow cytometry total cell counts (TCC) were used to quantify bacteria. ATP and TCC were measured as described by Magic-Knezev et al.[32] and Hammes et al.[34]

2.4. 454 Pyrosequencing. The DNA was extracted from the bulk water samples and the pretreated suspension of suspended solids, pipe wall biofilm, and loose deposits using FastDNA Spin Kit for Soil (Q-Biogene/MP Biomedicals, Solon, OH) according to the manufacturer's instructions[35,36] and was amplified with forward primer U515F (5′-Fusion A-Barcode-CA linker-GTGYCAGCMGCCGCGGTA-3′, covers 92.66% bacteria, 93.54% archaea) and reverse primer U1052R (5′-Fusion B-TC linker-TGCATGGYYGYCGYCAGYTC-3′, covers 95.10% bacteria, 90.95% archaea).[37] Pyrosequencing with titanium bulk sequencing methods (Roche, Branford, CT) was performed based upon the manufacturer's protocols developed at the Research and Testing Laboratory (Lubbock, TX). Following the sequencing and image processing, the sequences were binned into individual multifasta files based on tag sequences and used for data analysis. The obtained DNA sequences have been deposited into the DDBJ sequence read archive (accession no. DRA002220).

2.5. Pyrosequencing Data Analysis. The sequences generated from the pyrosequencing analysis of the 16S rRNA gene amplicons were processed (filtered, clustered, taxonomically assigned, and aligned) using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline with default settings.[38] The process consisted of quality checking and denoising and microbial diversity analysis. In short, the flow diagrams were denoised and the UCLUST algorithm was used for operational taxonomic unit (OTU) assignment. Representative OTUs were selected on the basis of the most abundant sequences, and the taxonomic assignment was conducted using the Ribosomal Database Project (RDP) classifier with data sets from Greengenes OTUs at a 0.8 minimum confidence level. Afterward, the sequences were aligned using the Phyton Nearest Alignment Space Termination Tool (PyNAST) alignment algorithm. Weighted and unweighted UniFrac distance matrices were constructed from the phylogenetic tree (built by FastTree algorithm) and used to conduct principal coordinate analyses (PCoA).

3. RESULTS

3.1. Bacterial Quantification. *3.1.1. Bacterial Quantification of Each Phase.* In this unchlorinated drinking water distribution system, the ATP concentration was an average of 2.7 ng L^{-1}, and the TCC values averaged 2.5×10^5 cells mL^{-1} in bulk water. As quantified by ATP, less than 20% of the biomass was found to be associated with suspended solids (Table S1, Supporting Information). The biofilm formed on pipe specimens ranged from 0.09 to 0.16 ng ATP cm^{-2} and $1.2-3.2 \times 10^5$ cells cm^{-2}. The amount of LD was 646 mg m^{-1}, 1386 mg m^{-1}, and 280 mg m^{-1} at L1, L2, and L3, respectively. Loose deposit microbial abundance quantified by ATP was found to range from 781 to 3993 ng ATP (g LD)$^{-1}$.

Due to the potential influence of particles/particle-associated bacteria revealed by cell numeration, the comparison of bacterial abundance in different phases was performed on the basis of ATP measurements. ATP values found in the different phases were normalized over one meter of water main (PVC, D = 110 mm), specifically, 534 (±23) ng ATP for BF, 9.6 (±0.7) ng ATP for water (suspended solids included), and 671−3738 ng ATP for loose deposits. The results are represented as relative abundance (percentage) in Figure 1 (details shown in Table S1, Supporting Information). As shown, biofilm and

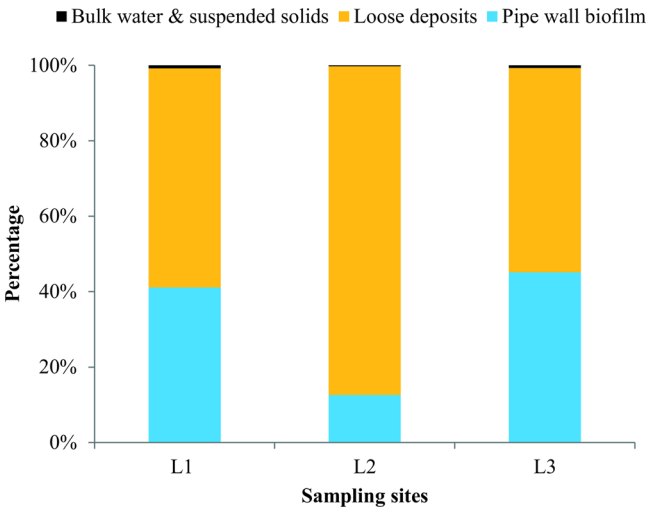


Figure 1. Comparison of bacterial abundance (comparison of biomass as inferred from ATP results) of different phases within a 1-m water main (PVC, 110 mm). Normalization was based on the surface area of the biofilm, the mass of loose deposits over 1 m, and the volume of water. The results are shown in the percentage of each phase to the overall amount of bacteria.

dx.doi.org/10.1021/es5009467 | Environ. Sci. Technol. 2014, 48, 5467−5476

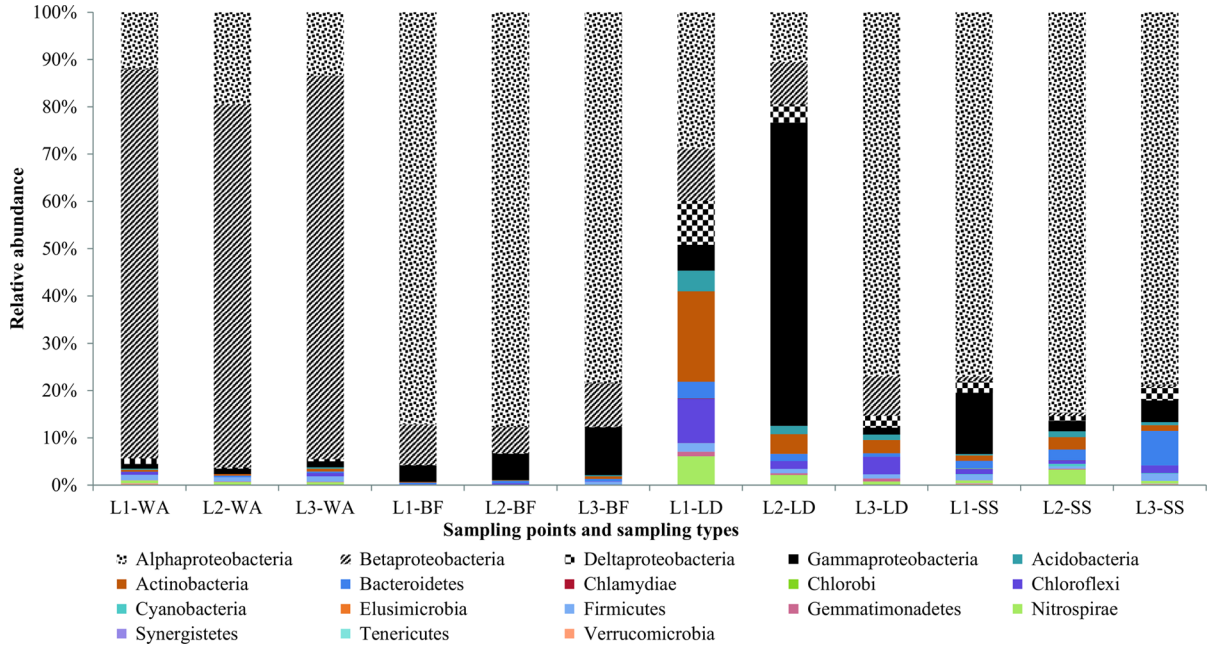


Figure 2. Relative abundance of different phyla and subclasses in *Proteobacteria* in all sampling phases and sampling locations. The phlyum of *Proteobacteria* is shown in subclasses of *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* (shown in black and white in the upper part of the column).

loose deposits contribute to ≥98% of the overall ATP, whereas bulk water and suspended solids account for ≤2%.

3.2. Bacterial Compositions. Diverse bacteria were detected (Figure 2 and Table S2, Supporting Information) in bulk water (19948 sequences, 11 phyla), pipe wall biofilm (19964 sequences, 9 phyla), loose deposits (30220 sequences, 12 phyla), and suspended solids (23143 sequences, 14 phyla). The 7 dominant phyla (averaging more than 1% of total OTUs) for all four phases, in descending order of their relative abundance, were *Proteobacteria* (90%), *Actinobacteria* (3%), *Chloroflexi* (2%), *Bacteroidetes* (2%), *Nitrospirae* (1%), *Firmicutes* (1%), and *Acidobacteria* (1%). Another 10 phyla represented approximately less than 1% of the total number of OTUs. Among the subclasses of *Proteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* accounted for approximately 56%, 23%, 2%, and 9% of the total number of OTUs, respectively. The OTUs identified within the different phases accounting for more than 1% and their abundance levels are shown in a heatmap (Figure S4, Supporting Information).

3.2.1. Bacteria in Different Phases. Bulk Water Bacteria. In bulk water (taken at the taps), *Proteobacteria* represented 97%, *Firmicutes* represented 1%, and another 9 phyla accounted for 2% of the total OTUs. Among the subclasses of *Proteobacteria*, the bulk water bacterial community was dominated by *Betaproteobacteria* (80%) and *Alphaproteobacteria* (15%). *Deltaproteobacteria* and *Gammaproteobacteria* together constituted approximately 2% of the total OTUs. The dominant bulk water bacterial genera for all sampling locations combined in descending order of their relative abundance were *Polaromonas* spp. (69%), *Sphingomonas* spp. (13%), *Acidovorax* spp. (5%), and *Janthinobacterium* spp. (4%).

Pipe Wall Biofilm Bacteria. In the biofilm from the pipe walls, Proteobacteria represented 99%, and another 8 phyla accounted for 1% of the total OTUs. Among the subclasses of Proteobacteria, the pipe wall biofilm community was dominated by Alphaproteobacteria (87%), Betaproteobacteria (7%), and Gammaproteobacteria (5%). The dominant bacterial genera in the pipe wall biofilm for all sampling locations combined in descending order of their relative abundance were *Sphingomonas* spp. (85%), *Janthinobacterium* spp. (4%), and *Pseudomonas* spp. (4%).

Suspended Solids Bacteria. Among the bacteria associated with suspended solids, Proteobacteria represented 90% of the total OTUs, followed by Bacteroidetes (3%), Actinobacteria (2%), Nitrospirae (2%), Chloroflexi (1%), and Firmicutes (1%). Another eight phyla contributed to 1% of the total OTUs. Among the subclasses of Proteobacteria, the bacteria in the suspended solids community were dominated by Alphaproteobacteria (80%), Deltaproteobacteria (2%), and Gammaproteobacteria (7%). The dominant bacterial genera in suspended solids for all sampling locations combined in descending order of their relative abundance were *Sphingomonas* spp. (66%), *Sphingopyxis* spp. (6%), *Pseudomonas* spp. (4%), *Novosphingobium* spp. (3%), *Flavobacterium* spp. (3%), and *Nitrospira* spp. (1%). Similar to results from pipe wall biofilm bacteria, *Sphingomonas* spp. and *Pseudomonas* spp. were found to be dominant in the bacteria associated with suspended solids.

Loose Deposits Bacteria. Among the bacteria in loose deposits, Proteobacteria represented 77% of the total OTUs, followed by the phyla Actinobacteria (8%), Chloroflexi (5%), Nitrospirae (3%), Acidobacteria (3%), Bacteroidetes (2%), and Firmicutes (1%). The remaining five phyla contributed to 1% of the total OTUs. Among the subclasses of Proteobacteria, the loose deposits bacterial community was dominated by Alphaproteobacteria (39%), Betaproteobacteria (9%), Deltaproteobacteria (5%), and Gammaproteobacteria (24%). The dominant loose deposits bacteria for all sampling locations combined in descending order of their relative abundance were *Sphingomonas* spp. (21%), *Alkanindiges* spp. (12%), *Pseudomonas* spp. (9%), *Sphingopyxis* spp. (4%), *Nitrospira* spp. (3%),

dx.doi.org/10.1021/es5009467 | *Environ. Sci. Technol.* 2014, 48, 5467−5476

Conexibacter spp. (2%), *Solirubrobacter* spp. (2%), *Caldilinea* spp. (2%), *Acidobacterium* spp. (2%), *Sphingobium* spp. (2%), *Rhodoplanes* spp. (2%), *Chloroflexus* spp. (2%), *Rubrobacter* spp. (2%), *Acidovorax* spp. (2%), *Pelobacter* spp. (2%), *Novosphingobium spp.* (1%), *Polaromonas* spp. (1%), *Janthinobacterium* spp. (1%), and *Geobacter* spp. (1%).

3.2.2. Shared OTUs among the Four Phases. In total, 36 OTUs are shared by all of the four phases, including a wide range of different genera. Specifically, 21 out of 36 shared OTUs belong to Proteobacteria. The remaining 15 OTUs belong to Actinobacteria (4), Firmicutes (3), Chloroflexi (2), Nitrospirae (2), Acidobacteria, Bacteroidetes, Gemmatimonadetes, and Verrucomirobia. *Sphingomonas* spp. is the most abundant OTU that accounts for 13%−85% of the total OTUs in the four phases. It is the only OTU that accounts for more than 1% of the total OTUs in all of the phases. *Pseudomonas* spp. are the most abundant OTU shared by pipe wall biofilm, suspended solids and loose deposits and account for more than 4% of the total OTUs. In total, these shared OTUs accounted for 90%, 88%, 61%, and 82% of the total bacteria for bulk water, biofilm, loose deposits and suspended solids, respectively.

3.2.3. PCoA Analysis of Bacterial Community Similarities. The three sampling sites have the same planktonic community (Figure 3, blue). Similar to the observation regarding bulk water bacteria, a comparable community structure was found in suspended solids-associated bacteria (Figure 3, black) and pipe wall biofilm bacteria (Figure 3, green) at different locations throughout the network. In contrast to all other phases, the bacterial communities of the loose deposits samples were very different across locations (Figure 3, brown). Comparing the bacterial communities from the different phases, three clusters with clear differences were noticed: bulk water; L1 and L2 of loose deposits; and suspended solids, pipe wall biofilm and loose deposits at L3.

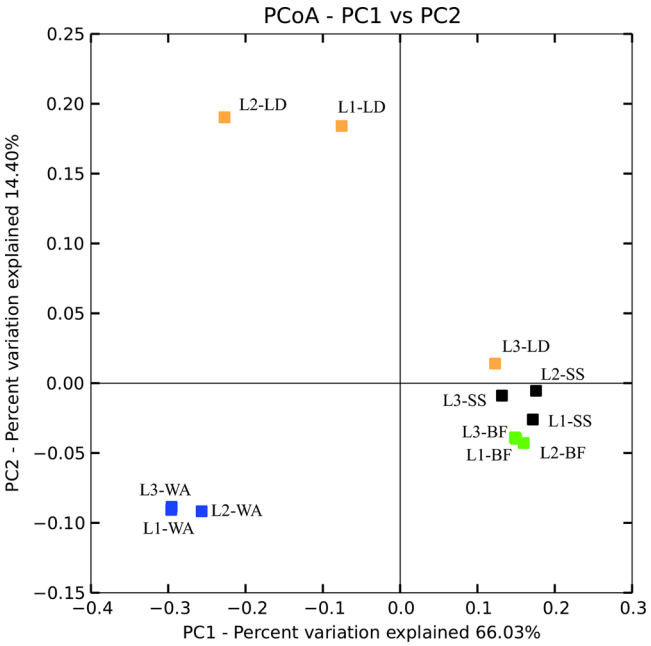


Figure 3. PCoA plot generated using WUnF metrics for all sampling locations and phases. The results of bulk water phase (WA) are shown in blue, pipe wall biofilm (BF) is shown in green, suspended solids (SS) are shown in black, and loose deposits (LD) are shown in brown.

4. DISCUSSION

4.1. Where Are Most of the Bacteria Located? To the best of our knowledge, no available research has previously has quantified ATP in loose deposits. The ATP per mass of loose deposits obtained in the present study in the unchlorinated drinking water distribution systems is comparable to (and even higher than) ATP concentrations found in granular activated carbon (GAC) used to treat drinking water (800−1830 ng ATP/g GAC).[39] This comparison indicates that DWDS loose deposits are biologically (very) active. Unlike the pipe wall biofilm, the quantity of the bacteria in the loose deposits was not evenly distributed and was highly dependent on the amount of loose deposits, which vary at different locations, depending on hydraulics. Compared to the reported values, the amount of loose deposits collected in the present study were in the lower range[24−26] due to the extensive treatment steps undertaken for drinking water purification, which result in a low particle load.

The comparison of different phases within 1 m of PVC pipe (diameter = 110 mm) showed that over 98% of the total bacteria was found in the pipe wall biofilm and the loose deposits. The exact contribution of loose deposits versus pipe wall biofilm depended on the amount of loose deposits. Generally, loose deposits contributed more biomass than the biofilm. This finding is true especially for the site L2 where most of the loose deposits were collected and the number of bacteria from the loose deposits was 7-fold higher than the number in the pipe wall biofilm.

Not surprisingly, bacteria were found everywhere in the DWDS. In addition to similar findings reported in the literature on bacteria in bulk water and pipe wall biofilm, a considerable amount of bacteria was associated with particulate matter. Both observed values and the ratio of ATP_{SS}/ATP_{WA} were higher than our previous research of Dutch systems (0.04−0.156 ng l^{-1} ATP, accounting for 1−2% of WA ATP),[32] which may be due to the previous study having collected suspended solids directly from treatment plants. However, in the present study, the suspended solids were collected from hydrants in the distribution systems. We noted that more cells were found in the suspended solids compared to the bulk water, which may be caused by the release/resuspension of cells from suspended solids. The observation that multiple cells per particle is important as reported here and elsewhere[32] is based on the attached cells being protected from disinfectant residuals (if applicable). Additionally, the cells per suspended particle will only be counted as one, which may lead to an underestimation of bacterial numbers as is the case of opportunistic pathogens.

4.2. Bacterial Community Composition and Structure. *4.2.1. Bacterial Communities of Bulk Water and Pipe Wall Biofilm.* *Polaromonas* spp. have been primarily observed in ultraoligotrophic freshwater environments.[40,41] Based on the study of predominant bacteria in drinking water filters at nine full-scale water treatment plants in The Netherlands, *Polaromonas* spp. were commonly found.[40] The finding that *Polaromonas* spp. were dominant in bulk water indicates that very low concentrations of substrates are available for microbial growth in the bulk water phase in these DWDSs (characterized by low AOC, Table 1). However, the bulk water bacteria may be shaped by the microbial community of the filter and dominated by bacteria released from the filter, which are both consistent with recent observations.[10] Considering the strong similarity among sampling locations and the slow growth rate of

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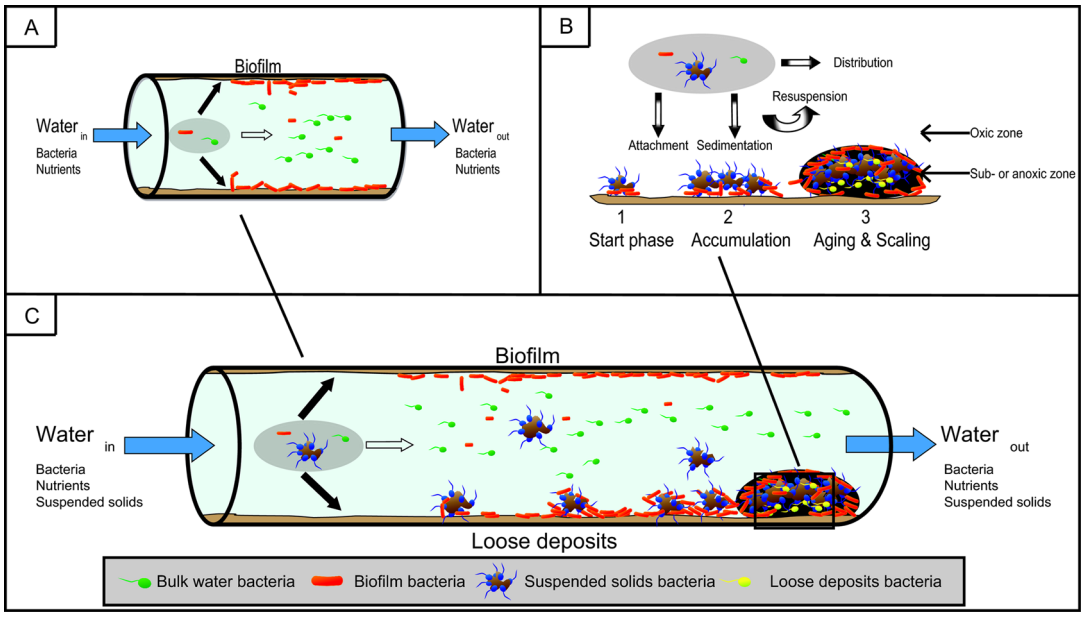

Figure 4. Microbial ecology present in drinking water distribution systems (DWDSs). (A) traditional understanding of DWDS ecology covered the phases of pipe wall biofilm and bulk water, with the main focus on pipe wall biofilm; (B) processes and contributions related to bacteria in suspended solids and loose deposits; (C) integral concept of DWDS bacteriology covered the contributions and interactions of the phases of pipe wall biofilm, bulk water, suspended solids, and loose deposits. Bacteria that are named by the phases refer to specific bacteria predominant in the phase and/or that only exist in the phase.

ultraoligotrophic bacteria, bacteria released from the filters may be more important for the bulk water bacteria in distribution systems.

Pseudomonas spp. and *Sphingomonas* spp. have been reported as the predominant bacterial genera detected in pipe wall biofilms.[1,13,42] *Pseudomonas* spp. have been reported as the most abundant bacterial organism in DWDSs[43,44] because of the ability to form biofilms.[45−48] *Sphingomonas spp.* are metabolically versatile bacteria that have high-affinity uptake systems under low-nutrient conditions; therefore, they have been commonly found in oligotrophic conditions, such as in DWDSs. The dominance of *Sphingomonas* spp. in biofilm is due to irreversible attachment of the bacteria to surfaces by producing exopolysaccharides around the cells to form the biofilm,[49−53] through the change from biofilm mode to planktonic mode, and the colonizing of new suitable environments[52] such as the pipe wall surface of downstream, suspended particles and loose deposits.

Although the water quality may deteriorate during transportation, consistent results have been observed in different field studies.[17,20] The results from the present study confirm the conclusion that the bulk water bacterial community remained stable during drinking water distribution. Similar to the observation regarding bulk water bacteria, a stable community structure was found in pipe wall biofilm bacteria at different locations (Figure 3, marked in green), which concurs with previous observations.[20,47] The homogeneous composition of the population in the mature biofilm throughout the network can be attributed to the mutual influence of pipe wall biofilms in which an exchange of bacteria caused by the adjacent, years-long coexistence: the pipe wall first covered by surface-colonizing bacteria and later over grown by a nearby biofilm community.[20]

In the present study, bulk water bacterial communities at different locations were stable (dominated by *Polaromonas* spp.) and different from the pipe wall biofilm (dominated by *Sphingomonas* spp.). This finding led us to question the reported conclusion that bulk water bacteria in distribution systems are the result of biofilm cell detachment rather than the growth and/or abundance of organisms in the water,[12,14] in which case the detected bacteria in bulk water should be dominated by pipe wall biofilm bacteria.

4.2.2. Bacteria Associated with Suspended Solids. Comparing the bacteria associated with suspended solids with the bacteria in bulk water and pipe wall biofilm, the former are more similar to pipe wall biofilm bacteria compared to the bulk water bacteria, which may be because the suspended solids originated from biofilm detachment. The richness and evenness of bacteria in suspended solids are greater than in pipe wall biofilm bacteria (Table S2, Supporting Information). This may be caused by the better mobility of suspended solids in bulk water coming in contact with bulk water bacteria.

4.2.3. Loose Deposits Hosted Bacteria. The spatial variability of the bacterial communities on the loose deposits is much higher than in other phases. At site L3, where the smallest amount of loose deposits was present, the bacteria in loose deposits were relatively similar to the suspended solids bacteria, suggesting that loose deposits are primarily the result of the sedimentation of suspended solids. However, that similarity was not observed at sites L1 and L2, where 2.5−5-fold more loose deposits were observed than at site L3.

Loose deposits bacteria have the highest diversity among the bacteria in all phases. They harbored 19 OTUs in fractions larger than 1% of the total OTUs, and the majority of the OTUs were considered to be of soil and sediment origin. Bulk water bacteria, pipe wall biofilm bacteria and suspended solids bacteria contained 4, 4, and 7 OTUs (>1%), respectively. The 19 OTUs can be divided into two groups, one group covers most of the dominant bacteria from the other three phases (7 OTUs), and the other group was only detected in the loose deposits (12 OTUs). The key bacterial genera that are unique to lose deposits include the following: (1) members of the

dx.doi.org/10.1021/es5009467 | *Environ. Sci. Technol.* 2014, 48, 5467−5476

Nitrospira spp., *Chloroflexus* spp., *Nitratireductor* spp., and *Rhodoplanes* spp., which are known to be involved in the biogeochemical cycling of nitrogen or sulfur; and (2) members of the *Rhodoferax* spp., *Geobacter* spp. and *Rubrobacter* spp., which are known to be involved in the biogeochemical cycling of iron and arsenic.[55,56] Identifying these specific groups of bacteria corresponds to the elemental composition of collected loose deposits as confirmed by the elemental composition analysis of loose deposits samples. The elements primarily detected were aluminum, calcium, iron, magnesium, manganese, and arsenic (Table S3, Supporting Information). When the biochemical processes occur, the accumulation of inorganic contaminants is accelerated. Arsenic(III) is oxidized more rapidly when both iron/manganese and related bacteria are present.[57] In the present study, the loose deposits were shown to contain iron and manganese as well as bacteria belonging to *Rhodoferax* spp., *Geobacter* spp., and *Rubrobacter* spp., which are involved in the biogeochemical cycling of iron and arsenic.

The following types of bacteria were collected from the loose deposits: aerobic, anoxic, anaerobic, and facultative anaerobic. Specifically, *Geobacter* spp. and *Rubrobacter* spp., which are anaerobic bacterial species, members of the *Rhodoferax* spp., which are facultative anaerobes, and members of the *Pelobacter* spp., which have a fermentative metabolism, were identified. Furthermore, members of the key group of sub- or anoxic bacteria and facultative anaerobes were primarily found at sites L1 and L2, such as *Geobacter* spp. and *Pelobacter* spp., as shown in Figure S4 (Supporting Information), suggesting that the availability of sub- or anoxic microenvironments depends on the amount of loose deposits.

The above-mentioned key features of loose deposits bacteria and their correlation with bacteria in other phases led us to hypothesize three stages of loose deposits formation: initiation, accumulation, and aging and scaling (Figure 4B). The formation of loose deposits starts with sedimentation of suspended solids on the pipe bottom where the surface may have biofilm bacteria attached. After the suspended solids and associated bacteria settle on (the bottom of) the pipe, the loose deposits continue to be enhanced by the exopolysaccharides produced by bacteria such as *Sphingomonas* spp. and *Pseudomonas* spp.[13,50] to form a structure with the potential development of sub- anoxic microenvironments inside. This process may occur over (tens of) years, with periodic interruption by hydraulic peaks that may resuspend loose deposits into the bulk water or bed transport moving in the pipes.

Regarding the bacterial community during the period of the first two stages, the community in the loose deposits is determined primarily by the bacteria present in the suspended solids; therefore, the bacterial community in loose deposits would have a similar community composition as the suspended solids bacteria (shown as L3-LD and L3-SS in Figure 3). Depending on the aging and scaling processes, different degrees of variation are found in loose deposits in their third phases (e.g., at L1 and L2). Chemically, the processes of loose deposit formation and accumulation are inorganic contaminant enrichment processes.

4.2.4. Similarity and Correlation of Bacteria across Different Phases. The similarity of bacterial communities across the four phases is compared in the complete DWDS environment (Figure 3, PCoA). A clear difference between bulk water bacteria and the other phases was observed, which is consistent with the previous comparison of bulk water bacteria and pipe wall biofilm bacteria.[20] The bacteria in bulk water may also be present in the other phases, but at levels too low to detect. The bacteria in the other phases are all a reflection of bacterial growth on a surface, which may cause the differences in detection. *Sphingomonas* spp., having a high tendency for attachment, are found most frequently in these phases. Another possible reason, as mentioned above, is that the suspended solids may have originated from biofilm, and the loose deposits may have originated from the sedimentation of suspended solids. Differences were also observed between the communities in suspended solids, loose deposits, and pipe wall biofilm, indicating different circumstances are available in these phases such as differences in nutrient levels, elemental composition (sulfur, iron, manganese) and oxygen availability (oxic, suboxic, anoxic-environments).

4.3. Integral Concept of DWDS Microbial Ecology. Based on the observations of differences and interrelations among the four phases, an integral concept of DWDS microbial ecology is proposed (Figure 4C). It suggests that a DWDS consists of four phases with different circumstances, including bulk water, pipe wall biofilm, suspended solids, and loose deposits. The treated water that enters distribution systems contains nutrients, planktonic bacteria, and suspended solids with their associated bacteria. During drinking water distribution, complicated processes occur simultaneously among all phases:

(1) **Bulk water**: the bulk water containing planktonic bacteria and suspended solids with associated bacteria flowing through the distribution system reach customers' taps. Bulk water bacteria are primarily controlled by the treatment plant;
(2) **Biofilm**: the bacteria with higher adhesion form a biofilm on the pipe surface; however, the biofilm may detach from the pipe surface due to the death of the biofilm and/or the changes in hydraulic conditions;[58]
(3) **Suspended solids**: suspended solids with associated bacteria either flow through the DWDS with bulk water or are retained in the distribution system by settling as loose deposits or attaching to the pipe surface forming a biofilm. In return, the settled loose deposits and formed biofilm become suspended when the hydraulic circumstances change such as with hydraulic peaks caused by firefighting;
(4) **Loose deposits**: settled loose deposits accumulate in the distribution system. The loose deposits may transport through distribution systems by bed transportation and/or resuspension and resedimentation.[59] Over time, the building up of a loose deposits layer, the microbial-generated EPS and the accumulated organic and inorganic nutrients create unique microenvironments within loose deposits for bacteria to inhabit.

Within this integral concept, the four phases are interrelated by water flow. The dissolved nutrients, particles, and bacteria keep feeding the distribution system due to the water flow. Biofilm (from pipe wall) and particle-associated bacteria (from loose deposits) need to become suspended into bulk water to be transferred to other phases or to flow out of the distribution system at the customers' taps. In all of these cases, the changes in hydraulic conditions (e.g., firefighting and morning water demand peaks) are essential dynamics of transferring one phase to another.

Quantitatively, most of the bacteria inhabit pipe wall biofilm and loose deposits, and the contribution of these two phases is comparable. Biofilm is generally evenly distributed throughout the distribution system, whereas the amount of loose deposits and the contained bacteria within the network are location-dependent. Qualitatively, considering the high similarity among bacterial communities at different locations within each phase (except for loose deposits), stable bacterial communities are expected throughout the network (the same pipe material and diameter). The amount of loose deposits contributed to the differences in shaping the bacterial community.

Results from the present study were obtained from an unchlorinated drinking water system. However, drinking water distribution systems are complicated systems with variable conditions. Factors such as seed bacteria,[10,20] disinfection strategies,[1] nutrients and element composition,[11,32,60−62] pipe material,[22] and hydraulic conditions[63] are essential for bacterial community development in the networks. In other systems, that chemical disinfection and disinfectant residual are maintained during drinking water distribution, the function of particle-associated bacteria may be more important than in the present system without disinfectant residual. The bacteria associated with particles that survived from disinfection and protected from disinfectant residual will seed the growth during distribution.[32] This finding is corroborated by Pinto et al.,[10] who reported that in the chemical disinfection system (ozonation was used before dual media filtration, and free chlorine was added prior to distribution), the bulk water bacteria are controlled by bacteria colonized in the final filter beds.

4.4. Importance of Loose Deposits Bacteria. As discussed above, bacteria in loose deposits clearly show bacteriological importance, both quantitatively and qualitatively. Depending on the hydraulic conditions, bulk water at the consumers' taps can be contaminated by the resuspension of loose deposits and/or detachment of the pipe wall biofilm. Hence, sampling methods and time will be essential for the collection of drinking water samples for microbiological studies. This finding is corroborated by Matsui et al.,[64] who reported the morning peaks of suspended solids at customers' taps and by Lautenschlager et al.,[65] who reported sharp peaks of cells and changes in communities after an overnight stagnation. They suggested taking water samples during low water demand and flushing the tap properly until the fresh water from the water main reached the tap. In the present study, the design of bulk water sampling was aimed at sampling bulk water without the influence of loose deposits. The samples were taken during the low demand period of the day. Under this circumstance, there is a higher probability that the opening of the sampling tap will not lead to contamination by the resuspension of loose deposits.

Confirmed by the abundant comparisons, if only considering pipe wall biofilm and bulk water, the evaluation of bacteria in a DWDS only covers half or even less than half of the total bacteria, or even less. As a result, bacteria growth during drinking water distribution has been considerably underestimated. For example, the methods to evaluate regrowth potential either cover the nutrients available in bulk water (such as assimilable organic carbon, AOC),[66,67] neglecting available nutrients in loose deposits or cover only the growth measured as the potential growth of biofilm (such as BFP)[68] without considering the surface area offered by accumulated particulate matter in DWDS. In The Netherlands, a further step has been taken to evaluate the growth potential by combining AOC and biofilm formation-potential measurements;[69] however, the contribution of loose deposits should also be included.

Similar to pipe wall biofilm, the loose deposits may protect microbes from disinfection (if applicable).[28,29] Loose deposits offer a surface area[70] and nutrients[28,29] for biofilm development. Nevertheless, different from pipe wall biofilm formation, the loose deposits-hosted bacteria may be formed by the growth of certain bacteria with available nutrients within the particulate matter,[54] and the aging and scaling processes of loose deposits may create different microenvironments inside the particulate structure. Both of these different mechanisms may lead to different community structures and compositions for loose deposits bacteria compared to pipe wall biofilm bacteria.

During sudden hydraulic changes (e.g., morning consumption peaks, firefighting, flushing or pipe bursts), loose deposits are resuspended.[27−29] Consuming resuspended loose deposits-containing drinking water (e.g., morning consumption) will significantly increase the consumers' intake of microbes and hazardous chemicals.[32] This event may be a more serious problem for special consumer groups (pregnant and lactating women, newborns, infants, children, and the elderly).

Further research is necessary to understand the DWDSs microbial ecology, especially for particle-associated bacteria such as the (opportunistic) pathogenic bacteria in loose deposits. The current study was conducted in a distribution system without disinfectant residuals; therefore, studying the influence of disinfection on DWDS microbial ecology is very valuable. The suspended solids bacteria and loose deposits bacteria will be even more important in these types of systems because suspended solids and loose deposits protect bacteria from disinfection, cause fast decay of the disinfectant residuals, and act as a transport vehicle to the customers' taps for bacteria that have better mobility than pipe wall biofilm.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional data, figures, and tables. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

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■ ACKNOWLEDGMENTS

We acknowledge support from the Chinese Scholarship Council (2008612022).

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