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Water impact statement

Rainwater intrusion, increasingly possible in systems with rainwater and greywater reuse, can compromise drinking water quality. Our results demonstrate both the temporary regulatory exceedances and impact on the microbiome as well as the natural recovery capacity of biostable distribution systems. These findings support the implementation of real-time microbial monitoring and proactive intrusion management to safeguard public health and water sustainability.

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ARTICLE

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From Contamination to Restoration: Microbial Dynamics After Rainwater Backflow into a Pilot-Scale Drinking Water Distribution System.

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Rainwater backflow caused by system cross-connection into drinking water distribution systems (DWDS), resulting from pipe breaks, leaks, manual installation errors, or misconnections associated with third-pipe systems for rainwater and greywater reuse, can disrupt biostability of the DWDS by introducing microorganisms and nutrients. Using a pilot-scale DWDS with a mature biofilm, this study simulated a conservative worst-case backflow scenario by supplying a 1:1 mixture of rainwater and tap water for two consecutive weeks in recirculation mode, followed by recovery through replacement with uncontaminated tap water without chemical disinfection or hydraulic flushing. This experiment was conducted twice under identical conditions. Microbial abundance, activity, and community composition in bulk water and biofilm were assessed using culture-based methods, total organic carbon (TOC), ATP, online flow cytometry, 16S rRNA gene amplicon sequencing, and continuous by Orbs in-line probe that measures fluorescence-based bio-load.

Rainwater backflow caused immediate impact with a one log increase in total cell concentrations, a two-fold increase in TOC values, peaks in ATP concentrations of 75.71 ng/L compared to 3.50 ng/L (experiment I) and 0.913 ng/L (experiment II) for regular tap water, alongside pronounced shifts in bulk water community composition, demonstrating the duration and scale of the backflow event. Rainwater-associated taxa were temporarily detected in the bulk and biofilm, even after 100% tap water was supplied, but remained at low relative abundances. The culture-based methods (CCA, PCN, ADA) demonstrated that regulatory limits regarding were exceeded during and after the backflow, reflecting the delay in compliance after a backflow event. These results highlight the resilience of biostable DWDS to short-term rainwater backflow and demonstrate the value of high-frequency and high-resolution microbial monitoring for rapid detection and management of contamination event.

1 Introduction

Receiving qualitative and safe drinking water is one of the basic human rights (1). The combined efforts of drinking water providers in both production and monitoring ensure the delivery of high-quality and safe drinking water to households, in compliance with Flemish and European legislation. Similar approaches are implemented internationally under comparable regulatory frameworks (2,3). However, considering that drinking water is transported from its source to the tap through a network spanning several kilometers, comprising pipes, reservoirs, water towers, and numerous connections, each with its own distinct microbiology, delivering high-quality, biostable water remains far from evident. The long and complicated distribution network is inherently susceptible to changes that impact the microbiological stability resulting in uncontrolled bacterial growth and associated safety, aesthetic or operational problems (4).

To avoid uncontrolled bacterial growth, one of the primary objectives of drinking water providers is to produce and distribute biostable water. The aim is to ensure that drinking water reaching the tap maintains a microbial abundance and community composition comparable to that at the point of production. By allowing only an “acceptable degree of change,” and the establishment of undesirable microorganisms, including pathogens and indicator species, is prevented within the distribution system (5–7).

Although the drinking water assets are designed to produce and distribute biostable drinking water, the distribution infrastructure remains prone to failures and environmental changes. The influence of temperature, the disinfection method

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and other operational parameters, such as pressure, flowrate, pipe materials and the water source from which the water was produced influence the microbial community and drinking water biofilm (8–11). Even the combination of several operational and environmental factors can impact the microbiology. More specifically, in chlorinated systems, changes in temperature can accelerate chlorine decay, favoring bacterial growth (10,12).

In general, biostability can also be directly affected by direct contamination from the environment, as a consequence of maintenance works, pipe breaks, leaks or improper connections with non-potable water sources. Household rainwater installations, to collect and distribute rainwater at the household level (e.g. gardening, flushing the toilet), represent a significant potential source of rainwater backflow into drinking water distribution networks. These systems frequently employ over-dimensioned pumps capable of generating pressures that exceed those of the potable water system. Consequently, in the event of an improper connection, the resulting pressure gradient may drive rainwater back into the drinking water network, referred to as backflow (13). Such misconnections pose substantial risks, as they can introduce unwanted microorganisms and/or additional nutrients into the system (14,15). Additionally, with the increasing adoption of household greywater and rainwater reuse, additional plumbing infrastructure, often referred to as "third-pipe" systems, is required (16,17). The installation of these supplementary networks introduces greater system complexity and necessitates updated regulatory frameworks. To prevent misconnections and the resulting cross-contamination between potable water, greywater, and rainwater systems, the design and installation of these additional pipes must be carried out with particular care and precision. These perturbations may temporarily disrupt the resident microbial community, compromise biostability, and ultimately affect both water quality and the integrity of the distribution infrastructure (18–21)

To protect both the drinking water infrastructure and the customer, drinking water providers monitor the production and distribution network heavily. Nowadays, to detect these disturbances, drinking water providers heavily rely on culture-based methods, which are the current legal standard (2). These techniques are often time-consuming, as results can only be obtained after the required incubation period, and they lack precision because they are limited to the detection of indicator organisms, making it difficult to identify the source of the problem and implement appropriate corrective measures. (22,23). High throughput and high resolution techniques, such as flow cytometry and sequencing could provide the drinking water providers with the right tools to identify a calamity and more specifically the impact on the microbiology almost instantaneously (24–27). Recently, novel monitoring technologies have been proposed, including fluorescence spectrometry, which is implemented in drinking water applications through commercial inline probes such as the Orb (Orb Limited, United Kingdom) that targets bacterial autofluorescence in real-time (28–31). With a lower overall cost in comparison to other techniques of around 9000 euro per probe, no need for extensive calibration and chemicals, this technique seems promising in full-scale DWDS monitoring (32,33). Monitoring, and in particular the application of novel analytical methods, is crucial, as appropriate tools enable the rapid and accurate identification of both the origin of a problem and the most effective mitigation measures.

Nowadays in case of a problem, one of the main procedures is a so-called "flush" in which a large amount of water is pumped through a certain part of the network with high velocity. This can be done with or a without the use of a chemical disinfectant, such as chlorine. The latter is often the method of choice in case of a microbiological issue (34,35). A flush with chlorine, however, has demonstrated to result in more regrowth on the long term, although it limits the invasion potential of unwanted organisms (21,34,35).

Currently, rainwater backflow, as a consequence of misconnections between the drinking water distribution network and third pipe, household rainwater installations or reuse systems, is not always detected in a timely and accurate manner. Moreover, when a backflow is identified, uncertainty often remains regarding the most appropriate actions required to effectively resolve the issue. As mentioned before, these systems often employ over-dimensioned pumps that can drive rainwater back into the low-pressure part of the distribution network. Since this section of the network uses small-diameter piping (80 mm), it can experience high mixing ratios, hereby affecting entire neighbourhoods or districts (14,15). To detect and look into system recovery on a relevant scale, the microbiological impact of rainwater backflow events on drinking water distribution systems, was investigated on a pilot-scale DWDS (36) with a mature biofilm to simulate a controlled contamination scenario. A 1:1 mixture of rainwater and drinking water was introduced to represent a conservative worst-case stress scenario, as has been the case for previous backflow events in the distribution network of local drinking water providers, mimicking the sudden backflow of non-potable water due to a pipe break, leak, or improper connection with a household rainwater installation. Although such a mixing ratio is unlikely under normal operating conditions, it provides a bounding framework to assess system resilience, recovery dynamics, and monitoring sensitivity. The objectives of this study are to quantify the immediate and short-term effects of rainwater backflow on microbial abundance, activity, and community composition in both bulk water and biofilm by means of different monitoring techniques; to evaluate the capacity of a mature drinking water biofilm to buffer or transiently retain rainwater-associated microorganisms; to assess the extent and rate of natural system recovery following simple operational remediation through replacement with uncontaminated drinking water in the absence of chemical disinfection or increased flow velocities; and to compare the performance of conventional and emerging monitoring

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techniques in detecting the onset, magnitude, and resolution of backflow events. Based on these results, we aim to provide drinking water providers with a better understanding of how rainwater backflow can be effectively monitored and managed. To ensure robustness and reproducibility, the experiment was conducted twice under identical conditions, integrating online, in-line, and offline monitoring approaches to capture high-resolution temporal dynamics and community-level responses.

2 Material & Methods

2.1 Pilot Design and Operation

A DWDS pilot with a mature biofilm (consistent cell concentrations and equilibrium between growth, attachment and detachment) (11,37) was supplied with 100% tap water (i.e., drinking water treated and chlorinated by the utility, without any additional treatment prior to use in the pilot system) with a residence time of seven days, followed by two consecutive weeks of a 1:1 mix of 50% tap water and 50% rainwater with each a residence time of seven days. This mixing ratio was specifically chosen to mimic a worst-case scenario, based on real-life cases of user errors in which substantial amounts of grey/rainwater impacted a DWDS and experience of the local drinking water providers (14,15). Because this study was designed to replicate conditions in the final, low-pressure section of the distribution network with smaller-diameter piping (80 mm), oversized pumps can introduce large volumes of rainwater into the system, resulting in high mixing ratios. After this mix of rain- and tap water, tap water was again supplied to the pilot. Every 7 days, the system was completely drained and refilled with the appropriate water type for the given experimental phase, namely tap water during the initial and final phases and a tap water–rainwater mixture during the intermediate phase. As the system operates in recirculation mode, this means that a residence time of 7 days was maintained throughout the experiment. The entire experiment was repeated twice. The tap water used for the experiments was chlorinated tap water supplied by Farys (Ghent, Belgium), sourced from the Albert Channel (surface water) in Antwerp (Belgium). The rainwater used in this study was collected at the building level and stored in the building's rainwater storage tank prior to use. As a result, its composition may vary depending on recent rainfall events. In addition, since the same storage tank supplies rainwater for regular building applications, routine usage and replenishment of the tank may also contribute to temporal variations.By the time the water reached Ghent, where the pilot facility is located, the free chlorine concentration was below the detection limit. The distribution pilot consists of three separate and identical subsystems (loops) of which two were used for this experiment. Each loop was connected to a 1 m³ non-transparent high density polyethylene intermediate bulk container (IBC), which was not disinfected beforehand, connected to 100 m of unplasticized polyvinylchloride pipes with a diameter of 80 mm for each loop (36) (Figure S1A). Water from the IBC was pumped into the loops, recirculated through the system, and subsequently returned to the IBC. Throughout the experiment, the flow velocity was maintained at 40L/min, the temperature at 16°C, and the pressure was kept around 0.5 bar, in line with previous studies (38).

2.2 Pilot Sampling

After five days from the start of the experiment, in the week in which tap water was added, samples were taken from each loop to establish a baseline, which were used for plating, TOC, ATP, and conductivity measurements (Figure 1). At the end of the baseline week biofilm coupons were collected to assess the baseline biofilm. At the beginning of each week, the pilot system was drained and refilled with the 1:1 mixture of 400 liters of rainwater and 400 liters of tap water or 100% tap water depending on the experimental phase. Every week samples of the added rainwater and tap water were taken for TOC, FCM, ATP and sequencing analysis. After one hour and six hours, samples for plating, TOC, ATP, and conductivity were taken. After 3 hours, samples for plating were also taken. After 24h of recirculation, samples were taken for ATP, and plating analysis. After four days (during the tap and rainwater mix and during the final week with just tap water), another round of samples was collected. After 7 days, samples were taken before the pilot system was drained and refilled. Samples for 16S rRNA gene-based amplicon sequencing were collected at the initial time point (after 1 hour) and at the final time point each week before the pilot system was refreshed. Samples for biofilm sequencing were collected at the end of each week. This sampling procedure was repeated weekly, with the pilot system being refilled each time. After two weeks in which the pilot was filled with a 1:1 tap and rainwater mix, all loops were refilled with tap water, while the sampling protocol remained unchanged for the remainder of the experiment. Each week after, the buffer tanks were refilled with tap water.

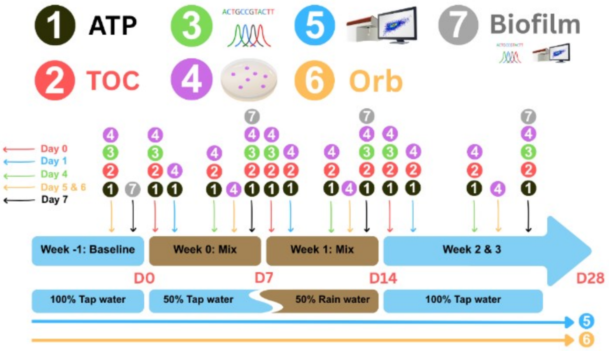

Figure 1: *Overview of the sampling campaign for each experiment. Arrows and numbered symbols indicate the sampling time points within each experimental week. Flow cytometry (5) and Orb (6) were monitored continuously online. ATP (1) and TOC (2) samples were collected on days 0, 1, 4, and 7 of each week. Plating samples (4) were obtained on days 0, 1, 5, and 6, while sequencing samples for the bulk (3) were collected at the beginning and end of each week. Biofilm samples for flow cytometry and sequencing (7) were taken at the end of each week. The blocks at the bottom indicate the type*

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of water used to fill the pilot system during each experimental phase.

2.1 Media Preparation

To assess the microbiological water quality, selective and differential culture media were used to target key indicators and opportunistic bacterial groups relevant to drinking water systems. Chromogenic Coliform Agar (CCA) was used for the detection and enumeration of coliform bacteria as indicators of fecal contamination and general water quality (Carl ROTH®). Ampicillin Dextrin Agar (ADA) was selected to target *Aeromonas* spp., which are commonly associated with aquatic environments and may proliferate in distributed water systems (HIMEDIA®). Pseudomonas Cetrimide Nalidixic Acid Agar (PCN) was used for the selective detection of *Pseudomonas aeruginosa*, an opportunistic pathogen of concern in premise plumbing systems (OXOID®). All media were prepared according to the manufacturers' instructions. All three media were poured into sterile Petri dishes under aseptic conditions in a laminar flow cabinet and allowed to solidify at room temperature.

For each sample 100mL of water was filtered over a 0.45µm MCE Membrane filter (Merck, Belgium) using a filtration unit consisting of six filtration funnels and a Microsart e.jet vacuum pump (Sartorius, Germany), after which the filters were placed onto the plates with growing media and incubated according to manufacturers' instructions.

2.3 Microbial monitoring

2.3.1 Bulk water phase

Online flow cytometry was applied to measure total cell concentrations and to perform phenotypic fingerprinting. To achieve continuous and automated measurements, an OnCyt© (OnCyt Microbiology AG, Switzerland) autosampler was coupled to an Accuri™ C6 Plus flow cytometer (BD Biosciences, Belgium) as described in Waegenaar et al. (2024) (11) (Figure S1B). Briefly, samples (200 µL) were taken in triplicate for each loop every 6 hours. Staining was performed using 200 µL SYBR Green I (10,000× concentrate in DMSO, Invitrogen, Belgium), 5000 times diluted in TRIS buffer (pH 8, 10 mM, Merck, Belgium). After mixing, the samples were incubated at 37°C for 20 min in the OnCyt chambers and sent to the flow cytometer for measurement. In between measurements, cleaning of the OnCyt sample lines was performed with a sodium hypochlorite solution (1 v% final concentration, Avantor, USA), after which the bleach solution was quenched with a sodium thiosulfate solution (50 mM final concentration, Merck, Belgium) and rinsed with ultrapure water (Milli-Q, Merck, Belgium). As sheath fluid, ultrapure water (Milli-Q, Merck, Belgium) was used. Manual samples, taken from the IBC in which the waters were stored, were collected during experiment II and measured with similar staining and incubation conditions on an Accuri™ C6 Plus flow cytometer (BD Biosciences, Belgium) in the lab. This was performed to compare the treated water supplied by the drinking water provider at the building inlet with the samples collected by the online flow cytometer, which monitored the recirculating water within the pilot system. To enable source attribution of microbial taxa, raw rainwater and tap water samples were collected and sequenced independently alongside the pilot system samples. This approach allowed comparison of the microbial communities present in the influent water sources and the recirculating water within the pilot system.

Samples for 16S rRNA gene-based amplicon sequencing were taken. For both experiments this was done on day 0 (after 1 hour of recirculation) and day 7. From each loop, 2 L was filtered over a 0.22 µm MCE Membrane filter (Merck, Belgium) using a filtration unit consisting of six filtration funnels and a Microsart e.jet vacuum pump (Sartorius, Germany), after which the filter was stored in a freezing tube at -21°C.

The total ATP concentration was measured to assess the active biomass at each sampling time point. ATP measurements were taken at the end of the baseline week and during the experiment after 1 hours, 6 hours, 24 hours, 4 days and on day 7 at the end of the week. For this, 400 µL of each sample was transferred into a sterile 1.5 mL Eppendorf tube. To prevent ATP degradation, the samples were immediately frozen and stored at -80°C until further analysis. For ATP analysis, the BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Belgium) was used. A 100 µL sample was transferred to a Greiner 96-well Flat Bottom White polystyrene plate, and an equal volume of BacTiterGlo™ reagent was added immediately prior to luminescence measurement using an Infinite® 200 Pro plate reader (Tecan, Switzerland). For the calibration curve, a standard series ranging from 10nM to 1 pM ATP was prepared using 10 mM ATP (Promega, Madison, Wisconsin, USA).

2.3.2 Biofilm sampling

To sample the biofilm, coupons composed of the same material as the pipes (PVC-U), were installed in the middle of each loop, oriented at a 45° downward angle relative to the pipe.(Figure S1C, S1D). To sample the mature biofilm, every time different coupons (n = 2) (coupons that were already in the pilot before) were taken before the start of the experiment (day 0), and every 7 days after. Biofilm cells were removed using the protocol described in Waegenaar et al. (2024) (11). In summary, biofilm cells were removed using an electric toothbrush (Oral-B Advanced Power, Procter & Gamble, Belgium) and collected in 15 mL of 0.2 µm filtered bottled water (Evian, France). The remaining volume was filtered using MCE Membrane filters (Merck, Belgium) and Polycarbonate syringe filter holder (Sartorius, Germany) to perform 16S rRNA gene-based amplicon sequencing.

2.3.3 Illumina Sequencing

Further processing for 16S rRNA gene-based amplicon sequencing (DNA extraction, PCR amplification, 16S sequencing) is in detail described in Waegenaar et al., 2024 (11). In brief, DNA extraction was performed using the DNeasy PowerSoilPro kit (Qiagen, Germany), following the

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manufacturer's protocol. 10 µL genomic DNA extract was send out to LGC genomics GmbH (Berlin, Germany) for library preparation and sequencing on an Illumina Miseq platform with v3 chemistry (Illumina, USA).

2.4 Chemical monitoring

2.4.1 Total organic carbon analysis

Samples for TOC analyses were collected in 40 mL TOC-free vials (Sievers, Germany) and stored at 6°C prior to analysis. TOC concentrations were measured in technical quadruplicate using a Sievers 900 Portable TOC Analyzer connected to a Sievers 900 Inorganic Carbon Remover (General Electric Company, Boston, USA). Sampling vials were rendered TOC-free prior to use by acid rinsing followed by combustion in a muffle furnace while wrapped in tin foil.

2.4.2 Ion chromatography

Samples for anions (NO_3^-, NO_2^-, SO_4^{2-}, Cl^-) and cations (Ca^{2+}, Mg^{2+}, K^+, Na^+) were collected in reusable IC tubes (PROMED, Italy), that were rinsed with ultrapure water (Milli-Q, Merck, Belgium),and stored at 6°C prior to analysis. The ions were separated using ion exchange chromatography (IC) by a 930 Compact IC Flex (Metrohm, Switzerland). The device is equipped with a Metrosep A Supp 5 150/4.0 column and a Metrosep A Supp 4/5 guard column/4.0, to protect the column from contamination, and 850 IC conductivity detector (Metrohm, Switzerland). As the mobile phase, a 1.7 mM HNO_3 (2 M, ThermoFisher Scientific, USA) and a 1.7 mM 2,6 pyridinedicarboxylic acid solution (Merck, Belgium) was used for the elution of the cations and a 1.0 mM $NaHCO_3$ (≥99.5%, Carl Roth, Germany) and 3.2 mM Na_2CO_3 (≥99.5%, Carl Roth, Germany) solution was used for the elution of the anions.

2.5 Orb probe analysis

Orb is an online monitoring technology specifically designed to detect changes in water quality within drinking water and purified water applications. The Orb probes were employed in this experiment to monitor the biological load of the water. The probes are highly sensitive and allow for continuous assessment of the biostability of a water network. In this study, the system was placed at each loop. The measurement principle is based on measuring bacterial autofluorescence was using inline fluorescence probes, following the principles described by Bhartia et al., 2010 (29). The bio-load is quantified using multi-channel spectrophotometry by combining signals from multiple fluorescence channels. These combined signals were benchmarked against extensive calibration experiments using L-tryptophan standards and cell counting as reference methods (28,30). The resulting spectral signatures are analyzed through Orb's digital Decision Intelligence Platform, where the multi-band data is combined into a quantitative measure of microbial presence. The platform enables continuous tracking of water quality events and provides a detailed overview of microbial activity within the piping system. The high-frequency scanning capacity allows for the analysis of up to 10,000 samples per week, ensuring a comprehensive and data-rich monitoring process (Orb monitor Ltd., 2025).

2.6 Data analysis

Data analysis was done in R (39) in RStudio version 4.3.0 (40). The Flow Cytometry Standard (.fcs) files were imported using the flowCore package (v2.14.0) (41). The background data was removed by manually drawing a gate on the FL1-H (green) and FL3-H (red) fluorescence channels as described in Props et al. (2016) (39) (Figure S8). Illumina data was processed using the DADA2 pipeline (v1.30.0) (43). Taxonomy was assigned using the Silva database v138 for the 16S rRNA gene-based amplicon sequencing (44,45). Further data analysis was performed using packages such as the phyloseq package (v1.46.0) and the vegan package (v2.6-4) (46,47). Data visualization was done using the ggplot2 (v3.4.4) and ggpubr (v0.6.0) packages (48,49). Shapiro-Wilk Test was used to test the data for normality. Further statistical analysis was done using the vegan package for ANOSIM tests (v2.6-4), and base R's stats package functions; wilcox.test() for Mann-Whitney (47). If numbers are reported as X ± Y, Y is the standard deviation. If only ± X is shown, X is the average.

2.7 Controls

To assess potential contamination and ensure analytical accuracy, Milli-Q (MQ) water was used as a negative control throughout all methods. For flow cytometry, MQ samples were measured prior to each analysis to verify instrument and procedural cleanliness. For sequencing, MQ water was filtered and subjected to DNA extraction following the same protocol as the experimental samples. For culture-based analyses, MQ samples were regularly included to monitor potential contamination during plating and incubation procedures.

For ATP measurements, standard controls and calibration curves were prepared to ensure accurate quantification. For total organic carbon (TOC) analysis, MQ water was measured as a reference and baseline sample to verify the reliability of the measurements.

3 Results

3.1 Current state-of-the-art monitoring techniques

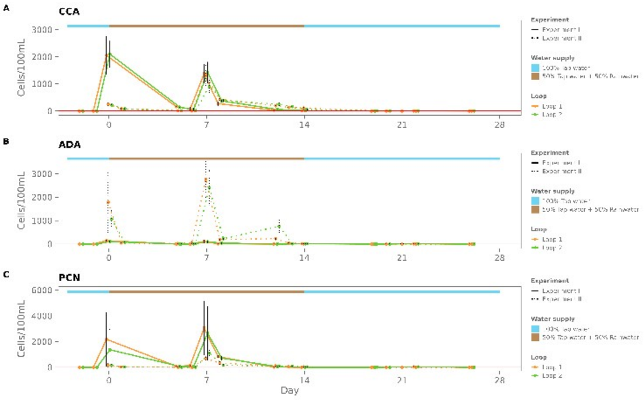

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Figure 2: Overview of the plating results for the different selective media and for experiment I (full line) and experiment II (dotted line) for each loop (loop 1: orange, loop 2: green). The dots display the average CFU/100mL and the error bars display the standard deviation (biological replicates, n=3). The red line determines the legislative threshold as for all cases less than 0 CFU/100mL should be detected. CCA, ADA and PCN represent the selective media were specifically chosen to monitor the coliform (CCA agar), Aeromonas (ADA agar) and Pseudomonas (PCN). The colored bars show which water type was added to the pilot.

To assess the immediate impact of rainwater backflow on regulatory microbial indicators and organic carbon levels in drinking water, culture-based plating and total organic carbon (TOC) analyses were applied throughout both experiments. This was achieved by combining high-frequency sampling shortly after backflow with repeated measurements during recirculation and subsequent replacement with uncontaminated tap water. Over the course of both experiments standard plating samples were taken (Figure 2). To evaluate the immediate impact of the rainwater backflow on water quality, samples were collected after 20 minutes, 1 hour, 3 hours, and 6 hours of recirculation. Additional samples were taken later in the week, on days 5 and 6.Three selective media were chosen to monitor coliforms (CCA agar), *Aeromonas* (ADA agar), and *Pseudomonas* (PCN agar)..It is important to note that, in accordance with drinking water regulations, none of these organisms should be detectable, with a standard limit of 0 CFU/100 mL. . This requirement was met in all samples taken from the pilot before the start of the experiment, when the loops were fed with 100% tap water (on days −2 and −1), which showed 0 CFU/100 mL. On the contrary, the mixture of rain and tap water resulted in an increase in overall coliform growth in both experiments, with concentrations exceeding the legislative limit. The rainwater used in this study was collected at the building level and is therefore subject to temporal variability in composition due to changing rainfall conditions. This variability likely contributed to differences in the detection and enumeration of coliform bacteria, *Aeromonas*, and *Pseudomonas* observed during the experiment. However, a decline in coliforms, *Pseudomonas* spp., and *Aeromonas* spp. was already observed within the first hours after introduction of rainwater into the pilot system and after the start of recirculation. This decrease continued throughout the week, with the samples collected at the end of the week showing low CFU counts per 100 mL. At the end of the rain-tap water mixture weeks, coliforms were still detected, indicating that the rain–tap water mixture did not comply with drinking water legislation. On day 14, when the pilot was refilled with 100% tap water, plating results revealed persistent coliform contamination, even in the direct absence of rainwater in the system: 103 and 54 CFU/100 mL were detected in loop 1 and loop 2, respectively, for experiment I, and 7 and 13 CFU/100 mL in loop 1 and loop 2 for experiment II. By the end of that week, on day 20, these values had decreased to 1–2 CFU/100 mL in all cases.Following an additional refreshment with 100% tap water, the regulatory limit of 0 CFU/100 mL was achieved by day 26.

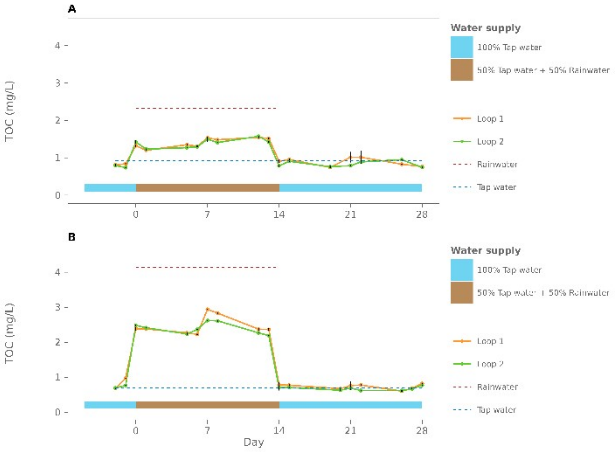

Figure 3: Average total organic carbon (mg/L) (technical replicates, n=4) measured in experiment I (A) and experiment II (B). The dotted lines represent the average TOC values of the rainwater (red) and tap water (blue) that was used to fill the pilot with. After the 1:1 rain–tap water mix was added, TOC values increased and remained elevated during recirculation. When 100% tap water was reintroduced, the values decreased to their initial levels. The error bars explain the standard deviation. The colored bars show which water type was added to the pilot.

Total organic carbon (TOC) measurements are a standard analysis to monitor drinking water quality and detect potential contamination. TOC samples were collected after 1 hour of recirculation, 24 hours, 5 days (except for week 3 in experiment II), and at the end of each week (after 7 days) (Figure 3). In both experiments, TOC values in both loops were significantly higher during the rainwater–tap water mixing phase than when the system was operated with 100% tap water.(for experiment I: Wilcoxon-test, Loop 1 $p = 2.07e^{-12}$, Loop 2 $p= 3.32e^{-10}$, for experiment II: Wilcoxon-test, Loop 1 $p = 1.95e^{-17}$, Loop 2 $p = 8.23e^{-16}$). During the weeks in which the 1:1 rainwater–tap water mixture was added, TOC levels reflected this mixing ratio, stabilising at values approximately intermediate between the mean TOC of the rainwater added during those weeks and the mean TOC of the tap water used throughout the experiment.After the rain-tap water mixture was replaced with 100% tap water, TOC levels in the pilot decreased to match those of the average of the supplied tap water throughout the experiment (indicated by the blue dotted line).

3.2 Cell concentration, cell activity and Orb bio-load measurements

To quantify changes in microbial abundance, activity, and overall microbial presence in response to rainwater backflow, total cell concentrations, ATP levels, and Orb bio-load were analyzed in the bulk water phase. This was done using online flow cytometry (total cell concentration), discrete ATP measurements, and continuous in-line Orb monitoring throughout the backflow and recovery phases.

Based on the online flowcytometry measurements the total cell concentration was determined (Figure 4A, 4B). For both of the experiments cell concentration significantly increased with one log in both biological replicates for both experiments when the 1:1 rain-tap water mixture was added and recirculated (for experiment I: Wilcoxon-test, Loop 1 $p = 6.14\ e^{-19}$, Loop 2 $p = 2.36\ e^{-19}$, for experiment II: Wilcoxon-test, Loop 1 $p = 7.84\ e^{-19}$, Loop 2 $p = 7.84\ e^{-19}$). In experiment I, during the two weeks in which the loop was filled with rain-tap water mixture, the cell concentrations remained nearly constant around 1×10^6 cells/mL. In experiment II, during week 2, in both Loop 1 and Loop 2, there was a decrease in cell concentrations.From day 14 onwards, when the pilot system was refilled with 100% tap water, cell concentrations immediately decreased by approximately one log and returned to levels comparable to those observed before the rainwater–tap water mixture was introduced. This pattern was observed in both experiments..

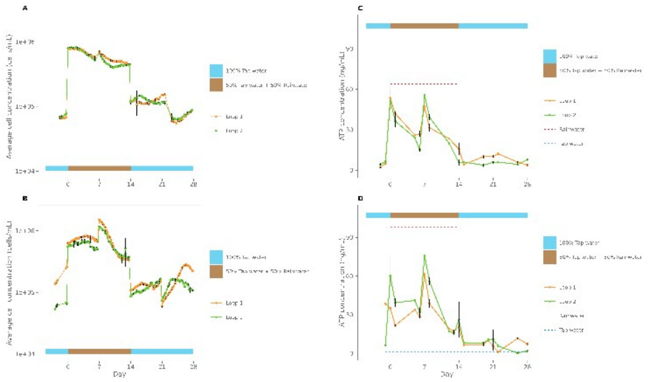

Figure 4: Average cell concentration (technical replicates, n=3) (cells/mL) measured by the online flow cytometer (A,B) and the average ATP concentration (biological replicates, n=3) (ng/mL) (C,D). Graph A and C represent the values from experiment I, while graph B and D represent values from experiment II. In graph C and D, the dotted lines represent the average ATP values of all rainwater (red) and tap water (blue) that was used to fill the pilot with. The cell concentration immediately increased after the 1:1 rain and tap water mix was added. The ATP concentration initially increases after the rain and tap water mix was added, but then decreases during recirculation. In both graphs, the error bars explain the standard deviation. The colored bars indicate what water type was added to the pilot.

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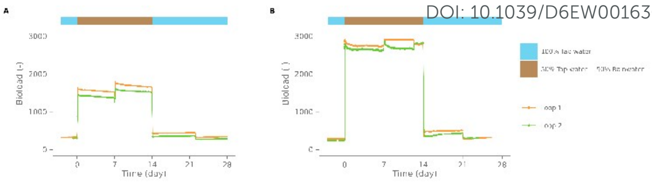

Figure 5: Bio-load (averaged for every two hours, biological replicates, n=24) measured by the Orb in-line probe for experiment I (A) and experiment II (B). In both experiments there is an increase in bio-load once the 1:1 rain and tap water mix is added to the system. Once 100% tap water is reintroduced, values return to the initial values. In experiment II there is a higher bio-load, compared to experiment I, similar to the TOC values of both experiments. The colored bars show which water type was added to the pilot.

ATP concentrations measured after 1 hour, 6 hours, 24 hours, 4 days, and 7 days exhibited a similar pattern across both experiments and both replicates (Figure 4C,4D). At the beginning of each week in which the tap water–rainwater mixture was introduced, ATP concentrations in Loop 1 showed a pronounced peak, reaching 35.00 and 61.56 ng/mL on D7 and D14, respectively, in experiment I, and 53.37 and 46.96 ng/mL, respectively, in experiment II.. With values of 59.98 ng/mL and 75.71 ng/mL, respectively, for D7 and D14 in experiment I and 51.30 ng/mL and 55.64 ng/mL, respectively, for D7 and D14 in experiment II similar peaks in ATP concentrations are seen when the rain - tap water mixture is added in Loop 2. At the end of those weeks the ATP concentration drops to concentrations as low as 5.79 ng/mL (Experiment I, Loop 2, D14). Despite the drop in ATP levels, the overall ATP measured for the weeks in which the tap and rainwater mix was added is significantly higher compared to the ATP levels of the weeks with regular tap water (for experiment I: Wilcoxon-test, Loop 1 $p = 0.0000198$, Loop 2 $p = 0.0000340$, for experiment II: Wilcoxon-test, Loop 1 $p = 0.000394$, Loop 2 $p = 0.000244$). In the weeks when tap water is reintroduced, ATP levels remain nearly constant with values ranging between 1.04 ng/mL and 11.59 ng/mL across both experiments, loops and weeks, closely resembling the average of the tap water of 0.91 ng/mL for experiment I and 3.50 ng/mL for experiment II, indicated with the blue dotted lines. This is the case for both experiments.

During the experiments, the Orb in-line probe was installed in both loops, and its continuous measurements were used to monitor the bio-load of the system (Figure 5). The bio-load tracks the microbial presence and thus represents unwanted/wanted growth in the system, meaning that an increase of this value indicates a change in biostability and microbiology. When the 1:1 rainwater–tap water mixture was introduced, the Orb bioload parameter increased significantly and remained relatively stable during the recirculation phase, thereby accurately reflecting an undesirable microbial shift in water quality. (for experiment I: Wilcoxon-test, Loop 1 $p = 2.2\ e^{-16}$, Loop 2 $p = 2.2\ e^{-16}$, for experiment II: Wilcoxon-test, Loop 1 $p = 2.2\ e^{-16}$, Loop 2 $p = 2.2\ e^{-16}$). Only when 100% tap water is

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reintroduced into the system, values returned to the initial values that were measured before the experiment. In experiment II, the bio-load values are double the values compared to experiment I, similarly to what was seen for the TOC values (Figure 3).

3.3 Sequencing results

To determine how rainwater backflow alters bulk water community composition and the persistence of rainwater-associated microorganisms, 16S rRNA gene sequencing was applied to bulk water samples collected during backflow and recovery and to the raw rain and tap water that was used to fill the pilot (Figure 6, Figure S2,S3,S4,S5,S6,S7). To specifically determine the impact of the rainwater backflow, ASVs that were uniquely associated with the introduced rainwater were tracked across successive mixing and refreshment phases (Figure 6). Taxa were classified as rainwater-associated when they were detected in the independently sequenced rainwater samples but were absent from both the tap water and pilot system samples. This comparative approach enabled the identification of microbial signatures specific to the rainwater source, despite the limited taxonomic resolution of 16S rRNA gene sequencing.

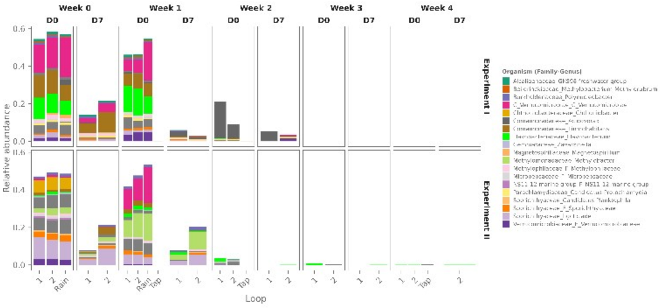

Figure 6: Top 20 rainwater associated ASV's found in the bulk samples during the experiment. To select the rainwater associated ASV's, all ASV's that were found in the tap water and in the last bulk samples taken before the start of the experiment were removed from the ASV's that were found in the rainwater that was used to make the first mix of rain and tap water. On the x-axis the origin of the sample is given. This was done for each week of the experiment on day 0 and day 7. This was done for both experiments separately.

For the bulk bacterial community in experiment II, two additional weeks in which tap water was supplied to the pilot were sampled (Figure 6). This was done because, in experiment I, ASVs originating from the rainwater were still detected in the bulk water after one week of refreshment with 100% tap water.

Based on the sequencing results, it is evident that during the on D0 of the weeks in which rainwater was mixed with tap water (weeks 0 and 1), as the 20 most abundant ASVs were detected with relative abundances comparable to those observed in the raw rainwater, even after mixing with tap water. In both experimental weeks, 7 days of recirculation led to a decrease in the relative abundance of rainwater-associated bacteria, while the overall taxonomic composition remained stable. In experiment II, the rainwater community introduced in the second week displayed minor compositional differences compared to one of the first week. Nevertheless, although the initial community composition differed between weeks, the community after one week of recirculation closely resembled that of the previous week, with *Methylobacter* and *Sporichthyaceae* persisting as the dominant rainwater-associated taxa in both loops. Upon reintroduction of tap water, rainwater-associated taxa remained detectable in both loops of both experiments. In experiment II, additional sequencing over two subsequent weeks demonstrated persistence of *Flavobacterium* in both loops, whereas by week 4, no rainwater-associated bacteria were detected in loop 1Notably, from D7 in week 2 onwards, *Flavobacterium* was consistently present at a low relative abundance of less than 0.6%, indicating that it constituted only a minor component of the total microbial community. (Figure S2,S3,S4,S5).

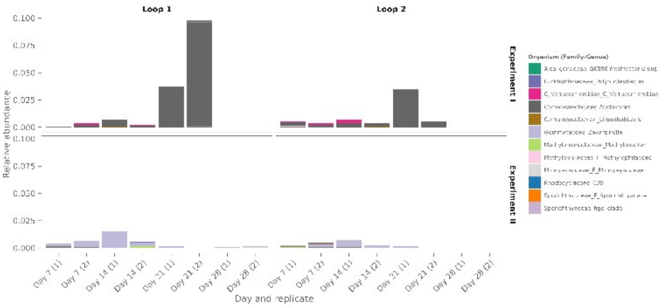

Figure 7: Top 20 rainwater associated ASV's found in the biofilm samples during each experiment for each loop. To select the rainwater associated ASV's, all ASV's that were found in the tap water and in the last biofilm sample taken before the start of the experiment were removed from the ASV's that were found in the rainwater that was used to make the first mix of rain and tap water. On the x-axis the days and replicates are given.

Similar to the bulk sequencing analysis, to determine whether rainwater-associated bacteria could be detected in the biofilm, the 20 most abundant ASVs that were exclusively present in the rainwater added during week 1 and absent from both the pre-experimental biofilm and the tap water were selected and screened for each experiment. (Figure 7). Overall, it should be noted that the relative abundances of rainwater-associated bacteria in the biofilm were substantially lower than those observed in the bulk community, indicating that the majority of the biofilm community remained largely unaffected. (Figure S6,S7). Nevertheless, in both experiments bacteria associated with the rainwater were found in the biofilm, with *Commamonadaceae acidovorax* indentified in experiment I and *Sporichthylaceae hgcl clade* in experiment II. In both experiments, no rainwater associated bacteria were found at the end of week 4.

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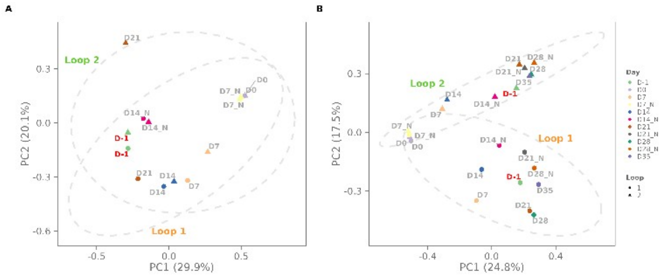

Figure 8: PCoA analysis (Bray-Curtis) of experiment I (A) and experiment II (B). Each day on which a sample was taken has a different color. All days with "_N" at the end represent the first sample after the refreshment taken on that day. The last day before the experiment, the baseline sample was marked in red. The shape of the dots and the ellipses (95% confidence) represent the loops.

3.3.1 Bulk bacterial community demonstrates microbiological recovery

The Bray–Curtis PCoA analysis of the bulk bacterial community revealed that both loops displayed comparable community compositions during the two rain–tap water mixing periods, as indicated by the clustering of D0 samples (week 0, the first week of rain–tap water mixing) and D7_N samples (week 1,, the second week of rain–tap water mixing) in the ordination space (Figure 8). During those weeks, the community gradually shifted toward the resident tap water community, as indicated by the migration of the D7 and D14 samples toward the D−1 samples, which were collected from the pilot prior to the experiment. All samples taken after the reintroduction of 100% tap water (D14_N and further) showed a microbial composition similar to that of the D−1 sample, as they clustered closely together. In experiment II, additional samples were collected over two extra weeks, and the community composition during these final sampling points remained nearly identical to that observed in the D−1 samples.

This indicates that after several refreshments, one in the case of experiment I and three in the case of experiment II, the bulk water community had returned to a composition comparable to that observed prior to the backflow.

4 Discussion

4.1 After contamination, the pilot-scale DWDS recovered and regained stability without intensive disinfection procedures.

Based on the plating results, after the mix of rainwater and tap water was introduced into the system, it took two weeks after the rainwater backflow, and thus at least two water refreshments, for the results to reach the legislative norm of 0 CFU/ 100mL for all plating methods. When comparing the plating results with the sequencing data, particularly the rainwater-associated ASVs in both the bulk and biofilm communities, a similar trend was observed. Even after three refreshments (experiment II), there were still rainwater associated bacteria (ASV's) detected, albeit in very low relative abundances.

The TOC values immediately increased following the addition of rainwater. Both the total cell concentrations and the TOC values in the rainwater were already higher than in the tap water, with the values in the mix reflecting the expected 1:1 ratio. The combination of the higher levels of carbon and higher number of cells, caused the system to reach its carrying capacity, as no additional growth was seen during the weeks in which the rain and tap water mix was added. During these experiments, no live/dead staining was performed. The inclusion of such an approach could have provided additional insight into the survival of rainwater-associated bacteria. To partially address this limitation, ATP measurements were included, which provide an indication of overall microbial activity. The entry of additional carbon is important as it has been seen that the amount of available carbon is one of the main drivers of regrowth (50–52). Over recent years, it has been hypothesized that the resident drinking water bacteria are K-strategists, while the unwanted bacteria in drinking water are r-strategists (27,53,54). The observed rapid decline of undesirable organisms based on the plating results, despite sustained high total cell counts during the backflow weeks, is consistent with the r- and K-strategist hypothesis. The high levels of activity (ATP) displayed by the bacteria when the rain and tap water is mixed, which also rapidly decreases during circulation could serve as additional proof that the unwanted bacteria, which are active when introduced, rapidly become inactive under the nutrient limited conditions within the pilot.

Despite the observed impact on the biofilm and bulk communities, the total cell concentrations, TOC, including the exceedance of legal standards and the backflow of rainwater-associated bacteria in the bulk and biofilm, the reintroduction of 100% tap water led to a rapid and autonomous recovery of the community structure, as supported by the plating results, PCoA ordination and immediate reduction in TOC and total cell counts. Despite the recreation of a worst-case scenario and the absence of any remedial measures such as flushing, chemical disinfection, or increased flow velocities, the system demonstrated a high degree of resilience, as replacing the rain–tap water mixture was sufficient to reduce total cell concentrations and unwanted organisms, restoring the total cell count to baseline levels. Based on the resilience supported by our results, it could be argued that natural recovery processes to maintain or restore biostability in drinking water distribution systems, even after disturbance, eliminate the need for intensive or disruptive interventions. This natural approach may be advantageous, as uncontrolled regrowth has been observed following chlorinated flushes, possibly due to necrotrophic growth (5,25,50,55–57). However, in practice, when time constraints and potential health risks are considered, drinking water providers typically opt for rapid and precautionary interventions. Since a flush with chlorine has proven to lower invasion potential, in case of a microbiological problem, this might still be the method of choice (21). In real-world situations, these worst-case scenarios (i.e. 50% rainwater in a DWDS) are uncommon and would likely be addressed differently. Given that the plating results exceeded the legislative standards by over two orders of magnitude, such a contamination event would probably lead to repeated chlorine flushing (58,59).

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4.2 The impact of rainwater backflow on bulk and biofilm dynamics

Sequencing results showed that both bulk water and biofilm community composition were affected by the addition of the tap and rainwater mix. This is supported by the shifts observed in the PCoA ordination and by the detection of rainwater-associated ASVs in both the bulk and biofilm communities. The impact of the rainwater backflow was more pronounced in the bulk water community, where rainwater-associated bacteria accounted for approximately 60% of the relative abundance at the beginning of the weeks. In contrast, within the biofilm community, these bacteria ranged between 0.1% and 1%, indicating a substantially lower impact on the overall microbial composition. Before the addition of treated water, the biofilm in the pilot-scale DWDS was cultivated for over 100 days, resulting in a stable and mature biofilm (11,36,60,61). Although previous studies have shown that a mature biofilm is less prone to short-term variations, in this experiment the biofilm community composition was clearly affected (62). Based on the relative abundance data, during the two weeks in which the rainwater was supplied, part of the biofilm community consisted out of rainwater associated bacteria. These results suggest that the biofilm may act as a temporary reservoir for incoming microorganisms, or alternatively that rainwater-associated bacteria are unable to persist within the biofilm, as they were no longer detected once regular tap water was reintroduced (63). To confirm this, additional research is needed.

In both experiments, rainwater-associated bacteria detected in the biofilm were present only at low abundances. In experiment II, the biofilm community was dominated by members of the *Sporichthyaceae* family, which have been linked to the production of taste and odor compounds (64). In previous studies, members of the *Sporichthyaceae* have also been detected in the outflow of wastewater treatment plants contaminating surface waters, as well as in contaminated groundwater sources used for drinking water production(65–67). The rainwater-associated taxa observed in the biofilm were among the most abundant rainwater-associated bacteria detected in the bulk water. In week 2 of experiment I, when the system was supplied with 100% tap water, *Comamonadaceae acidovorax* was the most abundant rainwater-associated bacterium present in both the bulk and biofilm communities. *Acidovorax*, a known freshwater bacteria, is in drinking water associated with contamination and capable of forming a biofilm (68–70). The increasing relative abundance of this bacterium in the biofilm during and following the addition of the rain–tap water mixture suggests possible reintroduction from the biofilm into the bulk phase (71,72). This release from the biofilm into the bulk water may be driven by the decrease in TOC levels following the reintroduction of 100% tap water, as nutrient-limiting conditions are known to induce the expression of genes associated with biofilm dispersal (71,73–75).

4.3 Techniques for rapid detection of unwanted change in (microbial) drinking water quality in case of a rainwater backflow event

In practice, drinking water providers rely heavily on culture-based methods to assess the microbiological status of distribution systems. However, during a contamination event, speed and accuracy become critical, as water utilities must ensure continuous operation of the distribution network while maintaining the delivery of safe, high-quality drinking water to consumers. With the increasing adoption of household greywater and rainwater reuse systems, including the associated third-pipe infrastructure, additional plumbing will be installed (16,17). This additional piping infrastructure may increase the risk of installation errors, potentially resulting in the backflow of rainwater or greywater into the drinking water distribution network. That means that in case of a backflow event, one of the kind that was simulated in this study, an ideal detection technique would rapidly give insight into the origin of the problem, as well as the scale of the contamination.

While the culture-based methods clearly indicated regulatory violations and showed that even after returning to 100% tap water there is a delay in compliance, the TOC, flow cytometry, and Orbs bio-load measurements reflect both the duration and magnitude of the backflow event. Additionally, culture-based methods rely on indicator organisms, which limits their accuracy and precision, and are subject to a considerable time delay, as results can only be evaluated after an incubation period of approximately 20–24 hours, making these techniques labour-intensive, slow, and comparatively imprecise (27).

TOC and total cell concentration shifted immediately following the addition of rainwater to tap water, with both parameters increasing to levels consistent with the 1:1 mixing ratio. During the period of rainwater contamination, TOC and cell concentrations remained elevated compared to the periods before contamination and after the reintroduction of regular tap water, whereas plating results and ATP values declined even during the weeks in which the rainwater – tap water mixture was applied. This underscores the potential of flow cytometry to rapidly and accurately monitor the microbiological quality of the drinking water (22,25,76,77). However, setting-up flow cytometric monitoring in practice is expensive, complex and needs trained personnel, thus practically less suited for every segment of a full-scale DWDS.

Therefore, newer techniques such as the relatively inexpensive and operationally simple Orb in-line monitoring probes show considerable promise and were installed on the pilot piping system. Because of the fact that no extensive calibration and/or chemicals are required to do the measurements, the Orb probe is capable of measuring the bio-load every minute. As a result, from the moment the rain and tap water mix was added, the bio-load values immediately increased and remained elevated over the course of the recirculation, making it an ideal monitoring tool to detect unwanted (microbial) quality changes in real-time, without the need for extensive data analysis. This enables early warning and facilitates a rapid response to

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deviations from baseline conditions. Similarly to the flow cytometry and TOC values, on day 14, when 100% tap water was reintroduced, the bio-load values decreased again. Based on these results, it is clear that the measured bio-load serves as a measurement for unwanted growth. When comparing the bio-load results, they closely resemble the TOC in this specific case, showing similar increases. In the Flemish drinking water directive it is stated that no undesired change in TOC values may be observed (2). As the Orb bio-load follows the TOC patterns and reflects similar trends, the Orb probe could serve as a valuable alternative, or at least a complementary addition, to existing in-line monitoring approaches for safeguarding overall drinking water quality. Especially, since its integration with an online platform allows multiple probes to be deployed at key locations throughout the network, enabling continuous and remote monitoring.

In contrast to the flow cytometry, TOC and Orb bio-load measurements, the ATP measurements only showed an increase immediately after the initial water was mixed a clear increase. In practice, this would mean that if a sample was taken further away from the leak/backflow point or after a couple of hours after the backflow event, the ATP levels might have already decreased again, and the contamination might remain unnoticed. However, other studies have shown that the use of ATP measurements could be a useful tool to monitor biostability and show correlations to the heterotrophic plate counting (HPC), especially in the case of water with a high microbiological activity (78–80).

4.4 Implications for full-scale DWDS operation in practice

In this experiment, 50% rainwater was introduced into the pilot-scale DWDS as a weekly one-time event, after which the same water was recirculated for seven days. Due to practical limitations of the pilot-scale setup, the water was recirculated and the entire system was drained and refilled weekly. To more closely mimic a real-life scenario, water would be continuously supplied while rainwater would be continuously added and mixed into the system, as this continuous backflow would have possibly led to a different response of the overall microbiology and biofilm. In that case, in contrast to our experimental setup, rainwater-associated bacteria may be continuously introduced, potentially allowing them to increase in relative abundance within the bulk bacterial community and to become more readily incorporated into the biofilm. This is particularly relevant given that our results show that bacteria associated with short-term rainwater exposure were dominant in the bulk community and were also detected in the biofilm.. Additionally, in the case of the pilot distribution system used for these experiments, which mimics the final, low-pressure part of the network, there are minimal dead-ends and stagnant water zones. The actual low-pressure distribution network on the other hand is a heavily branched network, which might have multiple stagnant zones and dead ends. These will inevitably complicate the problem detection, remediation procedure and its speed. In those cases, the use of a flush with chlorine would be beneficial as the dead ends or stagnant zones would be reached by the disinfectant or be flushed away by the high flow velocities. In all of the above mentioned real-life scenarios, the need for sensitive and accurate monitoring devices, such as those discussed in this study, will only increase. Although the pilot is representative for the real-life distribution network, caution is needed when extrapolating these results.

Additionally, given the large size and the complex, branched structure of real DWDSs, continuous monitoring across the entire network is not practically achievable. It is therefore important for drinking water providers to identify key locations within the network where the microbiology and drinking water quality can be regularly monitored with flow cytometers or similar devices, such as the Orb. An integrated approach that combines high-throughput screening methods, such as flow cytometry or Orbs in-line probes, at strategic points in the network with emerging technologies like Nanopore sequencing could provide a rapid and accurate means to identify the microbiological impact of rainwater backflow specifically (and/or other problems) and enable timely and effective corrective actions, in comparison to the current state-of-the-art (plating) (27).

5 Conclusion

This study demonstrates that drinking water distribution systems (DWDSs) exhibit a high intrinsic resilience to acute rainwater backflow events, even under conservative worst-case conditions. Using a pilot-scale DWDS with a mature biofilm, we showed that the introduction of a 1:1 rainwater–tap water mixture caused an immediate and measurable disruption of microbiological stability, reflected by elevated total cell concentrations, TOC levels, ATP activity, regulatory indicator organisms, and distinct shifts in community composition in the bulk water. Rainwater-associated microorganisms rapidly dominated the bulk phase, while the biofilm only retained a limited fraction of these taxa at low relative abundances.
Despite clear exceedance of legislative microbiological standards and a significant increase in total cell concentrations in the bulk, the system recovered autonomously following simple replacement with 100% tap water. Without applying flushing, chemical disinfection, or increased flow velocities, microbial abundance, activity and community composition returned to baseline conditions. Sequencing and principal component analyses confirmed that the resident drinking water community was restored after a limited number of refreshment cycles, with only minor and transient persistence of rainwater-associated taxa. These findings indicate that mature DWDS biofilms and bulk bacteria buffer short-term disturbances without undergoing long-term destabilization, supporting the concept that resident drinking water microbiota are well adapted to low-nutrient conditions and outcompete opportunistic intruders.

The combined use of conventional and high-resolution monitoring techniques revealed substantial differences in sensitivity and response time. While culture-based methods

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remain essential for regulatory compliance, they demonstrated that even after the rain-tap water mixture was replaced by 100% tap water, there is a delay in compliance with regulations. In contrast, flow cytometry, TOC, and in-line Orbs bio-load monitoring provided immediate, quantitative, and continuous insight into both the backflow events, reflecting both the magnitude and duration of the backflow event. Notably, the Orb in-line probe closely tracked changes in biostability and organic carbon availability, highlighting its potential as a cost-effective, real-time early warning tool for full-scale DWDS operation.

In general, these results suggest that, for short-term and localized backflow events, natural recovery processes may be sufficient to restore microbiological stability in biostable DWDSs, potentially reducing reliance on aggressive remedial measures such as chlorinated flushing that may induce long-term regrowth. However, given the complexity and heterogeneity of full-scale networks, precautionary interventions and specific treatment (chemical disinfectant, flushing procedures,…)will remain necessary under regulatory and public health constraints.

The findings of this study support a risk-based, monitoring-driven management strategy, in which rapid and high-resolution microbial surveillance will be important to future-proof drinking water monitoring. Especially since the adoption of rainwater and greywater reuse systems increases, such integrated monitoring approaches will become increasingly critical to safeguard drinking water quality and distribution system integrity.

Author Contributions

BDG conceived the original idea. TP and FW developed the theory and designed the experiments under the lead of BDG and NB. TP, FW, SP, BS and JF collected the data. TP performed the data analysis. TP, FW, SP, BS, JF, NB and BDG discussed the results critically. TP produced the final figures. TP wrote the manuscript with the input from the other authors. All authors agreed with the final version of the manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data Availability Statement

The datasets and code for this study can be found in the following Github repository: git@github.ugent.be:thpluym/Rainwaterintrusion.git

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Data availability statement

The datasets and code for this study can be found in the following Github repository: git@github.ugent.be:thpluym/Rainwaterintrusion.git