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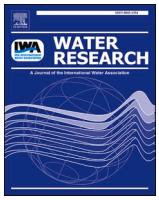

Long-term impacts of free chlorine and monochloramine on the development of drinking water biofilm

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ABSTRACT

Biofilm formation in drinking water distribution systems is primarily managed by disinfectants such as free chlorine (FC) and monochloramine (MC). However, there is limited understanding of their long-term and dynamic effects on biofilm development. To address this, a 56-week study was conducted to comprehensively assess biofilm development in terms of microbial quantity and community under different disinfection regimes: no chlorine (NC), FC (0.1 mg/L), and MC (0.4 mg/L). The results showed that both FC and MC significantly inhibited biofilm growth compared to the NC condition while shaping distinct biofilm communities. Notably, FC drastically reduced biofilm biomass and community diversity, resulting in a more uniform biofilm community predominantly composed of Proteobacteria (e.g., *Rhizobacter* spp., *Pseudomonas* spp., and *Hyphomicrobium* spp.), indicating stronger selection pressures on the microbial population. In contrast, though MC effectively reduced the biofilm biomass to a level comparable to that of FC, it maintained a high diversity comparable to that of NC (dominated by *Sphingobium* spp. and *Nocardioides* spp.), reflecting weaker selection pressure on bacterial community. Temporally, biofilm communities under all conditions started from nearly identical states. From week-19 and week-36 onwards, deterministic processes predominantly governed biofilm formation under FC and NC conditions, signifying that these biofilms reached a stable state. Differently, under MC condition, the community assembly was continually influenced by stochastic processes, with the biofilm not achieving stability until week-56. Overall, this study provides valuable insights into the long-term dynamics of biofilm development and evidenced that FC is better than MC in controlling biofilm formation, particularly from the community diversity perspective. This challenges classical views that MC is more effective than FC in penetrating and controlling biofilm, which may change the popularity of MC as a disinfectant in water utilities.

1. Introduction

Biosafety of drinking water is critical for all modern societies, for which the biofilm in distribution systems poses significant aesthetic and public health concerns, such as taste and odor issues (Zhou et al., 2017), discoloration problems (Boxall et al., 2023; Pick et al., 2021), pipe corrosion (Jia et al., 2025; Li et al., 2024), microbial regrowth (Cruz et al., 2020; Lin et al., 2025), and the potential to harbor opportunistic pathogens (Siponen et al., 2024; Waak et al., 2019b). To control biofilm formation in drinking water system, applying and maintaining disinfectant residual is the widely used approach worldwide (Dai et al., 2020; Waak et al., 2019a), though chlorine free water supply is achieved in some European countries by delivering biostable water with extremely low nutrients (e.g., AOC < 10 μg/L, the Netherlands) (Rosario-Ortiz et al., 2016; Smeets et al., 2009). However, a recent study indicates that 9–45 million Americans who rely on community water systems are affected annually by health-based water quality violations, with the majority of these violations caused by coliform bacteria (37 %) and disinfection by-products (DBPs, 25 %) (Allaire et al., 2018).

Free chlorine (FC) and monochloramine (MC) are the most popular

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disinfectants adopted worldwide. FC has been extensively used since the early 1900s (Galal-Gorchev, 1996), after which waterborne disease outbreaks became rare. MC is a weaker oxidizing agent than free chlorine. It has gained popularity, particularly in large megacities, because it forms fewer regulated DBPs, persists longer in water distribution systems (DWDSs), and penetrates better into biofilm (Lee et al., 2018, 2011). However, MC may promote nitrification due to ammonia presence during its formation or decay (Cruz et al., 2020; Furst et al., 2024), and high number of putative pathogenic bacteria could persistent in MC systems (Krishna et al., 2021). Therefore, for water utility, the choice between FC and MC has never been an easy decision. For example, for the megacities in China, Beijing transferred from MC to FC in 2019, Shanghai is using MC, while Tianjin is preparing to transfer from MC to FC.

There have been studies comparing FC and MC regarding their performance on controlling biofilm (Ke et al., 2024; Li et al., 2020; Siponen et al., 2025). A major limitation of available studies has been the lack of a long-term and dynamic assessment covering the development of both microbial quantity and community of biofilm. Previous studies mostly cultured biofilm without applying disinfectant for certain periods (5 – 10 weeks), after which FC and MC were added and the biomass and/or the biofilm community were analyzed (Ke et al., 2024; Siponen et al., 2025). Valuable knowledge has been obtained, but the biofilm formation process is fundamentally different from what happens in the field environment, such as the composition and concentration of assimilable organic carbon that biofilm grow on, and the selection and succession of community members. Moreover, from both microbiological and chemical perspectives, using dechlorinated water as blank is different from unchlorinated water. In this study, biofilm development under different disinfectant regimes (no chlorine, NC; free chlorine, FC; and monochloramine, MC) was followed for 56 weeks. The growth curve was assessed by quantifying biofilm with flow cytometry. The microbial community was analyzed by using targeted high-throughput 16S rRNA gene sequencing. The findings from this study are expected to offer new insights into biofilm development and management. More importantly, the comparison between FC and MC could provide actionable evidence for water utilities to optimize disinfectant selection for controlling biofilm proliferation and mitigating microbial risks in distribution systems, which are critical to sustaining water quality.

2. Materials and methods

2.1. Pilot drinking water distribution system

A new pilot system was built at one of the treatment plants of Oasen in the Netherlands. The pilot system consisted of 9 parallel pipelines with each of triplicate pipelines developed under unchlorinated (NC), free chlorine (FC, 0.1 mg/L) and monochloramine (MC, 0.4 mg/L) applied conditions, respectively (Fig. 1). Each pipeline was constructed with 40 segments of new 20 cm PVC pipes (D = 32 mm) with a total length of ~ 12 m. The system was operated at the flow velocity of 0.05 m/s (144 L/h, ~ 5 min retention time) in each pipeline and the pressure of ~ 2 bar, with the water flowing continuously from the inlet to outlet and being discharged directly. The system was supplied with treated water with extremely low AOC (10–15 μg/L) from the treatment plant, where groundwater is used as the source water and treated by conventional treatment processes (i.e., spray aeration, rapid sand filtration, pellet softening, carry-over submerged rapid sand filtration, granular activated carbon filtration and UV disinfection) without disinfectants. The system was maintained at 12~13 °C during the experiments, reflecting the stable temperature range typical of groundwater-sourced distribution systems (Agudelo-Vera et al., 2020). Prior to the start, the

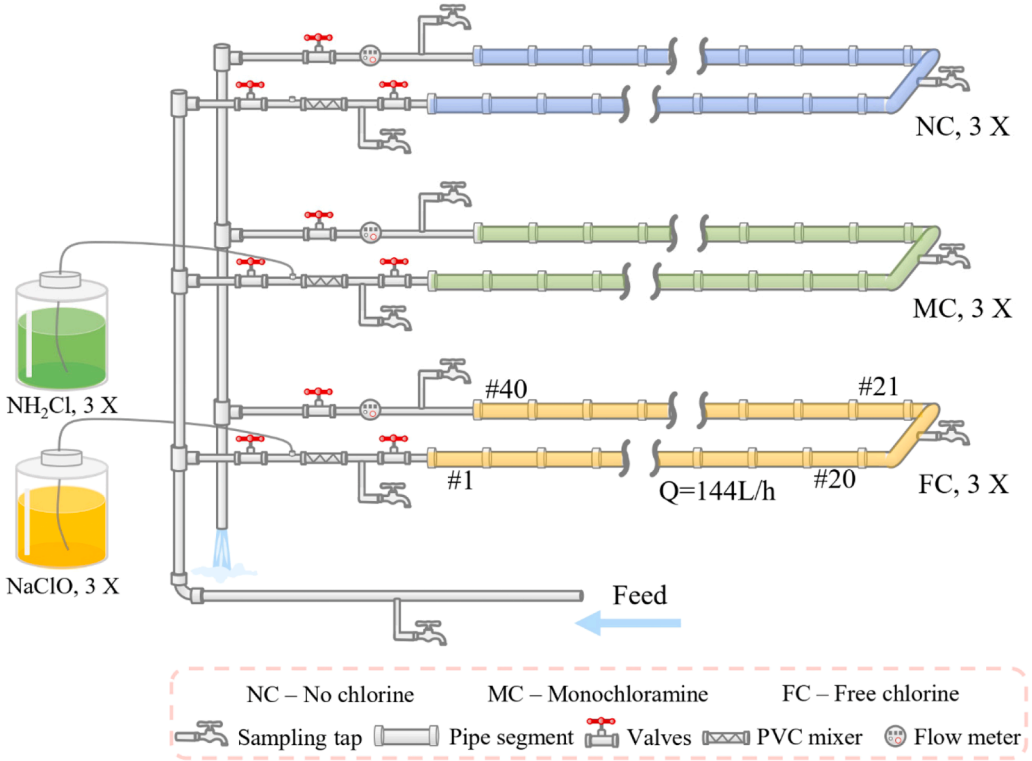

Fig. 1. The design of the pilot system. *The pilot system comprised nine parallel pipelines, arranged in triplicates for three treatment conditions: no chlorine (NC, 3X, blue), monochloramine (MC, 3X, green), and free chlorine (FC, 3X, yellow). Each pipeline consisted of 40 segments (#1–40) of new 20* cm *PVC pipes (D = 32* mm*), with a total length of ~ 12* m*. The system was operated at a flow rate of 144* L/h *in each pipeline and the pressure of ~ 2* bar*, with continuous water flow. Treated water with very low assimilable organic carbon levels (10–15* μg/L*) and no additional disinfectants, sourced from a groundwater treatment plant, was used. Stock solutions (NaClO and* NH_2Cl*, 3X) were refreshed every three days, and dosing flow rates were controlled at 250–300* mL/min *to maintain target free (0.1* mg/L*) and total chlorine (0.4* mg/L*) concentrations. PVC mixers ensured thorough mixing of chemicals and feed water, and sampling taps and pipe segments were utilized for water and biofilm sampling.*

pipelines were flushed with 20 mg/L sodium hypochlorite for 24 h at the maximum flow rate (~ 0.24 m/s) to disinfect the system and flushed afterwards with fresh treated water at the maximum flow rate until the chlorine was no longer detected. The physicochemical properties of the feed water were shown in Table S1.

The stock solution of free chlorine was prepared by directly diluting the commercial sodium hypochlorite (60 – 185 g/L active chlorine content), while monochloramine was prepared by the sequential addition of chlorine and ammonia at a Cl_2:N mass ratio of 4:1 and pH at 8 with slow stirs. The chemicals were prepared and refreshed every three days. The flow rate of dosing was controlled at 250–300 mL/min to obtain the target free (0.1 mg/L) and total chlorine (0.4 mg/L) concentration in the system (Fig. S1). The selection of concentrations for both free chlorine and monochloramine was based on the consideration of the low AOC content in the feed water (Ohkouchi et al., 2013). A static PVC mixer (~ 20 cm, Stock Schedule 80 Threaded PVC Mixer, Koflo Corporation, USA) was installed in each pipeline immediately after the dosing point to ensure complete mixing of the disinfectants and feed water. In addition, the free and total chlorine concentration was measured every 1–2 days to ensure the target concentration (Fig. S1). The system was operated for 14 months (56 weeks) during the experiments.

2.2. Sample collection

After the sampling of the bulk water for physicochemical analysis, the system was drained immediately for biofilm sampling. Biofilm samples were collected by swabbing the inner surfaces of the pipe segments every three to four weeks. At each sampling point, one pipe segment was removed for biofilm analysis and replaced with a new segment in each pipeline. The segments were collected sequentially, starting from the end (#40) and progressing toward the front (#21) of each pipeline, where free/total chlorine concentrations were maintained at their target levels (Fig. S1). Specifically, triplicate biofilm samples were collected under each condition at each time point, with each sample obtained from one of the triplicate pipelines per condition, ensuring reliability and representativeness. In addition, biofilm samples were swabbed in circles to avoid the uneven distribution of the biofilm on the inner pipes using sterile swabs in a short time (~ 5 min). For each pipe segments, the surface area swabbed for the intact cell count analysis was ~ 4 cm^2, while the rest of the surface area (~ 200 cm^2) was swabbed for DNA extraction.

2.3. Physicochemical and microbiological analysis

2.3.1. Water quality analysis

Free and total chlorine concentrations in the bulk water samples taken from midpoint (pipe segment #21) and outlet portal were determined by the N,N-diethyl-para-phenylenediamine (DPD) method using a Hach DR300 Pocket Colorimeter (Hach Company, Loveland, CO, USA) with a detection range of 0.02–2 mg/L Cl_2. Dissolved oxygen (DO), pH, temperature (T), and electrical conductivity (EC) were measured on site by WTW™ MultiLine™ 3420 Portable Digital Multiparameter (WTW GmbH, Germany). TOC was measured by TOC analyzer (TOC-V CPH, SHIMADZU, Japan). The concentrations of ammonia, nitrite, and nitrate were measured by ion chromatography using a Dionex IC-3000 system (Dionex, Sunnyvale, CA).

2.3.2. Intact cell counts measurements

All the biofilm samples were pre-treated through a low energy ultrasonic treatment performed 3 times, for 2 min each (Branson ultrasonic water bath, 43 kHz, 180 W power output, 10 L sonication chamber) before the intact cell count (ICC) measurements. Subsequently, the ICC was measured by using the Bactosense flow cytometry in manual mode with the LDC-Live/Dead Count cartridge, in accordance with the manufacturer's instructions (Manickum, 2020). The flow cytometer operates with a 488 nm laser diode and employs fluorescence detection channels FL1 (535/43) and FL2 (715 LP), along with side scatter (SSC) detection at 488/10. The instrument has a detection limit ranging from 100 to 5 million cells/mL.

2.4. DNA extraction and 16S rRNA gene sequencing

The DNA across all the samples was extracted through the FastDNA Spin Kit for Soil (Q-Biogene/MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. The V3-V4 hypervariable regions of the 16S rRNA genes were amplified before sequencing using the 341F-785R primer set (341F: 5′-CCTACGGGNGGCWGCAG-3′; 785R: 5′-GACTACHVGGGTATCTAATCC-3′). Paired-end sequencing of the amplicons (2 × 300 bp) was performed on an Illumina Miseq platform by BaseClear (Leiden, the Netherlands). The sequencing data have been deposited in the NCBI database, with reference code PRJNA966936.

2.5. Sequencing analysis

The bacterial 16S rRNA gene amplicons were processed using the Quantitative Insights Into Microbial Ecology (QIIME2, v2020.11) pipeline with default settings (Caporaso et al., 2010). DADA2 was used for filtering, dereplication, sample inference, chimera identification, and merging of paired-end reads (Callahan et al., 2016). As a consequence, unique amplicon sequence variants (ASVs) that were equivalent to 100 % similarity operational taxonomic units (OTUs) in the conventional practice were generated. The taxonomy assignment was complemented using the q2-feature-classifier with Silva SSU database release 132 (Quast et al., 2012). Multiple sequence alignment and phylogenetic tree construction were performed using the QIIME 2 plugin q2-phylogeny. Alpha and beta diversity analyses were performed using the QIIME 2 plugin q2-diversity with a threshold of 6095. Principal coordinates analysis (PCoA) was conducted based on Bray-Curtis distance to assess community dissimilarity within biofilm across sampling time periods and conditions. Significant differences in biofilm communities across different groups were assessed using PERMANOVA (Permutational multivariate analysis of variance) with 999 permutations calculated per test. The differences were considered significant when the p-value was lower than 0.05 ($P < 0.05$).

2.6. Null model analysis

To disentangle the relative importance of deterministic and stochastic processes underlying microbial community assembly, Raup-Crick (RC) based on Bray−Curtis dissimilarities were calculated (Stegen et al., 2013). The RC index values range between −1 and 1. |Values| > 0.95 represents that the community assembly was dominated by deterministic processes, whereas |values| < 0.95 indicates that stochastic processes dominated in the community assembly. The modified index-normalized stochasticity ratio (MST) was determined to further quantify the relative contributions of deterministic and stochastic processes in the community assembly, with 0.5 as the boundary point between more deterministic (<0.5) and more stochastic (>0.5) assemblies (Ning et al., 2019). The MST analysis was conducted in R using the package "NST" (Ning et al., 2019).

3. Results

3.1. Physicochemical water quality

In summary, there were no significant differences in the physicochemical parameters among these three conditions and from different sampling points (i.e., feed, inlet, outlet), except for ammonia. Specifically, TOC, water temperature, and pH were maintained relatively stable at 6.1 ± 0.6 mg/L, 12~13 °C, and 8.2 ± 0.1, respectively, during distribution under each condition for the entire experimental period. In

addition, nitrite and nitrate concentrations were 0.002 ± 0.002 and 13.8 ± 0.6 mg/L on average in the systems regardless of conditions. However, the ammonia concentration under the MC condition was significantly higher (29 times) than the other two conditions, with the increase observed immediately after the addition of monochloramine.

3.2. Development of biofilm with and without disinfectants quantified by ICC

As shown in Fig. 2, during the period of 56-week, the highest biofilm biomass was formed under NC condition (on average 1.7 ± 0.4 × 10^6 cells/cm^2), followed by FC (on average 2.6 ± 0.0 × 10^5 cells/cm^2) and MC (on average 1.4 ± 0.8 × 10^5 cells/cm^2) conditions. In general, the biofilm biomass increased over time, but with different trends under different disinfection conditions. Specifically, under NC condition, the ICC in biofilm consistently increased up to week-32, then slightly decreased to week-36, and remained relatively stable thereafter. While under FC condition, ICC increased slightly till week-19, after which the increase was consistent until week-36, and then decreased since week-40, whereafter ICC remained at a relatively low level. Similarly, in the MC condition, slight increases in ICC in biofilm were observed until week-44, then remained at a relatively low level that around the same range with FC condition.

3.3. Development of microbial communities of biofilm with and without disinfectants

In total, 3052,883 sequences were obtained from all the biofilm samples (n = 117). Rarefaction was performed prior to the alpha and beta diversity analysis by subsampling at an even sampling depth of 6095 sequences. The rarefaction curves reached a plateau after 3000 sequences, indicating enough sample coverage was obtained in this study (Fig. S2).

Alpha diversity. The number of observed ASVs was used as an indicator to represent the alpha diversity. As shown in Fig. 3A, the number of observed ASVs was the highest under NC condition (657 ± 33), followed by MC (644 ± 134) and FC (166 ± 44) conditions at week-56. Over time, the changes of observed ASVs numbers in biofilm showed different trends. Specifically, rapid increases were observed under both NC (595 ± 79) and MC (737 ± 123) conditions from week-0 to week-11, indicating the elevated diversity of biofilm communities during the initial development periods in both systems. Nevertheless, there was no such sharp increase under FC condition during the initial stage. Moreover, the differences in the temporal trends were more pronounced under different conditions. Under NC condition, a second peak in the number of observed ASVs was observed at week-32, followed by a rapid decrease till week-40 and waves during week-44 to week-56. For MC

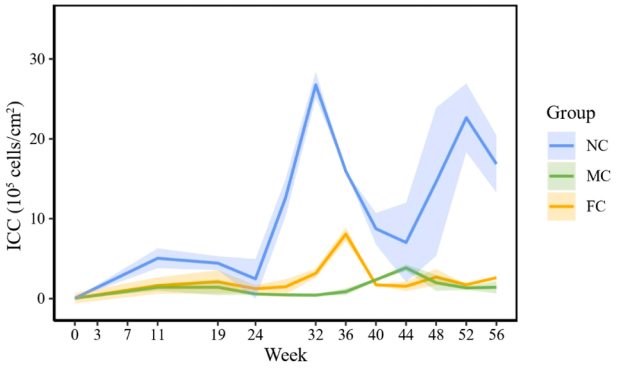

Fig. 2. Biomass in biofilm quantified by ICC. *Development of biofilm over the period of 56-week under unchlorinated (NC), monochloramine (MC), and free chlorine (FC) conditions. Line plots represent mean values with error bands (mean ± s.d., n = 6).*

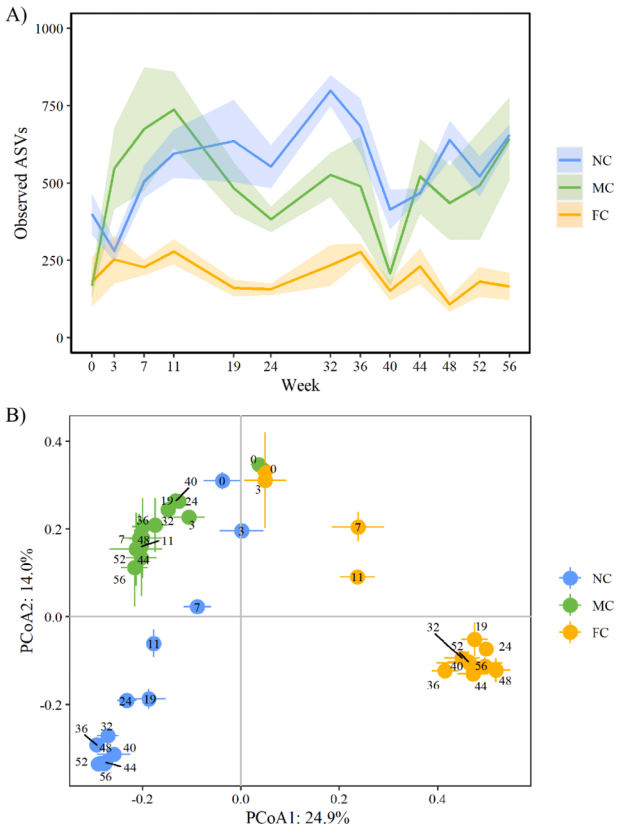

Fig. 3. Diversity of microbial communities over time under NC, MC, and FC conditions. *A) changes of the number of observed ASVs, represented as line plots showing the mean values with error bands (mean ± s.d., n = 3); B) PCoA plot based on Bray-Curtis distances, with numbers indicate the sampling time in weeks and error bars on data points represent the standard error of the mean (n = 3).*

condition, strong decreases were found from week 11 to 24, followed by waves thereafter, with comparable community diversity to the NC condition at the end of this study. Differently, throughout the experimental period of 56 weeks, the number of observed ASVs under FC condition remained relatively stable at a much lower level compared to the other two conditions.

Beta diversity. As illustrated by PCoA plot based on Bray-Curtis distances, the starting point of biofilms under different conditions showed highly similar microbial communities, reflecting the relatively undiversified biofilm communities established at the early stage of the development (Fig. 3B). With the increase of operation time, the changes of microbial communities happened earlier under MC and NC conditions, for which differences were already observed at week-3. Differently, the microbial communities under the NC condition stabilized at week-36, while those under the MC condition continued to change until the end of the experimental period. For the FC condition, the microbial communities showed no clear variations until week-7, indicating that their diversification occurred later than in both the MC and NC conditions. However, the microbial communities in the FC condition became stabilized starting from week-19, which was much earlier than in the MC and NC conditions. By the end of week-56, three clusters were formed for MC, FC, and NC, with samples from the MC clustered closer to those from the NC than those from the FC (Figs. 3B and S3A). Furthermore, the dissimilarities of microbial communities for samples from each two successive time points were progressively decreased over time regardless of conditions (Fig. S3B).

Community composition. As shown in Fig. 4, the microbial community composition of biofilms under different disinfection conditions were clearly different, which is consistent with the above-mentioned variations in community diversity. At phylum level, throughout the

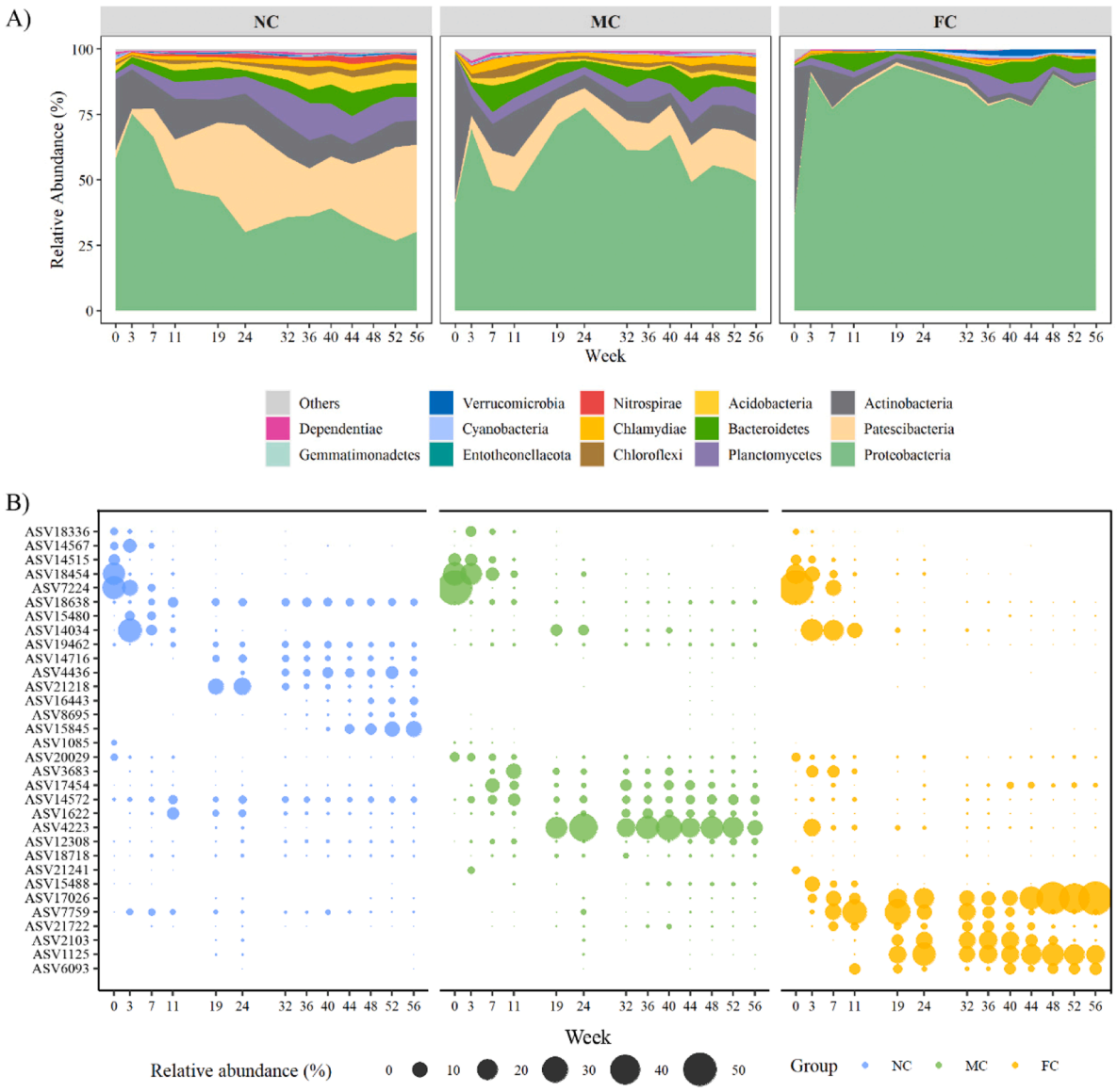

Fig. 4. Bacterial community composition at phylum and ASV levels. *(A) Variations in community composition over the course of time under unchlorinated (NC), monochloramine (MC), and free chlorine (FC) conditions at phylum level; (B) Variations in dominant ASVs (top 10) over the course of time under the three conditions* (i.e., *NC, MC, FC). The detailed taxonomy information of these ASVs were shown in Table S2.*

observation period, Proteobacteria (30 ± 1 %) and Patescibacteria (33 ± 2 %) were the two dominant phyla under the NC condition, while Proteobacteria dominated the biofilm communities under MC (50 ± 13 %) and FC conditions (88 ± 3 %) (Fig. 4A). Regarding succession dynamics in community composition, clear differences were observed among different disinfection conditions. Specifically, under NC condition, Proteobacteria dominated the early stage till week-19, whereas Patescibacteria progressively became dominant afterwards. In both MC and FC conditions, Proteobacteria dominated consistently over time, which sharply increased in the first 3 weeks. Differently, Proteobacteria maintained its dominant role throughout the study period (82 ± 14 %) under the FC condition. However, under the MC condition, although Proteobacteria remained dominant, its abundance fluctuated and was lower compared to the FC condition.

At ASV level, all biofilm started with same dominant ASVs (e.g., ASV7224, ASV14515, ASV18454), but succeeded by different ASVs under different conditions (Fig. 4B). Specifically, under NC condition, ASV14034 and ASV15480 (*Sphingobium* spp.), ASV7224 (*Rhodococcus* spp.), and ASV14567 (*Ferribacterium* spp.) dominated the first 7 weeks, followed by ASV21218 (o_Saccharomidales) and ASV14716 (*Pseudonocardia* spp.) since week-19, which were taken over by ASV15845, ASV4436, ASV8695, and ASV16443 (o_Saccharomidales) after week-44. Under MC condition, ASV14572 (*Nocardioides* spp.), and ASV17454 (f_Sphingobacteriaceae) predominated during the early stage until week-11, whereas ASV4223 (*Sphingobium* spp.) and ASV1622 (o_Saccharimonadales) became dominated since week-19, with ASV14572 and ASV17454 consistently dominated at the later stages. Under FC condition, ASV14034 and ASV4223 (*Sphingobium* spp.), ASV7759 (f_Sphingomonadaceae), ASV15488 (*Blastomonas* spp.), and ASV17026 (f_Burkholderiaceae) dominated the initial stage till week-11. Afterwards, ASV1125 (*Rhizobacter* spp.) and ASV2103 (*Hyphomicrobium* spp.) became dominant together with ASV17026 and ASV7759 until week 48. Thereafter, ASV17026 and ASV1125 and ASV6093 (*Rhizobacter* spp.) dominated. The detailed taxonomy information of the dominant ASVs is shown in Table S2.

3.4. Temporal dynamics of microbial community assembly and succession

The role of deterministic and stochastic processes in shaping microbial communities was assessed and shown in Fig. 5. Under NC

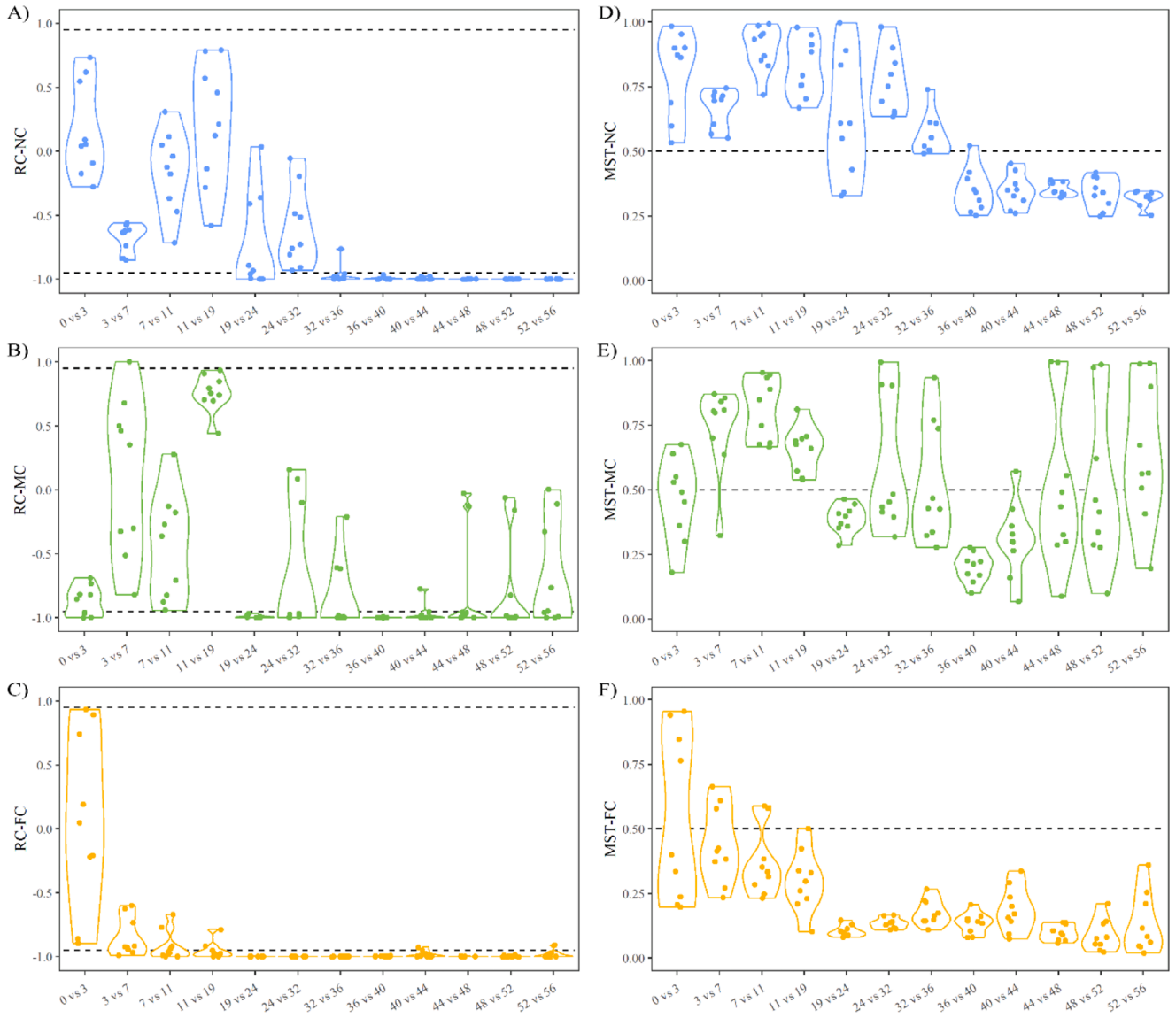

Fig. 5. Microbial community succession dynamics. *Raup-Crick dissimilarity (RC, A-C) and modified stochastic ratio (MST, D–F) based on Bray-Curtis distances under different conditions over time generated through the comparisons between two successive sampling times. Blue represents unchlorinated (NC), green and orange represents monochloramine (MC) and free chlorine (FC) conditions. Horizontal dotted lines indicate thresholds for significant deviations from the null expectation, −0.95 and +0.95 for RC and 0.5 for MST.*

condition, the stochastic processes played greater roles till week-36 with RC |values| ⟨ 0.95 and MST values ⟩ 0.5, while the deterministic processes progressively dominated thereafter. It should be noted that the increases in the relative contributions of deterministic processes were observed since week-19. Differently, under MC condition, stochastic processes remained relatively high throughout (RC |values| ⟨ 0.95 and MST values ⟩ 0.5), with more deterministic processes occurred since week-19 (RC values <-0.95 and MST values < 0.5). Interestingly, under FC condition, deterministic processes consistently dominated throughout the entire period (RC values <-0.95 and MST values < 0.5), although some stochastic processes were detected between week-3 to week-11 (RC |values| < 0.95 and MST values > 0.5). This means that chlorination changed the driving force, while MC and FC placed different influences on the microbial community assembly and succession. In other words, in the absence of chlorine, the microbial community assembly shifted from being primarily driven by stochastic processes to being dominated by deterministic processes after 36 weeks. Under the MC condition, community assembly was primarily influenced by stochastic processes, whereas deterministic processes dominated under the FC condition.

4. Discussion

In this study, we monitored biofilm development in simulated drinking water distribution systems (DWDSs) over 56 weeks under different chlorine conditions: no chlorine (NC), free chlorine (FC), and monochloramine (MC). To the best of our knowledge, this is the first study to systematically examine biofilm development over an extended period, focusing on the dynamics of bacterial quantity and community under NC, MC and FC conditions. The findings provide novel insights into our understanding of biofilm development and management.

4.1. Impact of disinfectants on the development of biofilm

Taking NC condition as control, this study demonstrated that the application of chlorine-based disinfectants, either MC or FC, significantly inhibited biofilm growth and shaped microbial communities. The capacity of FC and MC to suppress drinking water biofilm growth is well-

documented (Clayton et al., 2021; Shen et al., 2017). However, the long-term effects of these disinfectants on the development of microbial communities within biofilm are less understood. This study showed that MC supported more diverse microbial communities than FC (observed ASVs, Fig. 3A), suggesting that FC exerts stronger selective pressure on populations in biofilm, which might be attributed to its potent oxidizing property causing lethal cell membrane and DNA damages (Lee et al., 2018, 2011; Liu et al., 2016). Compared to FC, MC is less oxidative and damages bacterial membranes and DNA slowly, which may potentially drive cells into a dormancy state and result in lower cell proliferation but higher community diversity. Chen et al. reported that a higher percentage of cells entered the viable but non-culturable (VBNC) state under MC treatment than under FC treatment, using the same dose (Chen et al., 2018). Similarly, Ng et al. confirmed the presence of VBNC cells under MC conditions (Ng et al., 2021). However, further work is needed to confirm whether dormancy mechanisms (e.g., VBNC states) underlie this pattern. Interestingly, in the present study, the number of observed ASVs under MC condition was almost the same as that of NC condition, which is conflicted with some of previous studies (Aggarwal et al., 2018; Waak et al., 2019a). This may be due to the lower MC concentration, which resulted in higher diversity, or it may be because the low AOC concentration limited bacterial community diversity under the NC condition. As reported, higher MC concentrations have been associated with lower biofilm community diversity (Mi et al., 2015), while significantly reduced biofilm community diversity has been observed under low AOC concentrations (Pick, 2019).

Considering the composition of bacterial communities, FC and MC clearly shaped the dominant members overtime (Fig. 4B). Specifically, FC biofilm was rapidly dominated by Proteobacteria (e.g., *Rhizobacter* spp., *Pseudomonas* spp., and *Hyphomicrobium* spp.), which are known for their chlorine resistance (Gomez-Alvarez et al., 2012; Mi et al., 2015; Williams et al., 2004). MC supported a more diverse array of dominant species, including *Sphingobium* spp. and *Nocardioides* spp., which are known to be resistant to monochloramine (Gomez-Alvarez et al., 2012). The presence and dominance of *Sphingobium* spp. in MC biofilm may be linked to the increased ammonia concentration resulting from adding MC (Liao et al., 2015; Potgieter et al., 2020; Revetta et al., 2013). Notably, ammonia-oxidizing and nitrite-oxidizing bacteria were not abundant in the MC biofilm, which may be due to the well-controlled low temperature (12–13 °C), as these species generally prefer higher temperatures (25–30 °C) (Zhang et al., 2009).

Besides, this 56-weeks' study revealed the dynamics of microbial community assembly and succession under NC, MC and FC conditions. All conditions initiated from (almost) identical states with the attachment of bacteria that mainly affiliated to Proteobacteria (e.g., *Massilia* spp.) and Actinobacteria (e.g., *Rhodococcus* spp.) (Fig. 4B), which have been commonly found as initial colonizers in drinking water biofilms (Biggs et al., 2013; Douterelo et al., 2018; Fish and Boxall, 2018). It is likely that these pioneering microbes dispersed and attached to the pipe surfaces as stochastic process. Afterwards, the deterministic process dominated FC biofilm and NC biofilm since week-19 and week-36, indicating biofilms entered stable stage onwards. The observed stabilization timeline of NC biofilm aligns with previous study, which found the number of bacteria within biofilm formed under low AOC (6 μg/l) and NC condition stabilized after 200 days (Boe-Hansen et al., 2002). The much shorter stabilization time of FC biofilm might be attributed to the strong selection pressure of FC, and the lower microbial diversity might be easier to establish stable state (Coyte et al., 2015). Differently, for MC biofilm, the community assembly was consistently governed by stochastic process, which did not reach stable stage till week-56. This may be because of the weak selection pressure of MC, coupled with elevated ammonia levels that foster biofilm development (Cruz et al., 2020; Lipponen et al., 2002).

4.2. Free chlorine vs. monochloramine: which is better?

Quantitatively, compared to NC, both MC and FC effectively controlled biofilm formation. MC performed slightly better than FC, which reduced 7.1 % more (0.3 log) biomass as quantified by cell numbers. This agrees with previous studies that found MC penetrated biofilm better than FC, and therefore, remove 2 logs more biofilm (Türetgen, 2004). However, it should be noted that previous studies mostly colonized biofilm for certain period (30 – 70 days) before applying MC or FC (Gagnon et al., 2004; Ke et al., 2024). Such approach may result in different findings comparing to biofilm formed continuously under MC/FC from the very beginning, because the protection function of biofilm layer/thickness that prevent disinfectant penetration would not be in place. In field distribution system, the same as simulated in the present study, biofilm is formed under continuously MC or FC pressure.

From the perspective of microbial populations, the results were completely contradictory. FC sharply reduced the number of observed species (657 vs. 166 ASVs), while MC had no significant effects on the number of observed species (657 vs. 644 ASVs). This means that FC can reduce both the number of cells and species, but MC only reduced the number of cells while maintained almost the same number of species in biofilm. Previously, studies also reported a higher number of observed species (OTUs) in MC biofilm than the biofilms either under dechlorinated (Aggarwal et al., 2018) or FC condition (Li et al., 2020). The previous studies that found lower number of observed species in MC biofilm cultured biofilm first before removing biofilms by applying disinfectants (Ke et al., 2024; Williams et al., 2005), which were fundamentally different from biofilm formation under continuously MC condition from the very beginning.

Besides, the present dynamic study allowed us to explore community assembly mechanisms under different conditions. The deterministic process governed FC biofilm took only 19 weeks to establish stable state, which did not happen until 56 weeks later under MC condition. Such continued influence of stochastic processes under MC condition suggests that the community is still subject to random immigration events between biofilm and planktonic bacteria. Though very little is known about the potential impacts of bacterial diversity on biofilm pathogen survival (Revetta et al., 2013), the low selection pressure from MC may offer higher chance for (opportunistic) pathogen to live or maintain VBNC state, which pose potential risks to public health. This may necessitate more frequent management interventions to maintain biofilm stability.

4.3. Practical implications

This study is the first to examine and compare the effects of FC and MC on biofilm development in a parallel simulated distribution systems over an extended period, using Dutch drinking water that has not previously been exposed to disinfection-based selection pressures. In summary, both FC and MC efficiently controlled biofilm growth and markedly influenced its microbial community assembly. Specifically, FC dramatically reduced both biofilm biomass and diversity, while also accelerating biofilm stabilization, as indicated by changes in the biofilm community structure. In contrast, MC reduced biomass effectively to a similar level of FC biofilm but resulted in higher biofilm diversity and delayed stabilization, indicating weaker selection pressure on bacterial communities and raising concerns about pathogen disinfection efficiency. Altogether, comprehending the dynamics of biofilm succession is essential for the development of future strategies for monitoring and managing biofilms. Our results suggested that FC biofilm has a better predictability and manageability than MC biofilm. From a sustainability and safety perspective, minimizing reliance on residual disinfectants is highly beneficial. In countries like the Netherlands, biofilm growth is effectively controlled by delivering bio-stable drinking water. However, this requires access to high-quality source water, advanced treatment

technologies, and stringent management practices. When these conditions cannot be met, residual disinfectants remain essential. Based on the findings of this study, we argue that free chlorine could be better solution than monochloramine for biofilm control, particularly from the community diversity perspective. Given the challenges associated with DBPs produced by FC, the findings from this study suggest that reducing DBP precursors may be more effective than simply replacing chlorine with monochloramine. Recent advancements in artificial intelligence could offer precise control over chlorine concentrations, further optimizing the balance between effective disinfection and minimizing DBP formation.

However, the current study did not investigate the pathogenicity of chlorine/monochloramine-resistant species within biofilms, which could potentially be released into bulk water during distribution, leading to significant concerns. This limitation arises from the constraints of 16S rRNA sequencing (Dai et al., 2020; Sevillano et al., 2020). To address these issues more comprehensively, future research utilizing multi-omics approaches and qPCR techniques targeting waterborne pathogens and gene expression is strongly recommended. It is important to note that this study focuses on a single temperature regime (12–13 °C). While our approach allowed for a rigorous comparison of the impacts of FC and MC under stable conditions, it does not provide direct insights into biofilm behavior under warmer temperatures. Elevated temperatures may promote nitrification in MC-treated systems or accelerate disinfectant decay, thereby altering biofilm community assembly. Future investigations examining temperature-dependent effects on biofilm formation are needed to broaden the applicability of these findings. Additionally, while our experiments were conducted under stable hydraulic conditions, real-world distribution systems often face fluctuating water quality and hydraulic regimes (e.g., stagnation, flow surges). These dynamic conditions are known to influence biofilm detachment and subsequent contributions to bulk water (Chen et al., 2023). Building on this work, future investigations could explore how biofilms developed under FC or MC disinfection interact with bulk water during hydraulic disturbances, providing critical insights into system resilience and effluent quality under operational stressors.

5. Conclusion

A newly-built pilot drinking water distribution system was monitored over 56 weeks to comprehensively study biofilm development in terms of bacterial quantity, community composition, and community assembly under different disinfectant regimes (i.e., no chlorine - NC, free chlorine - FC, and monochloramine - MC). The following conclusions can be drawn from this study:

- Both FC and MC significantly inhibited biofilm growth and altered community composition, but their impacts differed substantially. FC exerted a stronger selective pressure, resulting in a more homogenous and less complex biofilm community. In contrast, MC allowed a more diverse microbial community to persist.
- Specifically, biofilms under FC were primarily dominated by Proteobacteria, such as *Rhizobacter* spp., *Pseudomonas* spp., and *Hyphomicrobium* spp.. Meanwhile, biofilms exposed to MC hosted a broader range of taxa, including *Sphingobium* spp. and *Nocardioides* spp..
- Temporally, biofilm communities across all disinfection conditions began similarly. However, from week-19 and week-36, deterministic processes primarily drove biofilm development under FC and NC conditions, leading to community stabilization. In contrast, the biofilm under MC remained influenced by stochastic processes, with the biofilm not achieving stability until week-56.

While this study provides valuable insights into the long-term dynamics of biofilm development under different disinfection conditions, challenging the classical view that MC is more effective than FC in controlling biofilms, some critical aspects remain unexplored. Future research should examine the formation of disinfection by-products, the dynamics of bacterial communities in the water phase, and the influence of temperature on biofilm development. These factors could further clarify the broader implications of disinfection strategies on water quality and potential health risks in drinking water distribution systems.

CRediT authorship contribution statement

Lihua Chen: Writing – original draft, Visualization, Validation, Methodology, Formal analysis. **Haoran Shi:** Validation, Investigation, Data curation. **Gertjan Medema:** Writing – review & editing, Supervision, Conceptualization. **Walter van der Meer:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Gang Liu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123566.

Data availability

Data will be made available on request.

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