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Proliferation of opportunistic premise plumbing pathogens in water treatment plants: Occurrence patterns and influencing factors

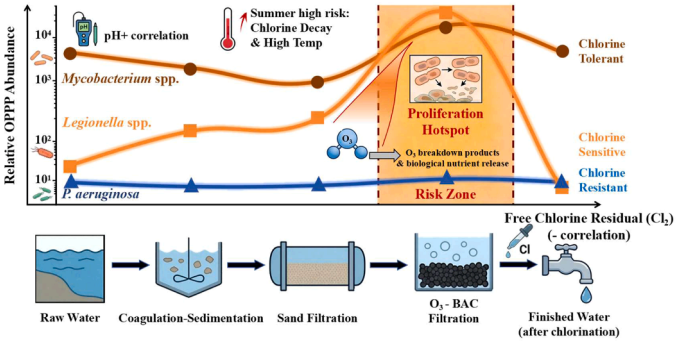
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HIGHLIGHTS

- Tracking the proliferation of OPPPs across full drinking water treatment processes.
- Identifying O_3-BAC filters as critical hotspots for OPPP proliferation.
- Revealing varying chlorine sensitivity: *P. aeruginosa* is highly resistant.
- OPPP occurrence is linked to water quality and microbial community dynamics.
- Providing insights for targeted OPPP control, emphasizing seasonal variations.

GRAPHICAL ABSTRACT



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ABSTRACT

Opportunistic premise plumbing pathogens (OPPPs), including *Mycobacterium* spp., *Legionella* spp., and *Pseudomonas aeruginosa*, pose emerging biosafety concerns in urban water supply systems, particularly for immunocompromised populations. While comprehensive quantitative microbial risk assessment (QMRA) requires dose-response data, this study systematically tracks the proliferation dynamics of these pathogens across full-scale treatment trains in four drinking water treatment plants (DWTPs) over four seasons. Water samples were collected from the intake, coagulation-sedimentation, sand filtration, activated carbon filtration, and finished water stages and analyzed via quantitative PCR (qPCR) with propidium monoazide (PMA) pretreatment to target membrane-intact cells. The results demonstrated that conventional treatment processes inadequately control OPPPs, with the ozone-biological activated carbon (O_3-BAC) stage identified as a critical hotspot for pathogen regrowth, which is likely driven by increased microbial activity and organic carbon utilization. Disinfection via chlorination exhibited variable efficacy as measured by PMA-qPCR: while *Legionella* spp. were relatively susceptible, *P. aeruginosa* demonstrated higher apparent persistence under chlorination, remaining detectable in finished water. Correlation analyses further linked the occurrence of OPPP to specific water quality parameters and shifts in the microbial community structure. These findings underscore the need for optimized treatment strategies that account for seasonal variations and target OPPP regrowth niches, thereby advancing biosafety control in the drinking water supply.

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1. Introduction

Among the pathogens that pose biosafety risks to water supply systems in urban areas, opportunistic premise plumbing pathogens (OPPPs) are frequently reported and widely distributed (Proctor et al., 2022). OPPPs primarily include *Mycobacterium* spp. (notably *nontuberculous mycobacteria*, NTM), *Legionella* spp. (represented by *Legionella pneumophila*), and *Pseudomonas aeruginosa*. Although they are generally less virulent than common pathogens such as *Escherichia coli* and *Staphylococcus aureus*, OPPPs can cause severe infections in vulnerable populations, including immunocompromised individuals, the elderly individuals and children (Falkinham, 2021).

During the water treatment process, which theoretically aims to continuously remove suspended solids and impurities, including microorganisms, the abundance of OPPPs does not monotonically decrease. Studies have reported that the abundance of Proteobacteria, a phylum to which OPPPs typically belong, increases in the effluent of grid flocculation tanks and sedimentation tanks (Hou et al., 2018). Compared with the coagulation-sedimentation and sand filtration stages, the ozone-biological activated carbon (O_3-BAC) stage significantly alters the community structure, enhancing the microbial utilization of various organic carbon sources and potentially promoting the proliferation of OPPPs (Thom et al., 2022). With respect to the BAC filters, the species richness and diversity increase, and the filters contain a high abundance and diversity of OPPPs. Additionally, after O_3-BAC filtration, the proportion of Actinobacteria first decreased but then increased, with *Citrobacter, Acinetobacter*, and *Pseudomonas* becoming the dominant genera in the BAC samples, which was consistent with the dominant flora in the finished water. This suggests that OPPPs are not effectively removed during the O_3-BAC filtration process (Hou et al., 2018). Certain *Legionella* spp. present in the source water during winter were selectively enriched after preoxidation, despite their sensitivity to subsequent disinfection steps, indicating a possible role of ozone in the enrichment of OPPPs (Zhao et al., 2024). During the disinfection stage, when chlorination is used as an example, although the species richness and diversity decrease sharply after chlorination, Firmicutes and Actinobacteria become the dominant phyla in the finished water. This may be because chlorination stimulates the ecological niche advantage of Firmicutes and Actinobacteria, which include typical OPPPs such as *Mycobacterium* spp. It is important to note that the health risks of OPPPs are not determined solely by their removal within treatment plants; regrowth in distribution mains and premise plumbing is a critical factor. However, the initial abundance and physiological state of OPPPs in finished water are directly shaped by treatment processes. In particular, certain treatment units (e.g., O_3-BAC filters) may inadvertently promote OPPP proliferation, creating a "seed source" that enters the distribution system. Therefore, understanding the occurrence and proliferation dynamics of OPPPs within drinking water treatment plants (DWTPs) is a prerequisite for assessing and mitigating risks along the entire water supply chain. Currently, research on the presence levels and patterns of OPPPs during various stages of water treatment is lacking, and biosafety measures for water treatment still need improvement (Li et al., 2016; Lytle et al., 2021). This study addresses this gap by systematically tracking OPPPs across full-scale treatment trains in four DWTPs over four seasons, identifying process-specific hotspots, and exploring correlations with water quality parameters and microbial community structure.

2. Materials and methods

2.1. Sampling points and methods

Two megacities in eastern China (City A, East China; City B, southeastern coast) were selected. Their climate characteristics are shown in Table S1.

Four drinking water sources were sampled: Y1 (river water, City A), Y2 (reservoir water, City A), Y3 (reservoir water, City A), and Y4 (river water, City B). Correspondingly, four full-scale DWTPs (C1-C4) fed by these sources were investigated. The treatment processes of each DWTP are summarized in Table S2, and the sampling point codes are listed in Table S3.

Sampling was conducted quarterly over four consecutive seasons (March, June, September, December). Water samples were collected in sterile glass bottles and plastic buckets. Prior to sampling, glass bottles were autoclaved at 121 °C for 20 min, and plastic buckets were cleaned with 1 mol/L sodium hypochlorite solution, left to stand for 24 h, and then emptied. During sampling, the tap was disinfected with alcohol cotton and allowed to run freely for 5 min. The sample container was rinsed three times with the water to be sampled before filling. Excess sodium thiosulfate solution was added to neutralize residual chlorine. Samples were stored at 4 °C within 2 h and analyzed within 24 h (Waak et al., 2019).

2.2. Water quality index detection methods

Physicochemical indicators were measured using both on-site and laboratory methods. Temperature, residual chlorine, and turbidity were detected onsite. Temperature was recorded with an AR8010 digital thermometer. Residual chlorine was measured with a HACH chlorine analyzer and DPD reagent kit; the average of three replicates was taken. Turbidity was measured with a HACH turbidity meter (average of three replicates). pH was measured using a pH meter (METTLER TOLEDO, Switzerland). Total dissolved solids (TDS) were measured using a conductometer (METTLER TOLEDO) and expressed in mg/L. For heavy metal analysis, water samples were filtered through 0.45 μm membranes and analyzed using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7900, USA) to determine the concentrations of Fe, Mn, Zn, Cu, and other relevant metals.

Heterotrophic plate count (HPC) was determined using R2A agar medium. After the medium was prepared, it was placed in an ultrasonic bath for 10 min to completely dissolve the reagents. The prepared medium was sterilized at 121 °C for 20 min, slightly cooled, and then poured into sterile Petri dishes. After the medium cooled and solidified, it was inverted and set aside. After the samples were returned, 100 μL of each water sample was directly aspirated via a micropipette and evenly spread on a Petri dish, with three parallel samples for each sample. For the raw water and prefinished water samples, gradient dilution with sterile water was performed before the above steps were repeated. After coating, the Petri dishes were inverted and placed in a 25 °C incubator for incubation in the dark for 7 days, after which they were removed and counted (Zhang et al., 2016).

2.3. DNA extraction and qPCR analysis

DNA extraction was performed via the Qiagen DNeasy® PowerWater® Kit. To exclude interference from dead bacteria and free DNA, propidium monoazide (PMA) pretreatment was applied prior to DNA extraction (Fittipaldi et al., 2012; Nocker et al., 2006). Briefly, PMA (Biotium, USA) was added to each water sample at a final concentration of 50 μM. The samples were incubated in the dark at room temperature for 10 min with gentle shaking to allow PMA penetration into membrane-compromised cells, followed by 15 min of photoactivation using a 650 W halogen lamp at a distance of 20 cm to cross-link PMA with DNA. After photoactivation, the samples were centrifuged, and the pellet was subjected to DNA extraction.

Quantitative PCR (qPCR) was performed to quantify target pathogenic DNA. The target microorganisms and their PCR primers and probe sequences (Liu et al., 2019b; Nazarian et al., 2008; Radomski et al., 2010; Suzuki and Giovannoni, 1996; Wang et al., 2012) are shown in Table 1. Standard curves for absolute quantification were constructed using ten-fold serial dilutions of plasmid DNA containing the target gene inserts, ranging from 10^1 to 10^8 copies/μL. The standard curve

equations, coefficients of determination (R^2), and amplification efficiencies (E) for each target are summarized in Table S4. The limits of detection (LOD) and quantification (LOQ) were determined as the lowest concentrations producing detectable amplification in all replicates (LOD) and with a coefficient of variation below 25% (LOQ), respectively. No-template controls (NTCs) were included in every run and remained negative throughout. Detailed qPCR reaction conditions, including the thermal cycler model, reaction volumes, primer and probe concentrations, reagent specifications, thermal cycling protocols, and the number of technical replicates, are summarized in Table S5.

All qPCR assays were conducted in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). A completed Environmental Microbiology Minimum Information (EMMI) Guidelines checklist (Borchardt et al., 2021) is provided as "Supplementary material-EMMI.xlsx". Extracted DNA was stored at −80 °C until analysis.

2.4. Assimilable organic carbon and intact cell count analysis

Assimilable organic carbon (AOC) was determined using the sequential inoculation method with *Pseudomonas fluorescens* P17 and *Spirillum* sp. NOX. Water samples were filtered through 0.45 μm membranes, and 40 mL of filtrate was pasteurized at 70 °C for 30 min. After cooling to room temperature, P17 bacteria were inoculated, and the samples were incubated at 25 °C in the dark for 3 days. After colony counting, the samples were pasteurized again, followed by inoculation with NOX and incubation for 4 days. AOC concentration was calculated from the colony counts and expressed as μgC/L.

Total cell count (TCC) and intact cell count (ICC) were determined using a flow cytometer (BD FACS Verse, USA). Water samples were vortexed for 5 s, and 500 μL was transferred into a detection tube. For TCC, 5 μL of SYBR Green I working solution was added. For ICC, 5 μL of SYBR Green I working solution and 5 μL of propidium iodide (PI) were added. The samples were incubated at 37 °C in the dark for 20 min, and then analyzed using a 488 nm (20 mW) excitation laser.

2.5. 16S rRNA gene amplicon sequencing

Bacterial community structure was analyzed by 16S rRNA gene amplicon sequencing. DNA was extracted from water samples using the PowerWater® DNA Isolation Kit (MOBIO, USA). The V3-V4 region of the 16S rRNA gene was amplified using primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′) (Muyzer et al., 1993; Yu et al., 2005). PCR products were purified and sequenced on an Illumina MiSeq platform (Illumina, USA) using paired-end 2 × 300 bp reads. Raw sequence data were processed using QIIME 2 software (version 2023.2) (Bolyen et al., 2019). Sequences were denoised, dereplicated, and resolved into amplicon sequence variants (ASVs) using the DADA2 plugin, which performs error correction and sequence inference rather than clustering by similarity thresholds. Taxonomy was assigned using the SILVA reference database (version 138).

To evaluate the stability of bacterial communities across treatment stages, the Average Variation Degree (AVD) was calculated based on the relative abundance of genera obtained from 16S rRNA amplicon sequencing. AVD quantifies the compositional variation of a community across samples within a given group; lower AVD values indicate higher community stability, whereas higher AVD values reflect greater compositional fluctuation. The AVD was computed as follows:

$$\mathrm{AVD} = \frac{1}{n}\sum_{j=1}^{n}\left(\frac{1}{k}\sum_{i=1}^{k}\left|\frac{x_{ij}-\bar{x}_j}{\sigma_j}\right|\right)$$

Where x_{ij} is the relative abundance of genus j in sample i, $\bar{x}_j$ is the mean abundance of genus j across all samples within the group, σ_j is the standard deviation of genus j across samples, k is the number of samples, and n is the number of genera. This index has been previously applied to assess microbial community stability in drinking water treatment systems (Thom et al., 2022).

2.6. Data analysis

The experimental data were statistically analyzed via IBM SPSS Statistics 27. The Kruskal-Wallis rank sum test was employed to compare seasonal differences in OPPP concentrations within each treatment stage. When a significant difference was detected ($p < 0.05$), pairwise comparisons were performed using Dunn's post-hoc test with Bonferroni correction. For correlation analyses, Spearman's rank correlation coefficients were calculated due to the non-normal distribution of the qPCR data, as confirmed by Shapiro-Wilk tests. Statistical significance was defined at $p < 0.05$, $p < 0.01$, and $p < 0.001$. All statistical analyses were performed using IBM SPSS Statistics 27. Spearman rank correlation coefficients were used to test correlations between data, with the same significance level judgments. Beta diversity distances were calculated via QIIME 2 (Bolyen et al., 2019), and community composition analysis-related figures were drawn via Python 3.9 (using libraries such as matplotlib, seaborn, and pandas). Occurrence-related figures were drawn via Origin 2022 (Peng et al., 2020; Soler et al., 2021).

3. Results and discussion

3.1. Occurrence and patterns of OPPPs in DWTPs

The occurrence of OPPPs in the DWTPs is shown in Table 2. *Mycobacterium* spp., *Legionella* spp., and other typical OPPPs were detected in all the DWTPs. *Mycobacterium* spp. had a 100% detection rate in all

Table 1
Target microorganisms, target genes, primer or probe sequences, amplicon lengths, and references.

Target microorganism	Target gene	Primer or probe sequence	Amplicon length	Reference
Mycobacterium spp.	16S rRNA	110F: CCTGGGAAACTGGGTCTAAT I571R: CGCACGCTCACAGTTA H19R: FAM-TTTCACGAACAACGCGACAAACT-TAMRA	462 bp	(Radomski et al., 2010)
Legionella spp.	23S rRNA	Leg23SF: CCCATGAAGCCCGTTGAA Leg23SR: ACAATCAGCCAATTAGTACGAGTTAGC Probe: VIC-TCCACACCTCGCCTATCAACGTCGTAGT-TAMRA	92 bp	(Nazarian et al., 2008)
Mycobacterium avium	16S rRNA	MycavF: AGAGTTTGATCCTGGCTCAG MycavR: ACCAGAAGACATGCGTCTTG	180 bp	(Wang et al., 2012)
Legionella pneumophila	*mip*	LmipF: AAAGGCATGCAAGACGCTATG LmipR: GAAACTTGTTAAGAACGTCTTTCATTTG Probe: FAM-TGGCGCTCAATTGGCTTTAACCGA-TAMRA	78 bp	(Nazarian et al., 2008)
Pseudomonas aeruginosa	*oprl*	F: GACGTACACGCGAAAGACCT R: GCCCAGAGCCATGTTGTACT	99 bp	(Liu et al., 2019b)
Total bacteria	16S rRNA	341-F: CCTACGGGAGGCAGCAG 534-R: ATTACCGCGGCTGCTGG	194 bp	(Suzuki and Giovannoni, 1996)

process effluents except finished water, with a 97.2% detection rate in finished water, indicating limited removal rates by water plants, possibly related to the chlorine resistance of *Mycobacterium* spp. *Legionella* spp. showed a 100% detection rate in all process effluents except finished water (83.3%), with three samples undetected in autumn and winter, suggesting relatively higher chlorine sensitivity than *Mycobacterium* spp. *P. aeruginosa* was widely present in all treatment processes, with detection rates of 97.2% in carbon-filtered water and finished water and 100% in other process effluents, but at low detection concentrations (4–78 copies/mL). *Mycobacterium avium* was detected sporadically (<10% across all stages). *Legionella pneumophila* was detected only in the sedimentation and BAC effluents (maximum 26 copies/mL).

Mycobacterium spp. concentrations (Fig. 1a) were significantly higher in spring ($p < 0.001$) across all process effluents except finished water, likely due to higher source water levels. Finished water concentrations in summer were significantly higher than in other seasons ($p < 0.05$), possibly because high temperatures accelerate sodium hypochlorite decomposition, reducing effective chlorine.

Legionella spp. (Fig. 1b) showed relatively small seasonal differences across processes. In the coagulation-sedimentation stage, *Legionella* increased in summer, autumn, and winter, with the greatest increase in autumn (more than two orders of magnitude above raw water; $p < 0.05$). This may be related to its mesophilic nature. In all four seasons, BAC filtration significantly increased *Legionella* concentrations (3.6-, 4.5-, 2.5-, and 8-fold, respectively), indicating that BAC filters are a risk site for *Legionella* regrowth. In winter, despite lower raw water concentrations, BAC effluent levels rose to similar levels as other seasons, possibly due to the insulating effect of BAC filters providing a warm environment. Finished water concentrations remained low across all seasons, confirming that *Legionella* is relatively chlorine-sensitive (Lytle et al., 2021; Zhao et al., 2024).

P. aeruginosa (Fig. 1c) showed low but nearly ubiquitous concentrations (detection rates ≥97.2%). The variability in concentration patterns across seasons and processes may reflect interactions with dominant bacterial species. The relatively large standard deviations may also indicate sampling or detection challenges at trace levels.

Total bacterial concentrations (Fig. 1d) generally decreased through treatment, with a sharp decline after chlorination. However, in autumn, although raw water total bacteria were significantly lower than in spring ($p < 0.001$), concentrations after coagulation-sedimentation increased by approximately 3.8-fold, coinciding with increases in *Mycobacterium* spp. (≈ 35-fold) and *Legionella* spp. (≈ 180-fold). This suggests a higher risk of bacterial regrowth during coagulation-sedimentation in early autumn. Similarly, total bacteria increased in the BAC stage during autumn and winter. During disinfection, the log10 reduction of total bacteria exceeded 2.0 in spring, autumn, and winter, but was significantly lower in summer (approximately 0.8-log) as measured by PMA-qPCR.

3.2. Influence of water quality in DWTPs on OPPPs and their correlations

In the DWTPs, TDS was significantly negatively correlated with *Mycobacterium* spp. (Fig. 2), possibly due to coagulant addition increasing TDS while removing bacteria. However, TDS showed no significant correlation with other OPPPs. The correlation between turbidity and OPPPs changed from negative in the source water to positive. This may reflect that residual flocs serve as attachment sites for OPPPs. pH also showed a positive correlation trend with OPPPs, similar to that in the source water, where the concentration of *Mycobacterium* spp. was significantly positively correlated with pH. The free chlorine concentration was significantly negatively correlated with *Mycobacterium* spp., *Legionella* spp., and total bacteria but not with *P. aeruginosa*. These findings indicate that differential chlorine susceptibility among OPPPs as measured by PMA-qPCR: *P. aeruginosa* exhibited the highest apparent chlorine resistance, *Legionella* the lowest, and *Mycobacterium* intermediate.

Notably, *Legionella pneumophila* was not included in this correlation analysis due to its sporadic detection across the sampling campaign. As shown in Table 2, *Legionella pneumophila* was detected only in sedimentation effluent (2.8% detection rate) and carbon filtration effluent (2.8%), with a maximum concentration of 26 copies/mL. The low detection frequency (overall 11.3%, n = 180 across all stages) precluded meaningful statistical correlation with water quality parameters.

We acknowledge that PMA-qPCR, while providing a more conservative estimate of viable cells than conventional qPCR (PMA selectively excludes DNA from membrane-compromised cells), does not fully equate to culturability. Membrane integrity is an indicator of viability but does not necessarily reflect the ability to form colonies (Nocker et al., 2006). Moreover, while PMA-qPCR is a successful approach for detecting membrane-intact cells, it has practical limitations when applied to environmental samples and does not equate to culturability (Fittipaldi et al., 2012). Consequently, the apparent susceptibility patterns reported here should be interpreted as a risk assessment rather than absolute infectivity. Future studies using culture-based methods are warranted to validate these findings. Nevertheless, given that PMA-qPCR detects membrane-intact cells, which represent the fraction of cells capable of initiating regrowth under favorable conditions, our results provide a conservative but meaningful assessment of OPPP persistence risk across treatment trains.

In DWTPs, the extensive removal of metal ions by treatment processes was associated with a shift in the correlation between Fe/Mn concentrations and OPPP abundance: negative correlations in source water (high concentrations) shifted toward positive trends in treated water (low concentrations), suggesting a potential concentration-dependent effect that warrants further experimental validation (Zhang et al., 2021). The manganese content was significantly positively correlated with the *Mycobacterium* spp. concentration (Fig. 3). Zinc and copper remained negatively correlated with OPPPs, indicating that these ions continued to play an inhibitory role in bacterial physiological

Table 2
Detection rates and concentrations of OPPPs in DWTPs.

Target microorganism	Detection rate (%)				
	Raw water (n = 36)	Sedimentation effluent (n = 36)	Sand filtration effluent (n = 36)	Carbon filtration effluent (n = 36)	Finished water (n = 36)
Mycobacterium spp.	100.0	100.0	100.0	100.0	97.2
Legionella spp.	100.0	100.0	100.0	100.0	83.3
Mycobacterium avium	5.6	2.8	5.6	8.3	2.8
Legionella pneumophila	0.0	2.8	0.0	2.8	0.0
Pseudomonas aeruginosa	100.0	100.0	100.0	97.2	97.2
Total bacteria	100.0	100.0	100.0	100.0	100.0

Note: n = 36, representing the total number of samples collected from four DWTPs across four seasons, covering five common treatment stages (raw water, sedimentation, sand filtration, carbon filtration, finished water).

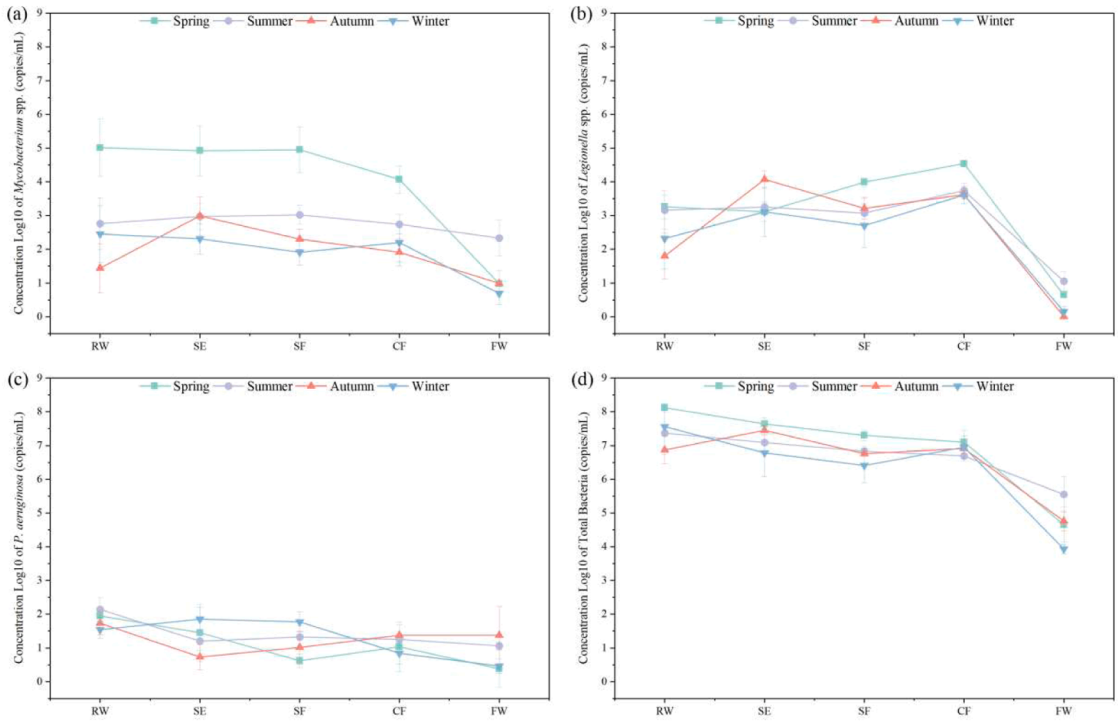


Fig. 1. Copy number concentrations of OPPPs detected during various processes in DWTPs. (a) *Mycobacterium* spp.; (b) *Legionella* spp.; (c) *P. aeruginosa*; (d) Total bacteria.
Abbreviations: RW, Raw Water; SE, Sedimentation Effluent; SF, Sand Filtration Effluent; CF, Carbon Filtration Effluent; FW, Finished Water.
Data are presented as mean ± standard deviation (SD). The Kruskal-Wallis test (with Dunn's post-hoc tests and Bonferroni correction) was used to compare seasonal differences within each treatment stage ($p < 0.05$ was considered statistically significant). Significant seasonal differences are described in Section 3.1.

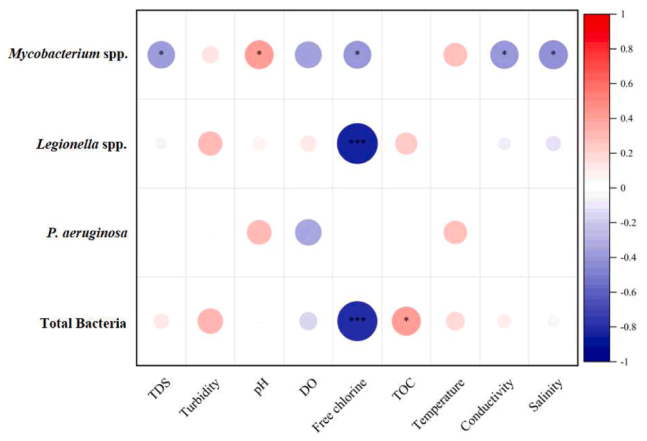


Fig. 2. Correlations between conventional physicochemical indicators and OPPP abundance in DWTPs.

activities. HPC value still showed a positive correlation with OPPPs, and a positive correlation with ICC also appeared in the finished water stage. This positive correlation may be explained by the efficient utilization of AOC by *P. aeruginosa* in the oligotrophic DWTP environment.

Metal ions showed process-dependent effects (Fig. 3) (Zhang et al., 2021). Iron and manganese exhibited a concentration-dependent correlation pattern with OPPPs: negative correlations were observed at the higher concentrations typical of raw water, whereas positive (though weak) correlations appeared at the lower concentrations found in treated water. This observational pattern is consistent with previously reported hormetic effects of metal ions on bacterial growth (Rhoads et al., 2017), but causality cannot be inferred from correlation alone. Manganese was significantly positively correlated with *Mycobacterium* spp. Zinc and copper remained negatively correlated with OPPPs. HPC and ICC showed positive correlations with OPPPs, particularly in finished water. Notably, AOC tended to be positively correlated with *P. aeruginosa*, possibly because *P. aeruginosa* efficiently utilizes AOC in oligotrophic DWTP environments.

3.3. Bacterial community structure in DWTPs and its influence on OPPPs

Bacterial community structure was analyzed at five stages: SE, SF, CF, MF (only applicable to DWTP C2), and FW (Fig. 4). *Hydrogenophaga* was the most abundant genus, followed by *Ralstonia*; both are widely distributed in freshwater and participate in organic matter degradation (Tang et al., 2023). *Sphingomonas* was abundant in sedimentation and sand filter effluents, reflecting its ability to utilize diverse organic carbon sources under oligotrophic conditions. *Pseudomonas* abundance was high in sedimentation and sand filter effluents, decreased after BAC filtration, but increased sharply in finished water, indicating chlorine tolerance and post-disinfection dominance. *Chryseobacterium* increased sharply in finished water, also indicating strong chlorine tolerance; this genus includes opportunistic pathogens such as *C. indologenes*, which is highly drug-resistant. Other chlorine-tolerant genera, including *Bacteroides*, also increased after disinfection (Abbas et al., 2024).

AVD decreased progressively from coagulation-sedimentation through membrane filtration, indicating gradually increasing community stability, but increased after chlorination (finished water), suggesting that disinfection exerted a stabilizing effect on the bacterial community. A Venn diagram (Fig. 5) showed 43 common genera across all stages, with finished water harboring the largest number of unique genera (117), indicating that some chlorine-resistant bacteria proliferated after disinfection (Thom et al., 2022).

K-W tests (Fig. 5) revealed that the abundances of *Hydrogenophaga, Novosphingobium, Erythrobacter*, and *Flavobacterium* differed significantly across the processes. *Hydrogenophaga* was significantly more

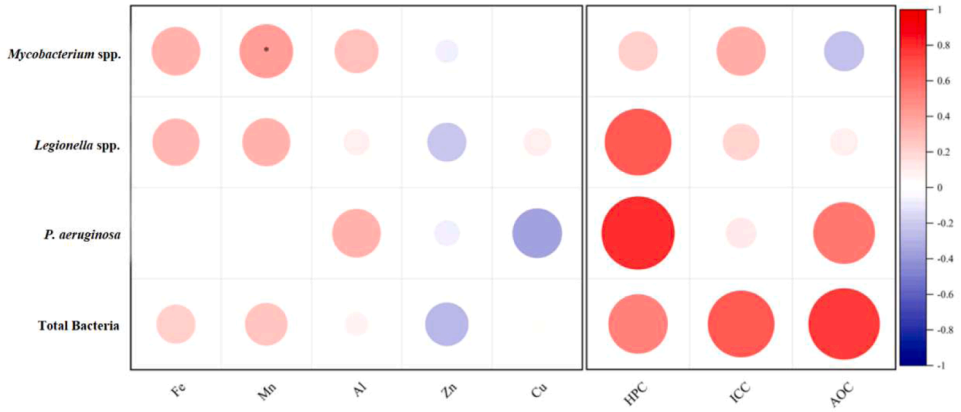


Fig. 3. Correlations between metal contents and microbial indicators in DWTPs and OPPP abundance.

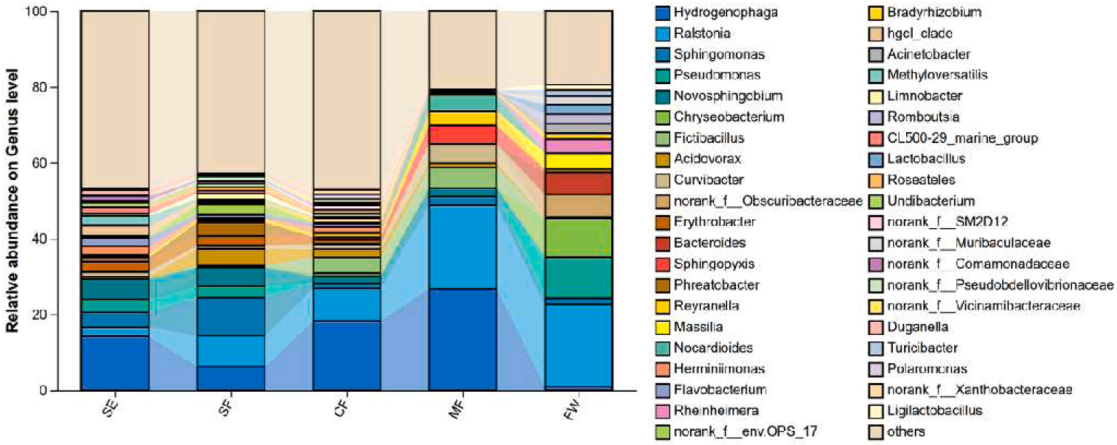


Fig. 4. Hierarchical bacterial community structure in the DWTPs. Abbreviations: MF, Membrane Filtration Effluent (only applicable to DWTP C2; MF samples were not included in cross-plant statistical comparisons, n = 12).

abundant in the effluent in the finished water than in the other processes, whereas *Novosphingobium* and *Erythrobacter* were significantly more abundant in sedimentation and sand filter effluents.

Mycobacterium spp. presented significant negative correlations with *Romboutsia, Lactobacillus, Turicibacter*, etc. All of the above genera belong to Bacillota, which are important probiotics in the digestive tract. *Lactobacillus* has been found to regulate the gastrointestinal pH and inhibit the growth of pathogenic bacteria (Kullar et al., 2023), thereby playing an important role in maintaining intestinal health. The inhibitory effect of digestive tract probiotics on OPPPs may stem from their secreted metabolites, such as lactic acid and acetic acid, which block the proliferation of OPPPs (Wang et al., 2013). Moreover, competition for ecological niches due to different nutritional requirements may also be a reason. *Mycobacterium* spp. showed significant positive correlations with *hgcI_clade, Methyloversatilis, CL500-29_marine_group, Limnohabitans*, etc. These genera are all common in freshwater environments, with strong environmental adaptability and metabolic diversity.

Correlation analysis between dominant genera and OPPPs (Fig. 6) showed that *Mycobacterium* spp. were significantly negatively correlated with *Romboutsia, Lactobacillus*, and *Turicibacter* (all belonging to Bacillota). *Lactobacillus* is known to produce organic acids that inhibit pathogenic bacteria (Kullar et al., 2023). *Mycobacterium* spp. were positively correlated with *hgcI_clade, Methyloversatilis, CL500-29_marine_group*, and *Limnohabitans*, all common in freshwater environments with high metabolic versatility. This suggests that some *Mycobacterium* spp. share ecological traits with dominant natural water bacteria, enabling their persistence in DWTPs (Waak et al., 2019).

For *Legionella* spp., significant negative correlations were observed with *Pseudomonas, Chryseobacterium, Acinetobacter*, and *Bacteroides*. *Pseudomonas* spp. can inhibit *Legionella* via the quorum-sensing autoinducer 3-oxo-C12-HSL (Kimura et al., 2009). Positive correlations with *Hydrogenophaga* and *Roseateles* suggest that these genera may support *Legionella* by providing small-molecule organic substrates through complex organic matter decomposition. *P. aeruginosa* was significantly positively correlated with *Flavobacterium, Novosphingobium, Sphingomonas, Limnobacter, Undibacterium*, and *Duganella*. Most of these genera possess nitrate-metabolizing capabilities and may form synergistic relationships with *P. aeruginosa* (Falkinham et al., 2015).

3.4. Integrated mechanisms driving OPPP proliferation in DWTPs

Building on the above results, we propose an integrated mechanistic framework that explains why OPPPs proliferate in specific treatment stages (Fig. S1).

First, the coagulation-sedimentation stage also poses a risk, particularly in early autumn. During this stage, flocs formed by coagulants act as physical carriers, allowing bacteria to attach and potentially multiply before being partially removed. This interpretation is supported by the observation that the correlation between turbidity and OPPPs shifted from negative in raw water to positive in treated water (Fig. 2), implying that residual flocs serve as attachment sites. The significant autumn increases in *Legionella* spp. (≈ 180-fold) and *Mycobacterium* spp. (≈ 35-fold) after sedimentation (Section 3.1) coincide with higher water temperatures favorable for these mesophilic bacteria.

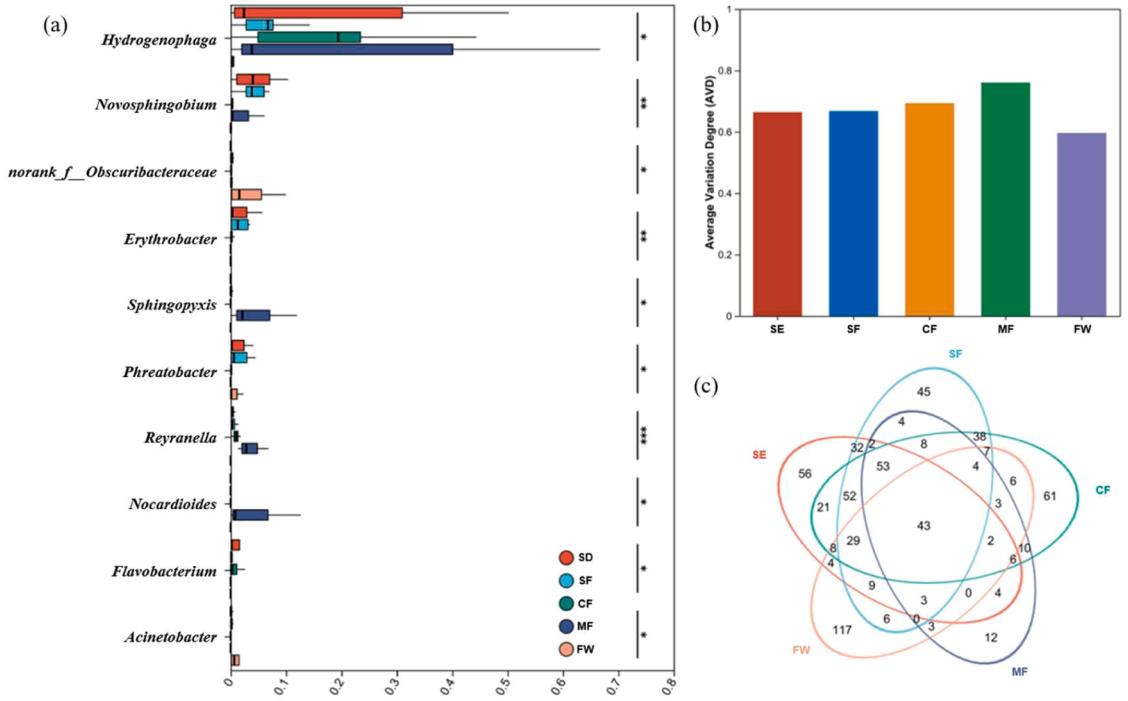


Fig. 5. Bacterial communities in DWTPs. (a) Kruskal-Wallis test showing genera with significant abundance differences across treatment stages; (b) AVD across treatment processes; (c) Venn diagram showing shared and unique genera among stages. MF samples were only available from DWTP C2 (n = 12) and were included in the community analysis; however, MF was not uniformly present across all four DWTPs.

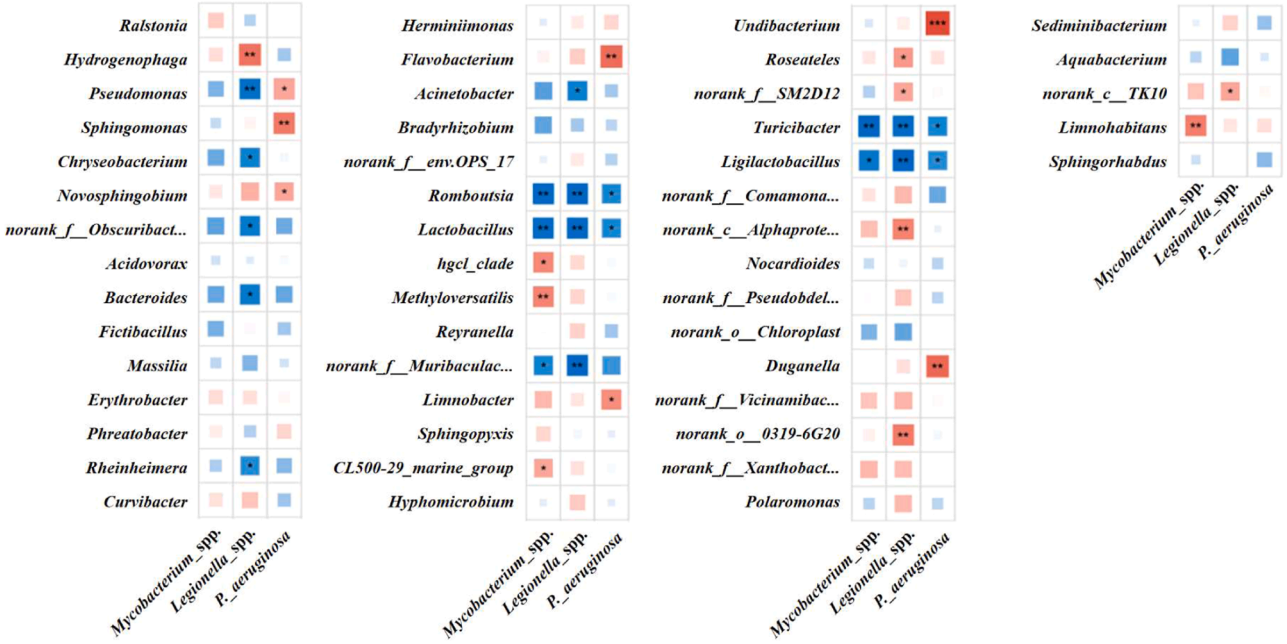


Fig. 6. Correlations between the dominant bacterial genera in the bacterial community and OPPPs in the DWTPs.

Second, the O_3-BAC stage acts as a critical hotspot. Ozonation oxidizes large organic macromolecules into smaller, more bioavailable compounds, thereby increasing AOC. This was supported by the positive correlation between AOC and *P. aeruginosa* (Fig. 3). The subsequent BAC filter, colonized by active biofilms, provides a protected microenvironment and a continuous supply of small-molecular-weight organic substrates from microbial metabolism. Notably, genera such as *Hydrogenophaga*, which were significantly enriched in BAC effluents and positively correlated with *Legionella* spp. (Fig. 6), may facilitate OPPP proliferation by breaking down complex organics into utilizable carbon sources (Tang et al., 2023). These combined factors explain the marked increase in *Mycobacterium* spp., *Legionella* spp., and total bacteria after BAC filtration (Fig. 1).

Third, differential chlorine susceptibility is driven by physiological traits. *Mycobacterium* spp. possess a lipid-rich, hydrophobic outer membrane that limits chlorine penetration (Falkinham, 2021). *P. aeruginosa* can exhibit enhanced cell membrane permeabilisation and a strong oxidative stress-response under chlorine exposure, as reported

in chlorine-tolerant bacteria including *P. aeruginosa* (Jin et al., 2020). In contrast, *Legionella* spp., although protected within biofilms, are more vulnerable when freely suspended; previous work has similarly shown that opportunistic pathogens within biofilms were tolerant to upstream disinfection, whereas those in the bulk water were more effectively controlled (Liu et al., 2019a). Notably, our PMA-qPCR results reflect the risk posed by membrane-intact cells, which, even if not culturable, may retain the ability to regrow under favorable conditions.

Fourth, microbial interactions modulate OPPP abundance. We observed both inhibitory and facilitative relationships. For example, *Lactobacillus* and certain *Pseudomonas* spp. were negatively correlated with OPPPs, possibly due to the production of organic acids or quorum-sensing molecules such as 3-oxo-C12-HSL, which is known to inhibit *Legionella* (Clements et al., 2024). In contrast, *Hydrogenophaga* and *Roseateles* showed positive correlations with *Legionella*, suggesting that they may supply growth-limiting substrates (e.g., short-chain organic acids) that *Legionella* cannot produce on its own (Falkinham et al., 2015).

Comparison with established mechanistic frameworks. The integrated framework proposed here, identifying O_3-BAC as a proliferation hotspot, differential chlorine susceptibility among OPPPs, and the modulating role of microbial interactions, is broadly consistent with existing conceptual models (Falkinham et al., 2015). delineated four common traits of OPPPs, including chlorine resistance, biofilm formation, amoeba association, and oligotrophic growth, that enable their persistence in drinking water systems. Our findings extend this framework by quantifying process-specific proliferation magnitudes (e.g., 2.5- to 8-fold increases of Legionella spp. across BAC filters) and by identifying the O_3-BAC stage as a more dominant hotspot than previously recognized in full-scale plants. The WHO Guidelines for Drinking-water Quality (2004) emphasize multi-barrier approaches for microbial safety (Organization, 2004). Our results reinforce this principle by demonstrating that conventional chlorination alone is insufficient against OPPPs, particularly *P. aeruginosa*, and that upstream treatment stages, especially BAC, can serve as "seed sources" that undermine downstream disinfection. Furthermore, the negative correlations we observed between OPPPs and probiotic genera (*Lactobacillus, Romboutsia*) align with the "probiotic approach" proposed by Wang et al. (Wang et al., 2013), although our study provides only correlational evidence. We acknowledge that the mechanistic inferences drawn here are primarily hypothesis-generating. Controlled experiments, such as co-culture assays and gene knockout studies, are needed to establish causative relationships.

4. Conclusion

This study systematically tracked the occurrence and proliferation of OPPPs (*Mycobacterium* spp., *Legionella* spp., and *P. aeruginosa*) across full-scale treatment trains in four DWTPs over four seasons. The following conclusions are drawn:

1. OPPPs were consistently detected throughout the treatment process, with variable abundances across stages. The O_3-BAC stage was identified as a critical hotspot for OPPP proliferation, likely due to enhanced microbial activity and increased AOC availability. This stage can serve as a "seed source" that releases OPPPs into finished water and downstream networks.
2. Conventional treatment processes, including coagulation-sedimentation, sand filtration, and chlorination, showed limited effectiveness in completely removing OPPPs. Finished water still contained detectable levels of membrane-intact OPPPs, highlighting the need for enhanced treatment strategies targeting regrowth niches.
3. The limited removal of OPPPs by conventional treatment contrasts sharply with the high removal efficiencies typically achieved for traditional fecal indicator bacteria. For physical barriers, coagulation-sedimentation and sand filtration can achieve approximately 1–2 log removal of bacterial pathogens and protozoa under optimized conditions (LeChevallier, 2013). In contrast, our data showed that these processes exhibited poor and inconsistent OPPP removal, with regrowth observed — *Legionella* increased ~180-fold after sedimentation in autumn (Fig. 1). For chlorination, the disparity is even more striking: chlorine typically achieves >4-log reduction of *E. coli*, whereas the log reductions observed here for OPPPs ranged from only 0.8 to 2.3 (Fig. 1), consistent with reports that *Mycobacterium avium* requires 571- to 2318-fold higher chlorine Ct values than *E. coli* (Taylor et al., 2000). These findings underscore that compliance with traditional indicator standards does not guarantee OPPP biosafety, and that OPPP-specific monitoring and treatment optimization are essential.
4. Seasonal variations significantly influenced OPPP abundance and distribution. Spring and summer were associated with higher OPPP concentrations by PMA-qPCR, likely due to elevated water temperatures and microbial activity. DWTP operations should consider seasonal adjustments (e.g., chlorine dosage, backwashing frequency) to mitigate seasonal peaks.
5. Water quality parameters such as TDS, turbidity, pH, and free chlorine showed varying degrees of correlation with OPPP abundance. Free chlorine was negatively correlated with *Mycobacterium* spp. and *Legionella* spp. but not with *P. aeruginosa* as measured by PMA-qPCR, indicating that *P. aeruginosa* possesses higher apparent persistence under chlorination. These findings should be interpreted as an assessment of relative risk rather than absolute infectivity; future studies integrating culture-based validation are needed to confirm the chlorine susceptibility patterns of viable but non-culturable populations.
6. The microbial community structure shifted significantly through the treatment train. Certain genera, such as *Lactobacillus*, exhibited inhibitory effects on OPPPs, suggesting potential biological control strategies. Understanding these microbial interactions may inform targeted interventions (e.g., probiotic biofilm management) to reduce OPPP risks.

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CRediT authorship contribution statement

Songkai Cai: Writing – review & editing, Writing – original draft, Visualization, Data curation. **Weiying Li:** Writing – review & editing, Visualization, Validation, Project administration, Funding acquisition. **Yu Zhou:** Visualization, Validation. **Boyuan Deng:** Writing – review & editing, Project administration, Data curation.

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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2026.126801.

Data availability

Data will be made available on request.

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