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Opportunistic pathogens and their health risk in four full-scale drinking water treatment and distribution systems

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ABSTRACT

This study investigated the occurrence of various opportunistic pathogens (OPs) through four drinking water treatment and distribution systems in eastern China. Conventional treatment trains involving coagulation/sedimentation, filtration, and disinfection efficiently removed total coliforms from 1700 to 2300 CFU/L in the influent to undetectable levels in treated and tap water. However, culture-independent qPCR analysis detected *Legionella* spp., *Mycobacterium* spp., *Mycobacteria avium*, *Pseudomonas aeruginosa* and the amoeba *Acanthamoeba* spp. in all water samples, reaching maximum tap water concentrations of 5.33, 4.87, 1.63, 3.85, and 4.32 log (gene copies/mL), respectively. Thus, OPs were abundant in tap water despite total coliforms met applicable microbiological standards in China (GB 5749–2006). Occurrence of OPs correlated positively with turbidity and chemical oxygen demand (COD), and negatively with chlorine residual. Turbidity removal by coagulation and COD removal by ozonation (O_3) followed by biological activated carbon (BAC) filtration was the treatment train with the highest OPs removal efficiency, and ClO_2 was a more effective disinfectant than NaClO. OPs significantly rebounded in the tap water (up to 11-fold for *P. aeruginosa* and 21-fold for *M. avium* in tap water). However, quantitative microbial risk analysis (QMRA) showed that the potential infection risks in tap water were still below WHO (10^{-3}) and even EPA (10^{-4}) benchmarks. Overall, likely underestimation of the pathogenic risk by culture-dependent quantification of indicator organisms makes it prudent to use molecular approaches to periodically revisit the safety of water distribution systems.

1. Introduction

Current drinking water safety standards use total coliforms as indicator microorganisms to monitor pathogenic conditions (Wang et al., 2013a). Opportunistic pathogens (OPs) in drinking water systems have been recognized by the Center for Disease Control as a leading source of disease outbreaks (Garner et al., 2019). Estimated incidence of Legionnaires' disease is increasing at 5% per year, and mycobacterial infections are increasing at 8–10% in the U.S. (Falkinham III, 2015). Furthermore, "fatal brain-eating" amoeba such as *Naegleria fowleri*, which host bacterial OPs, was reported having a mortality rate over 90% (Khan et al., 2017).

Drinking water treatment plants (DWTPs) use treatment trains commonly including coagulation/flocculation, sedimentation, filtration and disinfection. These combined processes remove OPs to provide potable water (Hu et al., 2018). However, OPs generally possess several adaptive features to survive in drinking water treatment and distribution systems, including oligotrophy, resistance to disinfection and heat, slow growth and decay rates, and tendency to form protective biofilms (Falkinham III et al., 2001; Wang et al., 2012b; Wang et al., 2013a). Chlorine has long been used as a water disinfectant due to its strong oxidation capacity; however, it may select for some chlorine-resistant OPs such as

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Mycobacteria spp. and *Pseudomonas* spp. (Shi et al., 2013; Sevillano et al., 2019). Alternative current approaches to control OPs include biological activated carbon (BAC) filtration which mitigates OPs growth by degrading assimilable organic carbon (AOC) (Liu et al., 2019b), followed by disinfection with chlorine, chlorine dioxide (ClO_2) or chloramine (Vicuna-Reyes et al., 2008; Li et al., 2018). However, OPs may proliferate in drinking water distribution systems (DWDSs) depending on residual disinfectant, antibiotic and AOC concentration, water age, and pipe material (Falkinham III et al., 2001; Wang et al., 2012a; Wang et al., 2012b; Ling et al., 2018; Wang et al., 2018b; Liu et al., 2019a). For example, whereas chloramines offer residual disinfection capacity to control *Legionella* spp. in DWDSs, it may increase the occurrence of *Mycobacteria* spp. (Moore et al., 2006; Wang et al., 2012b).

Although many lab-scale efforts have focused on improving the effectiveness of water treatment process for eliminating OPs (Wang et al., 2013b), studies that systematically characterize the effectiveness of full-scale DWTPs and corresponding distribution systems at controlling OPs are relatively scarce. Several full-scale sampling studies have corroborated that OPs concentrations generally increase from the effluent of drinking water treatment plants through distribution systems (Lu et al., 2016). However, there is limited quantitative knowledge about which OPs are the main health risk drivers in different full-scale systems, and the variability of such risks (Wang et al., 2019).

Microbial risk was usually analyzed by quantitative microbial risk assessment (QMRA) approach, including steps of pathogen identification, dose-response modelling, exposure assessment and risk characterization (Haas et al., 1999). QMRA analysis has been employed to quantify the microbial risk exposed by reclaimed water, surface water, and recirculated cooling water (Cui et al., 2017; Hamilton et al., 2018). However, the associated risks posed by tap water are location-specific, influenced by confounding biogeographical and infrastructure factors that include system-specific network infiltration rates, residual disinfectant concentrations and sinks, pipeline materials used, hydrodynamic properties and redox and substrate gradients (Ling et al., 2018; Liu et al., 2019a; Sevillano et al., 2019). This underscores the need for site-specific QMRA evaluation of specific OPs from full-scale systems across different regions to scrutinize water safety (George et al., 2015; Amoueyan et al., 2017).

In this study, we considered four full-scale DWTPs in one city of eastern China and investigated the occurrence of OPs from influent water to tap water. OPs detected by quantitative polymerase chain reactions (qPCR) include *Legionella pneumophila*, *Mycobacteria avium* and *Pseudomonas aeruginosa* as well as their amoeba hosts, *Acanthamoeba* spp., *Naegleria fowleri* and *Hartmannella vermiformis*. We also assessed the efficiency of different treatment processes at controlling these OPs and used QMRA to estimate the infection risks from specific OPs after drinking water treatment and distribution.

2. Materials and methods

2.1. Site locations and samples collection

This investigation was conducted at four DWTPs in a city of eastern China. Together the DWTPs serve a population of about one million. Surface water from the Beijing-Hangzhou Grand Canal is used as the influent for DWTP1, DWTP2 and DWTP4, and surface water from the Huaishu River is used as influent for DWTP3. The water treatment processes for DWTP1 were coagulation/sedimentation, BAC filtration, and ClO_2 disinfection. The water treatment processes for DWTP2 were coagulation/sedimentation, ozone (O_3), BAC, and chlorine (NaClO) disinfection. The water treatment processes for DWTP3 were coagulation/sedimentation, sand filtration, O_3, BAC, and ClO_2 disinfection. The water treatment processes for DWTP4 were coagulation/sedimentation, sand filtration, and ClO_2 disinfection. Details of the water treatment trains for the four DWTPs are given in the supplemental material (Table S1, Fig. S1).

Collected samples consisted of 2 L each of influent water (IW), effluents of sedimentation tank (ES), sand filtered water (SFW), biological activated carbon filtered water (BACW), disinfected water (DW), and tap water (TW). For each sampling location in different DTWPs, three sampling campaigns were carried out within one week in 2018 using sterile bottles. The tap water samples were collected inside the apartments which were about 5 km away from the respective DWTPs. They were used for cooking and washing. No tap water sample was from the calorifier.

2.2. Water quality analysis

Temperature, pH, residual chlorine and turbidity were measured in situ at the time of collection. The pH was monitored using a portable pH 110 series meter (Oakton Research, Vernon Hills, IL). The chlorine residual and chlorine dioxide were measured by the DPD method using a Pocket Colorimeter II (HACH, USA). The turbidity was measured using a portable 2100Q parameter meter (HACH, USA). Chemical oxygen demand (COD) was determined as permanganate index (COD_{Mn}) with the Chinese national standard method GB 11892–89. The culturable bacteria including total coliforms and heterotrophic plate counts (HPC) were enumerated according to Standard Methods (APHA/AWWA/WEF, 2005). All the chemical parameters were measured in triplicate for each sample.

2.3. DNA extraction from the sampled water

Water samples were filtered through sterile 0.22 μm polycarbonate filters (Millipore Isopore™, USA) to obtain intracellular DNA, according to the protocol in a previous study (Jäger et al., 2018). The filters were then fractured using sterilized tweezers and placed in 2 mL Lysing Matrix A tubes (MP Biomedicals, Solon, USA). DNA was extracted using a FastDNA® SPIN Kit (MP Biomedicals, Solon, USA) according to instruction of the manufacturer. The extracted DNA was finally concentrated into 100 μL DNase/Pyrogen-free water. The DNA was quantified with Nanodrop (ND-1000, NanoDrop Technology, USA).

2.4. qPCR assays for selected OPs

Legionella spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *P. aeruginosa*, *Acanthamoeba* spp., *H. vermiformis*, *N. fowleri* and total bacteria (16S rRNA genes) were quantified by qPCR using a 7300 qPCR system (ABI 7300, Applied Biosystems, Singapore). All primers, probes and qPCR amplification programs were used as previously described (Liu et al., 2019b; Wang et al., 2018a), and are listed in Table S2. For the TaqMan assay, 12.5 μL of Premix Ex Taq (Takara, Dalian, China), 0.5 μL of 10 μmol/L forward and reverse primers, 1.0 μL of 3 μmol/L TaqMan probe, 8.0 μL of distilled water, 0.5 μL of ROX reference dye (50×), and 2.0 μL of DNA template were used. For the SYBR Green assay, 12.5 μL of SYBR Ex Taq (Takara, Dalian, China) and 9.0 μL of distilled water, 0.5 μL of 10 μmol/L forward and reverse primers, 0.5 μL of ROX reference dye (50×), and 2.0 μL of DNA template were used. Each qPCR reaction was run in triplicate. For each run, a melt curve analysis was conducted to verify the specificity of the primers by increasing from 75 to 95 °C with 20-s holds. Standard curves were generated by serial ten-fold dilution (10^9–10^2 copies/μL) of the plasmids. Amplification efficiencies and the limits of quantification (LOQ) are given in Table S3. The amplification efficiency values ranged from 92.5% to 99.6%, and the LOQ ranged from 1 to 70 gene copies/reaction for different OPs.

2.5. QMRA analysis of M. avium

In this study, *M. avium* and *P. aeruginosa* are detected at species level. However, it lacks the dose–response model for *P. aeruginosa* by the route of oral ingestion linked in this study (http://qmrawiki.org), failing to quantify its infection risks by QMRA analysis (Haas et al., 1999). Thus,

only *M. avium* was selected as potential pathogenic species for QMRA analysis. For *M. avium*, oral ingestion was considered to be the main exposure route in the QMRA analysis due to our focus on drinking water safety and the lack of dose–response model for other exposure routes (http://qmrawiki.org). The exponential model was selected as best fit does-response model for *M. avium* (Cui et al., 2017). Dose-response parameters of *M. avium* were obtained from the QMRA wiki (http://qmrawiki.org).

In China, because few people drink tap water directly (i.e., water is commonly boiled before drinking), the risk of tap water intake mainly considered residues from tooth-brushing as well as food and dish washing. The ingestion volume (*Vi*) of such residual water was reported to range from 7 to 71 mL per person-day (An et al., 2011). Gene copies of *M. avium* (determined by qPCR) were considered as its cell number (*Ci*) because 16S rRNA of *M. avium* is a single-copy gene (Wang et al., 2012a; Fang et al., 2018). Similar to previous QMRA evaluations, one-half of the LOQ was used as input for dose-response models if the samples were detected below the LOQ (Cui et al., 2017). Thus for the QMRA assessment of *M. avium* with *Ci* below the LOQ (i.e., 4.10 gene copy/μL), 2.05 gene copy/μL was applied (Cui et al., 2017).

QMRA requires viable and infectious microorganism concentrations as input for the dose-response models, and qPCR data may be used when viability and infectivity information is not available (Hamilton et al., 2018). In such cases, a correction factor must be applied to account for potential over-counting by qPCR of DNA fragments or freshly killed bacteria as infectious agents (Wang et al., 2017a). Here, to assess the risk of viable *M. avium*, qPCR measurements were multiplied by a correction factor that was determined experimentally as the ratio of viable plate counts to qPCR counts for a pure strain of *M. avium* ATCC 25291, yielding a "live fraction" $Li = 3.92\times10^{-4}$. *Li* would be much lower immediately after disinfection due to the predominance of recently killed bacteria counted by qPCR, but we used the above value because it is more representative of re-growth in distribution systems and is more conservative risk assessment. Infectivity to humans (*Ii*) was assumed to be 0.1%, based on literature (Fang et al., 2018).

Ranges of possible OPs concentrations (*Ci*, cell/mL) and the ingestion volume (*Vi*, mL) of disinfected or tap water were given above. Within these ranges, daily dose of *M. avium* (*D*, viable and infectious cell) was calculated according $D = Ci \times Li \times Ii \times Vi$. Then, one thousand pathogen condition profiles of *M. avium* were randomly generated to create a daily infection probability ($P_{(inf,\ d)}$) distribution (Eq.1):

$$P_{(inf,d)} = 1 - e^{-kD} \quad (1)$$

where $k = 6.93\times10^{-4}$ (viable and infectious cell $^{-1}$) (Cui et al., 2017).

This value was used to estimate the annual infection risk ($P_{(inf,\ a)}$) (Eq. 2):

$$P_{(inf,a)} = 1 - \left(1 - P_{(inf,d)}\right)^{n} \quad (2)$$

where $n = 365$ for annual risk estimations, and $P_{(inf,\ d)}$ are the daily infection probability.

Note that for disinfected water samples, gene copies of *M. avium* were below the limit of qPCR quantification (LOQ, 4.1 gene copy/(μL DNA)); thus, one-half of the LOQ were used as the input of dose-response models (Cui et al., 2017; Fang et al., 2018).

2.6. Statistical analysis

qPCR data was $\log_{10}$-transformed prior to analyses. Parametric one-way analysis of variance (ANOVA) was used to compare the log-transformed qPCR data, and $p < 0.05$ was considered as statistically significant. Canonical correspondence analysis (CCA) was used to correlate the OPs and water qualities using the CANOCO program (CANOCO 4.5 for Windows). Spearman rank correlation analysis was conducted with SPSS software (IBM statistics, version 22) to identify the associations of target OPs with turbidity, COD and chlorine residual based on a 95% confidence level.

3. Results and discussion

3.1. Culture-based microbial safety standards were met, but OPs were present

All four water treatment systems completely removed total coliforms from 1700 to 2300 CFU/L in the influent water (IW) to undetectable levels in the disinfected water (DW) and tap water (TW) (Fig. 1a). Thus, chlorine residual concentrations in DWs (0.17–0.39 mg/L) and in TWs (0.08–0.11 mg/L) were enough to control total coliforms and mitigate their significant regrowth. Heterotrophic bacteria plate counts (HPC) were reduced from 864 to 1020 CFU/mL in IWs to 4–12 CFU/mL in DWs, but re-bounded to 32–80 CFU/mL in TWs (Fig. 1b). Disinfection removed 90%–100% of HPC, and was (as expected) more effective than sedimentation and filtration (33.3% to 73.3%) ($p < 0.05$). Consistent with other studies, the chlorine residual in the tap water decreased significantly from the value immediately after disinfection, and turbidity, COD and HPC increased (Table S4; Liu et al., 2017; Miller et al., 2015). All DWTPs met the required drinking water standards in China (GB 5749–2006) and effectively controlled total coliforms, the traditional pathogen indicator.

We also used culture-independent qPCR to investigate the concentration levels of OPs through four DWTP trains and in tap water (Fig. 2). Note that our analysis predominantly excluded extracellular DNA released from dead, lysed cells. Nevertheless, some internal DNA may persist after cells have lost viability (Kim and Wuertz, 2015). Thus, DNA-based quantification methods such as qPCR tend to overestimate the number of viable cells (Perrin et al., 2019), even though most bacterial cells in water distribution systems without significant chlorine residual are alive (van Nevel et al., 2017). Therefore, our qPCR measurements represent a conservative upper bound estimate of OP concentrations. Accordingly, our DNA measurements are appropriate to discern the main OPs risk drivers and assess the relative efficacy of different unit processes to remove them, as well as potential increases in OPs concentrations in distribution systems.

OPs including *Legionella* spp., *Mycobacterium* spp., *M. avium*, *P. aeruginosa*, and the amoeba *Acanthamoeba* spp. were detected in all samples. In influent water samples, abundances of total bacteria (16S rRNA), *Legionella* spp., *Mycobacterium* spp., *M. avium*, *P. aeruginosa*, and the amoeba *Acanthamoeba* spp. were 7.52–7.78, 4.81–5.33, 5.33–6.38, 1.55–2.37, 4.44–5.50, and 4.90–5.80 log (gene copies/mL), respectively. After treatment, these OPs in disinfected water (DWs) were significantly removed, but rebounded in the distribution system by the time it reached tap water (TWs) (Fig. 2), as detailed below (see Section 3.3). Thus, OPs were present in tap water despite full compliance with applicable microbiological standards (Fig. 1).

3.2. Sedimentation and filtration reduce OP concentrations while removing turbidity and COD

Quantitative correlation by canonical correspondence analysis (CCA) between water quality parameters, environmental variables and gene copies of different OPs in four drinking water systems showed that turbidity and COD were positively correlated with the abundance of OPs in both influent and treated water (Fig. 3, $p < 0.05$, Table S5). COD includes carbon sources for microbial growth (Chandy and Angles, 2001) and turbidity reflects suspended solids that bacteria (including OPs) are prone to attach to and use as shelters against disinfection (Yao et al., 2014; Wang et al., 2018b). Accordingly, COD and turbidity were also positively correlated with HPC, and 16S rRNA for total bacteria ($p < 0.05$, Table S5).

All four investigated DWTPs systems included coagulation/sedimentation, filtration, and disinfection to remove turbidity, organics (COD) and OPs (Table S4). For turbidity removal, coagulation/

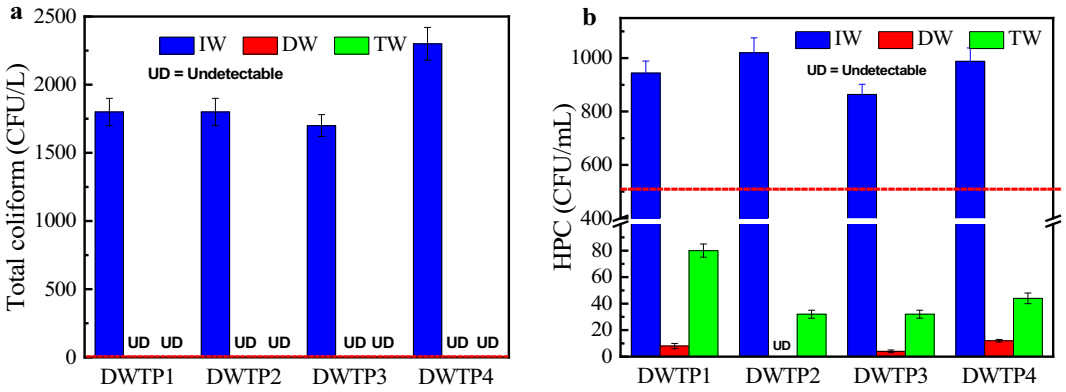

Fig. 1. The total coliform counts (a) and heterotrophic plate counts (b) in influent water (IW), disinfected water (DW) and tap water (TW) in the four drinking water treatment plants (DWTPs). Red dash line is the safety standard (a: National Standard of the People's Republic of China: standards for drinking water quality, GB 5749–2006; b: National Primary Drinking Water Regulations, U.S, EPA 2012). Note: Drinking water met China's current microbial safety standards. Error bars represent mean values ± one standard deviation, $n = 3$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

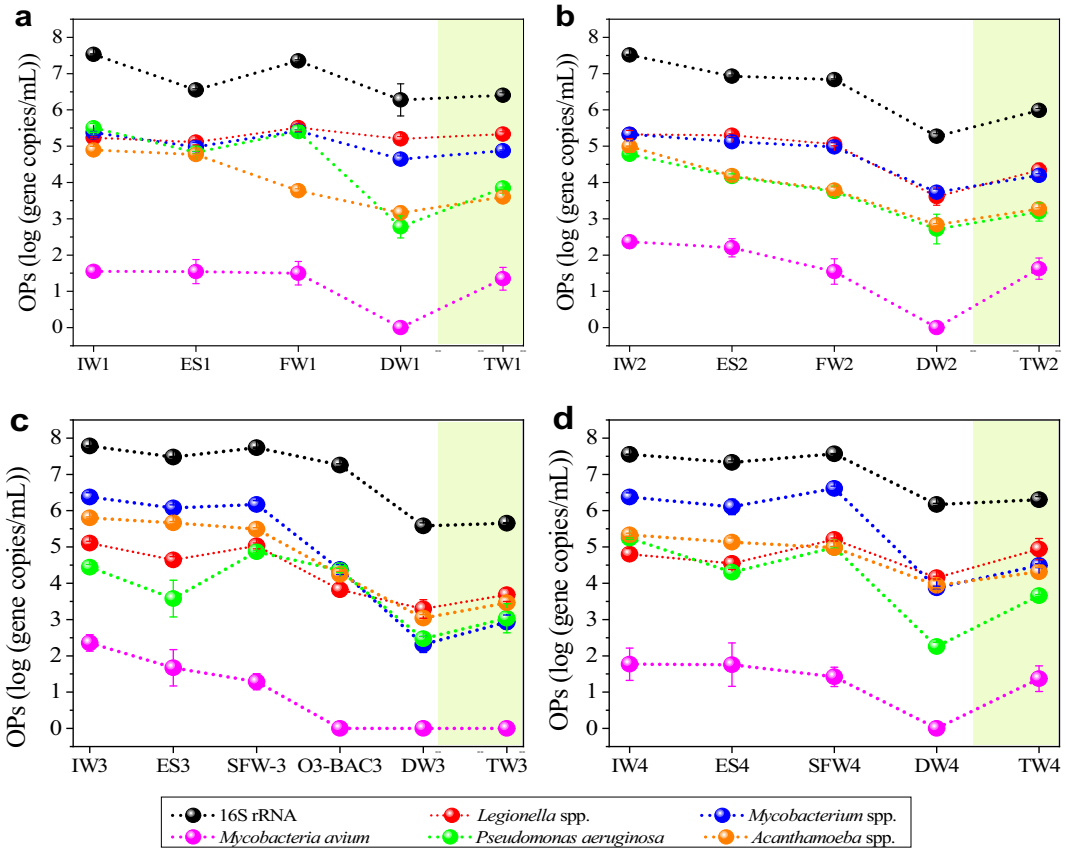

Fig. 2. Occurrence of different opportunistic pathogens through four water treatment trains and distribution systems (panels a, b, c and d). Concentrations were measured by qPCR in samples from influent water (IW), after coagulation and settling (ES), sand filtration (SF), biological active carbon filtration (BAC), disinfection (DW), and tap water (TW). Note: OPs were mainly removed by disinfection, but rebounded in tap water. Error bars represent mean values ± one standard deviation ($n = 3$), and bars smaller than symbols are not visible.

sedimentation was most effective, reducing turbidity by 93.5%–94.9%, BAC filtration only reduced turbidity by 61.5%–72.4%, and sand filtration by only 43.2%. For COD, the removal rate by coagulation/sedimentation was from 18.4% to 34.3%, sand filtration was from 8.92% to 20.9%, and BAC filtration was highest from 50.2%–72.4%. Thus, coagulation/sedimentation was the main treatment process to reduce turbidity while BAC filtration was the main process for COD removal.

Sedimentation removed total bacteria (16S rRNA) by 39%–89%, *Legionella* spp. by 4.4%–65%, *Mycobacterium* spp. by 37%–59%, *M. avium* by 1.7%–79%, *P. aeruginosa* by 75%–88%, and the amoeba *Acanthamoeba* spp. by 25%–84%, respectively (Fig. 2). Removal of 16S rRNA and *P. aeruginosa* by sedimentation was significant ($p < 0.05$, Table S6), but their removal efficiency varied for other OPs in different DWTPs (Fig. 2, Tables S6). Removal of *M. avium* by sedimentation in DWTP1 (1.7%) and DWTP4 (2.4%), and removal of *Legionella* spp. in DWTP2 (4.4%) were negligible. In DWTP3, all the OPs were

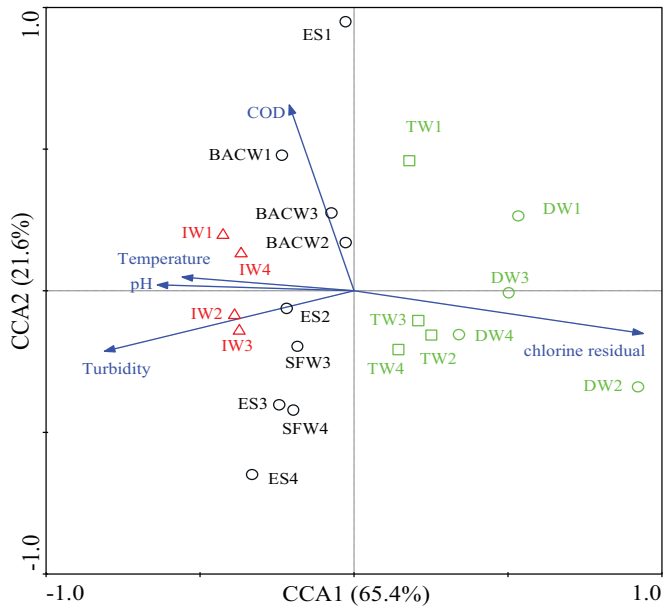

Fig. 3. Quantitative correlation between water quality parameters and gene copy numbers of different OPs in influent water (IW) and after coagulation and settling (ES), sand filtration (SF), biological active carbon filtration (BAC), and disinfection (DW). Correlation analysis was conducted by canonical correspondence analysis (CCA). Note: the gene copies of OPs positively correlated with turbidity and COD, while negatively correlated with residual chlorine.

significantly reduced after sedimentation ($p < 0.05$). This could be attributed to lower influent turbidity, COD, HPC and total coliforms. Turbidity-causing particles can enhance survival and proliferation of OPs such as *Mycobacterium* spp. and *M. avium* (Falkinham III et al., 2001). Thus, turbidity removal by sedimentation decreases the availability of attachment and growth sites for OPs.

After sedimentation, BAC filtration and sand filtration are used independently or together in DWTP1, DWTP3 and DWTP4. These filtration processes removed *M. avium* (10%–58%) and *Acanthamoeba* spp. (29%–89%). However, the abundance of other OPs (*Legionella* spp., *Mycobacterium* spp., *P. aeruginosa*) and total bacteria (i.e., 16S rRNA) increased by 23%–1855% (Fig. 2, Table S7). This is because the filter media contains organic matter that supports bacterial growth (Pinto et al., 2012). In DWTP2, O_3-BAC treatment decreased the gene copies of all OPs by 20%–78%, better than with just BAC treatment in DWTP1, which only significantly removed *Acanthamoeba* (89%, $p < 0.05$, Table S7, Fig. 2). In DWTP3, sand filtration followed by O_3-BAC significantly removed all OPs and total bacteria, ranging from 0.48–1.79 log (gene copies/mL) (66%–98%, $p < 0.05$, Table S7). Thus, O_3-BAC is effective at removing OPs. This could be due to O_3 having the dual function of inactivating OPs as well as removing COD, the carbon source for regrowth.

3.3. OPs were significantly removed by disinfection but rebounded in tap water

Chlorine and chloramines can induce OPs into a viable but non-culturable (VBNC) state (Chen et al., 2018; Shaheen and Ashbolt, 2018), resulting in significant underestimation. For example, Wang et al. (2013b) detected *Legionella* spp. at considerable levels using q-PCR, but could not detect them using standard culturing methods. Therefore, qPCR was used in our study to quantify OPs after water disinfection and distribution. Gene copies of total bacterial and all OPs significantly decreased (51%–99%) after disinfection (Fig. 2). As expected, CCA analysis indicates that occurrence of OPs, HPC, and 16S rRNA in each DWTP is negatively correlated with residual chlorine (ClO_2 or NaClO) (Fig. 3, Table S5). Thus, disinfection is the most important process for OPs removal. However, the efficacy of different disinfectants and pretreatment processes at controlling different OPs and their regrowth in distribution systems are not well understood. For example, chloramine disinfectants control *Legionella* (Moore et al., 2006), but may enhance *Mycobacteria*, though the mechanism is unclear (Moore et al., 2006; Pryor et al., 2004). Therefore, system-specific selection and dosing of appropriate disinfectants (informed by qPCR analysis or other culture-independent measurements of OP abundance) is necessary for effective management of OPs.

In DWTP1 and DWTP4, ClO_2 was the only disinfectant with residual chlorine (ClO_2) in disinfected water (DW), at 0.17 to 0.21 mg/L (no significant difference between DW1 and 4, $p > 0.05$, Table S4). In DWTP1, ClO_2 removed *P. aeruginosa* by 99%, *M. avium* by 97%, total bacteria by 91%, *Mycobacterium* spp. by 83%, amoeba *Acanthamoeba* spp. by 75%, and *Legionella* spp. by 50%, respectively (Fig. 2). DWTP4 removed these OPs better than DWTP1 (Fig. 2, Table 1), particularly *Legionella* spp., which was removed by 1.05 log (gene copies/mL) in DWTP4 compared to only by 0.31 log (gene copies/mL) in DWTP1. The overall removal efficiency for *Legionella* spp. was 77.3% in DWTP4, which is much higher than 6.7% in DWTP1 (Table 2). This suggests OPs in the effluent of sand filtration (DWTP4) were inactivated by ClO_2 more easily than OPs in the effluents of BAC filtration (DWTP1). Previous study also indicated that sand filtration better controlled the bacterial antibiotic resistance rather than BAC filtration, attributing to the faster biofilm proliferation attached in the surface of activated carbon (Bai et al., 2015).

Due to the potential regrowth of OPs in DWDS (Garner et al., 2018; Garner et al., 2019), The abundance of all OPs increased while treated water traveled about 5 km through distribution pipelines to the tap, achieving maximum tap water concentrations of 5.33, 4.87, 1.63, 3.85, and 4.32 log (gene copies/mL) for *Legionella* spp., *Mycobacterium* spp., *Mycobacteria avium*, *Pseudomonas aeruginosa* and the amoeba *Acanthamoeba* spp., respectively (Fig. 2). In the tap water (TW) of DWTP1, gene copies of 16S rRNA and all OPs rebounded by 1073% (*P. aeruginosa*), 2130% (*M. avium*), 33% (total bacteria), 71% (*Mycobacterium* spp.), 175% (amoeba *Acanthamoeba* spp.) and 34% (*Legionella* spp.), respectively. Four OPs: *Mycobacterium* spp., *M. avium*, *P. aeruginosa* and the amoeba *Acanthamoeba* spp. showed greater increase than others ($p < 0.05$ for each OP, Table S8). Unexpectedly, OPs gene copies in tap water from DWTP4 increased more than that from DWTP1. In TW4, *Legionella* spp. increased by 0.79 log (gene copies/mL) (39.0%) but only by 0.13 log (gene copies/mL) (25.7%) in TW1 (Fig. 2, Table 1).

O_3-BAC pretreatment was followed by disinfection using NaClO in DWTP2 or ClO_2 in DWTP3. Maintaining disinfectant residual is a common strategy to control bacterial rebound in distribution systems (Waak et al., 2019). Residual chlorine (NaClO) in the effluent of DWTP2 (DW2) was 0.39 mg/L while ClO_2 residual in DW3 was 0.21 mg/L ($p < 0.05$). Both disinfectants (NaClO and ClO_2) were effective at removing OPs ($p < 0.05$ for each pathogen, Table S8). Results corroborated that despite lower residual chlorine in DW3, ClO_2 used in DTWP3 is more effective for OPs inactivation than NaClO used in DWTP2. This could be attributed to the higher oxidation capacity of ClO_2 (5 e^-) than NaClO (2 e^-) (Hinenoya et al., 2015).

These OPs also rebounded in tap water (TW) from both DWTP2 and DWTP3 ($p < 0.05$ for each OP except *P. aeruginosa*, Table S8). Despite the rebound, the concentrations of OPs were the lowest among the four TWs. Furthermore, *M. avium* was under the limit of quantification (LOQ) in the effluents of O_3-BAC treatment, DW3, and TW3. Total bacteria (assessed per 16S rRNA) also did not significantly rebound in TW3 ($p = 0.208$, Table S8). Thus, without considering potential confounding factors such as pipeline leakage, infiltration and corrosion in distribution systems (Wang et al., 2017b), residual ClO_2 apparently mitigates better against OP regrowth than NaClO (Fig. 2).

Table 1
Removal efficiencies after treatment and distribution by four different systems.

OP	DWTP1 (%)		DWTP2 (%)		DWTP3 (%)		DWTP4 (%)	
	DW1	TW1	DW2	TW2	DW3	TW3	DW4	TW4
16S rRNA	94.4	92.5	99.4	97.0	99.4	99.3	95.8	94.3
Legionella spp.	6.7	−25.7 *	98.1	89.6	98.5	96.2	77.3	−39.0 *
Mycobacterium spp.	81.4	68.2	97.5	92.6	99.9	99.9	99.7	98.7
Mycobacteria avium	97.2	37.4	99.6	81.9	99.6	99.6	98.3	59.9
Pseudomonas aeruginosa	99.8	97.8	99.1	97.4	98.9	96.1	99.9	97.4
Acanthamoeba spp.	98.2	95.0	99.3	98.1	99.8	99.5	95.9	90.4

Note: "DW" means disinfected water, and "TW" means tap water for treatment trains depicted in Fig. S1.
* Negative removal efficiency denotes OP concentration rebound in TW1 and TW4.

3.4. Potential infection risks of *M. avium*

Annual infection probabilities of *M. avium* were estimated for the four drinking water treatment and distribution systems, using QMRA with our experimentally-determined "live fraction" of 3.92×10^{-4} and an infectivity of 0.1% from the literature (Fang et al., 2018). In all cases, influent *M. avium* posed annual infection probabilities that exceeded the EPA benchmark risk level (10^{-4}) and even WHO benchmark (10^{-3}) (Fig. 4), but treated and disinfected water samples showed significant OP removal (>97%), easily meeting WHO and the more stringent U.S. EPA risk level (10^{-4}) (Pecson et al., 2017). Annual infection risks in tap water were higher than those in the disinfected water due to significant rebounding of OPs in the distribution systems (Fig. 2). Drinking water distribution systems are complex, and many confounding factors including water age, water chemistry, leakage, infiltration, and pipeline corrosion may affect the regrowth of OPs (Garner et al., 2018). Huang et al. (2020) indicated that the attenuation of residual chlorine and the prolonged stagnation would not efficiently suppress the growth of OP, increasing the QMRA quantified L. *pneumophila* infection risk. Nevertheless, all tested tap water samples in this investigation met the WHO and U.S. EPA risk benchmarks for *M. avium*. Overall, periodic testing using more sensitive culture-independent molecular approaches is recommended to ensure continued compliance of microbial safety at the point of use.

4. Conclusions

This investigation of the occurrence of various OPs through four full-scale drinking water treatment plants shows that whereas these systems meet microbial safety standards for total coliforms and total heterotrophic bacteria counts, there are still OPs present in the finished water and tap water, posing an overlooked (though relatively small in this case) public health risk. Coagulation/sedimentation reduced the gene copies of OPs while removing turbidity. O_3-BAC mainly removed COD (i.e., potential carbon sources for OPs), which ultimately resulted in lower OPs abundance. However, just filtration (BAC or sand filtration) resulted in regrowth of some OPs including *Legionella* spp., *Mycobacterium* spp. and *P. aeruginosa*. Disinfection was corroborated to be the most effective process to remove OPs in DWTPs, and O_3-BAC-ClO_2 disinfection was the most effective treatment train. OPs were shown to rebound significantly

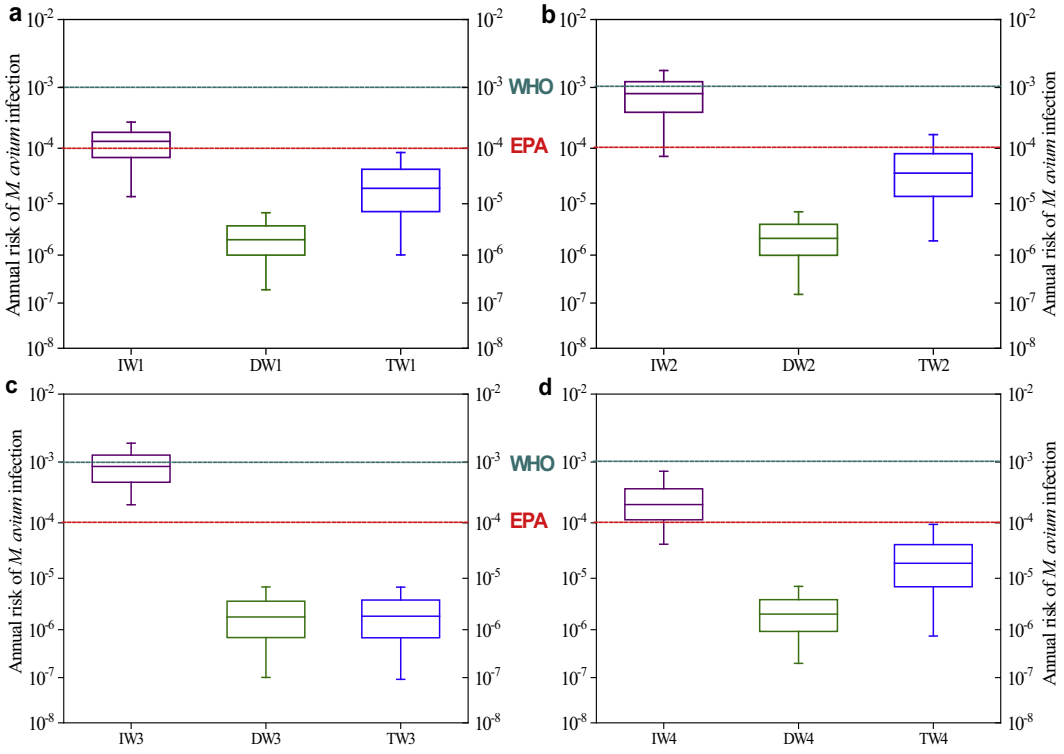

Fig. 4. Annual risk of *M. avium* infection through influent water, disinfected water and tap water in four drinking water treatment systems (panels a, b, c and d). The horizontal line in the box is the median value ($n = 1000$); box boundaries represent the 1st and 3rd quartiles. Error bars represent the 95% confidence interval. All DWTPs easily reduced the annual infection risks of *M. avium* efficiently. Despite OP rebounds in distribution systems, all potential infection risks in tap water were below WHO and even EPA benchmarks.

in the four distribution systems under consideration, although QMRA indicated that potential infection risks of *M. avium* in tap water were still below WHO (10^{-3}) and EPA (10^{-4}) benchmarks. Overall, culture-dependent quantification of indicator organisms that prevail in China and other parts of the world tend to underestimate the pathogenic risk of drinking water and overestimate the effectiveness of some treatment processes for controlling OPs. Thus, it is prudent to periodically scrutinize the safety of drinking water using molecular approaches, and to develop more efficient approaches to mitigate the rebound of OPs in water distribution systems.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoleng.2020.106134.

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