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# One-year survey of opportunistic premise plumbing pathogens and free-living amoebae in the tap-water of one northern city of China

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## ABSTRACT

In this study, qPCR was used to quantify opportunistic premise plumbing pathogens (OPPPs) and free-living amoebae in 11 tap water samples collected over four seasons from a city in northern China. Results demonstrated that the average numbers of gene copies of *Legionella* spp. and *Mycobacterium* spp. were significantly higher than those of *Aeromonas* spp. ( $p < 0.05$ ). *Legionella* spp. and *Mycobacterium* spp. were 100% (44/44) positively detected while *P. aeruginosa* and *Aeromonas* spp. were 79.54% (35/44) and 77.27% (34/44) positively detected. *Legionella pneumophila* was only detected in 4 samples (4/44), demonstrating its occasional occurrence. No *Mycobacterium avium* or *Naegleria fowleri* was detected in any of the samples. The average gene copy numbers of target OPPPs were the highest in summer, suggesting seasonal prevalence of OPPPs. Average gene copy numbers of OPPPs in the taps of low-use-frequency were higher than in taps of high-use-frequency, but the difference was not significant for some OPPPs ( $p > 0.05$ ). Moderate negative correlations between the chlorine concentration and the gene copy numbers of OPPPs were observed by Spearman analysis ( $r_s$  ranged from  $-0.311$  to  $-0.710$ ,  $p < 0.05$ ). However, no significant correlations existed between OPPPs and AOC, BDOC, or turbidity. Moderate positive correlations were observed between the target microorganisms, especially for *Acanthamoeba* spp., through Spearman analysis ( $p < 0.05$ ). Based on our studies, it is proposed that disinfectant concentration, season, taps with different-use frequency, OPPP species, and potential microbial correlations should be considered for control of OPPPs in tap water.

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## Introduction

Opportunistic premise plumbing pathogens (OPPPs) like *Legionella pneumophila*, *Mycobacterium avium*, and *P. aeruginosa* in drinking water could be closely linked to waterborne diseases such as

Legionnaires' disease, Pontiac fever, and pulmonary diseases (Wang et al., 2013a; Hoge and Breiman, 1991). In August 2015, Legionnaires' disease, caused by the OPPP *Legionella*, attacked people in New York City, killing 7 and sickening 86 people (Almendrala, 2015). In fact, OPPPs have been reported to share a

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variety of environmental habitats with humans, including drinking water distribution systems (DWDS) (Delafont et al., 2014), premise plumbing systems (PPS) (Wang et al., 2013a), hospital therapy pools (Stout et al., 1998), shower hoses (Proctor et al., 2016), garden hoses (Thomas et al., 2014), and cooling towers (Mouchtouri et al., 2010). Therefore, it could be deduced that OPPPs possibly cohabit with humans. Among all the colonization points of OPPPs, taps are the points of frequently used and contacted and are especially accessible to human due to aerosol generation. Generally, taps are part of the distal parts of DWDS. Due to the consumption of chlorine (caused by intermittent stagnation and long water age) (Erickson et al., 2017; Kumpel and Nelson, 2016), OPPPs may regrow in the distal parts of DWDS. Also, the growth and detachment of biofilms in the DWDS could increase the number of OPPPs transferred to the taps (Shen, 2016). The proliferation of OPPPs and free-living amoebae (FLA) in the DWDS may pose risk to certain susceptible groups such as the old, the sick, and the injured, mainly through inhalation of aerosols and direct contact (Wang et al., 2013a). *Legionella*-bearing aerosols detected in proximity to taps have been reported (Bolin et al., 1985), manifesting that taps could be a source of OPPPs-infection. To date, many hospital-acquired pneumonia infections and community-acquired pneumonia infections associated with the occurrence of OPPPs have been reported (McEachern and Campbell, 1998; von Baum et al., 2010). Hospitals and hotels have been identified as sensitive points for OPPPs (Liu et al., 1995). 29,636 cases of OPPP diseases costing \$850 million were discovered per year (Collier et al., 2012).

Typically, OPPPs are natural inhabitants of drinking water, and the commonly tracked OPPPs in drinking water are *Legionella* spp., *Mycobacterium* spp., *Aeromonas* spp., and *P. aeruginosa* (Wang et al., 2017). The genera listed above may harbor many pathogenic species such as *L. pneumophila*, *M. avium* and others. Many kinds of these clinically relevant species have been isolated from patients. In addition to DWDS, *Legionella* spp. can also colonize the hot water network (Leoni et al., 2004; Borella et al., 2004; Saby et al., 2005). As eukaryotic hosts for OPPPs, FLA such as *Acanthamoeba* spp. and *Naegleria* spp., often isolated from drinking water (Delafont et al., 2013), represent another risk to the drinking water. FLA can survive in the DWDS and PPS, serving as the hosts for *Legionella*, *Mycobacteria*, and *P. aeruginosa* through internalization, increasing their chance of survival in drinking water. (Wang et al., 2017; Garcia et al., 2013). The colonization and regrowth of FLA in DWDS and premise plumbing tanks have been previously reported (Thomas and Ashbolt, 2011). In fact, OPPPs like *Legionella*, *Mycobacteria* and *Pseudomonas* are amoebae-resistant bacteria (ARB). These OPPPs could grow and survive inside the FLA.

In summary, OPPPs colonizing drinking water are relatively disinfectant-resistant, oligotrophic, and FLA-internalized (Pruden et al., 2013), making them advantageous competitors in the drinking water. For example, *M. avium* is about 500 times more resistant to chlorine than *E. coli* (Taylor et al., 2000). Although residual chlorine is maintained at the end of DWDS, OPPPs could still survive and multiply (Marciano-Cabral et al., 2010; Lehtola et al., 2007; Dantec et al., 2002). In addition, biofilms formed in the inner surface of DWDS pipes have been identified as a source of protection and ideal niche for OPPPs (Buse et al., 2014). The detachment and release of OPPPs from pipe wall biofilms may increase the migration of OPPPs to tap water. The pathogens in

the drinking water passing through the DWDS could regrow with disinfectant decay, which may increase the pathogens in tap water. Therefore, tap water may be a sensitive point for the inhabitation of OPPPs.

In this study, typical opportunistic pathogens occurring in drinking water such as *Legionella* spp., *Mycobacterium* spp., and *Aeromonas* spp. were targeted. Three representative pathogenic species, *Legionella pneumophila*, *Mycobacterium avium*, and *P. aeruginosa*, were chosen. Typical FLA, acting as hosts and protection for many OPPPs, *Acanthamoeba* spp. and *Naegleria fowleri*, were also targeted. In addition, the 16S rRNA genes representing total bacteria were also quantified. The aims of this investigation are as follows: (1) profiling the prevalence and incidence of OPPPs in drinking tap water and their changes with seasons; (2) comparing the distribution characteristics of OPPPs in the taps of high-use-frequency and low-use-frequency; (3) evaluating the correlations of critical water parameters like chlorine, assimilable organic carbon (AOC), and turbidity with OPPPs in the taps. The potential microbial relationships between the target OPPPs were analyzed with Spearman correlation analysis. Quantitative polymerase chain reaction (qPCR) was used to quantify the target OPPPs due to its high specificity, high throughput, and low detection limit (Wang et al., 2013b).

## 1. Materials and methods

### 1.1. Water sample collection and processing

The studied city is located in the north of China (Appendix A Fig. S1). The groundwater and surface water are treated with different processes by different drinking water treatment plants (DWTP). For the groundwater, the water is treated by sand filtration and chlorination prior to entering the DWDS. For the surface water, the water is treated with precipitation and flocculation, settling, sand and biological activated carbon filtration prior to chlorination. Poly aluminum chloride and ferric sulfate are used as flocculants. The speed of filtration for sand filtration is approximately 7 m/hr, and the empty bed contact time is 15 min. The water is disinfected with sodium hypochlorite for at least 30 min of contact time, and a chlorine residual of >4 mg/L is maintained prior to entering the DWDS.

All the collected tap water samples were cold water sampled at the same time of day during normal operation and usage. A total of 11 sampling sites dispersed across the city were monitored (Table 1). Four sampling events (Autumn: Sep, 2015; Winter: Dec, 2015; Spring: Apr, 2016; Summer: Jul, 2016) were performed. The taps were wiped with 75% ethanol and run for 15 sec prior to water collection (Donohue et al., 2015). At each tap, 3 L of water was sampled in three 1-L sterile glass media bottles, respectively. 1 L of water was used for the testing of water quality parameters and the other 2 L of water (bottles containing sterile sodium thiosulfate) was used for DNA extraction (Appendix A Fig. S2). All the water samples for DNA extraction were stored at 4°C and transported to the lab and processed within 6–12 hr.

### 1.2. DNA extraction

For DNA extraction of each water sample, two aliquots (1 L) of water were separately filtered through a 0.20-μm polycarbonate

**Table 1 – General description of the sampling events.**

Sampling numbers	Location type	Sampling type	Sampling time
1	Public office*	Wash room tap water	autumn, winter, spring, summer
2	Resident kitchen	Kitchen tap water	autumn, winter, spring, summer
3	Hospital*	Wash room tap water	autumn, winter, spring, summer
4	Private dining room	Washroom tap water	autumn, winter, spring, summer
5	Middle school*	Washroom tap water	autumn, winter, spring, summer
6	Middle school*	Washroom tap water	autumn, winter, spring, summer
7	Hotel*	Washroom tap water	autumn, winter, spring, summer
8	Private dining room	Kitchen tap water	autumn, winter, spring, summer
9	Public office*	Washroom tap water	autumn, winter, spring, summer
10	Private residence	Washroom tap water	autumn, winter, spring, summer
11	Private office	Washroom tap water	autumn, winter, spring, summer

The locations with asterisk (\*) represented the taps of high-use-frequency.

filter (0.20- $\mu$ m GTTP, Millipore Isopore™, USA) (Appendix A Fig. S2). The filters were fractured and subjected to DNA extraction with a FastDNA SPIN Kit for soil (Solon, MP Biomedicals, USA) following the manufacturer's protocol. The extracted DNA for each water sample (2-L water aliquot) was pooled to minimize the bias caused by variation in the DNA extraction process (Li et al., 2015). DNA concentrations were measured with a spectrophotometer (ND-1000, NanoDrop, USA) and stored at  $-80^{\circ}\text{C}$  before use.

### 1.3. Quantitative PCR (qPCR) assay

All the primers and probes (if necessary) were acquired from published studies and listed in Appendix A Table S1. qPCR was performed using an Applied Biosystems 7300 qPCR system (ABI 7300, Applied biosystems, USA). *Legionella* spp., *Mycobacterium* spp., *L. pneumophila* and 16S rRNA genes were quantified with 25  $\mu$ L of TaqMan-qPCR mixture containing 10.2  $\mu$ L dd H<sub>2</sub>O, 12.5  $\mu$ L premix Ex taq (TaKaRa, China), 0.5  $\mu$ L ROX (50 $\times$ , TaKaRa, China), 0.25  $\mu$ L of each primer (10  $\mu$ mol/L), 0.3  $\mu$ L of probe (10  $\mu$ mol/L) and 1- $\mu$ L DNA template. For the Synergy Brands (SYBR) Green assays used to quantify the rest of the OPPPs, 25- $\mu$ L mixture was comprised of the following: 9.5- $\mu$ L double-distilled H<sub>2</sub>O (dd H<sub>2</sub>O), 12.5- $\mu$ L SYBR Premix Ex taq (TaKaRa, China), 0.5- $\mu$ L ROX (50 $\times$ , TaKaRa, China), 0.25- $\mu$ L of forward and reverse primer (10  $\mu$ mol/L), 2- $\mu$ L template DNA. For each qPCR run, a six-point 10-fold diluted plasmid standard curve, a negative control and the samples were run in triplicate. A melt curve was produced in each qPCR run to verify the specificity of the primer. The qPCR products were randomly selected for agarose gel electrophoresis to visualize the product size (Appendix A Fig. S4). Information on inhibition, limit of detection, and recovery efficiency of filtration for the qPCR tests is listed in Appendix A Text S1 and Fig. S3.

### 1.4. qPCR standards

The plasmids used for standard curves of qPCR were prepared through TA cloning based on a previous study (Oster et al., 2014). Briefly, target gene fragments were amplified from *Pseudomonas aeruginosa* (ATCC 15692), *Aeromonas hydrophila* (ATCC 7966), *Acanthamoeba* spp. AC362, *Legionella pneumophila* (ATCC 33155) and *Mycobacterium* spp. (directly isolated from the water) genomic DNA. Plasmids DNA concentration was measured with the spectrophotometer (ND-1000, NanoDrop

Technology, America). The gene copy numbers were determined by the following formula: gene copy numbers/ $\mu$ L =  $6.02 \times 10^{23} \times \text{plasmid DNA concentration (ng/ $\mu$ L)} / (\text{molecular weight of plasmid} + \text{molecular weight of gene insert})$  (Oster et al., 2014). A (1:10 volume) serial dilution of plasmid DNA was performed, acting as the qPCR standard.

### 1.5. Water quality parameter test

Temperature and pH were measured by a pH meter (Five Easy Plus, Mettler Toledo, Switzerland) at the sampling site. Free chlorine was determined by the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method. Alkalinity was tested through hydrochloric acid titration using Methyl Orange as indicator. The turbidity was measured by reading the absorbance of each sample at 680 nm through a spectrophotometer (U-3900, HITACHI, Japan). The heterotrophic plate count (HPC) for water was enumerated by spread plating on R2A agar and CFUs were counted after 7-day incubation at  $25^{\circ}\text{C}$ . dissolved organic carbon (DOC) was measured via a total organic carbon analyzer (TOC-V<sub>CPH</sub>, SHIMADZU, Japan). The biodegradable dissolved organic carbon (BDOC) was measured using sand-fixed bacteria following a published procedure (Escobar et al., 2001; Escobar and Randall, 2001). The AOC was tested by measuring the growth of *Pseudomonas fluorescens* strain P17 and *Spirillum* strain NOX according to the literature (Liu et al., 2002; Lechevallier et al., 1993) and the growth yields for acetate were  $7.85 \times 10^6$  and  $1.00 \times 10^6$  CFU/ $\mu$ g C for P17 and NOX, respectively. Further details are presented in Appendix A Text S2. Each water quality parameter was measured thrice.

### 1.6. Data analysis

The gene copy numbers were  $\log_{10}(x + 1)$  transformed to enable plotting (OriginPro 2016). Prior to statistical analysis, the normality of data sets was tested with Shapiro–Wilk test (Wang et al., 2012b). One-way analysis of variance (ANOVA) or the nonparametric Wilcoxon test (Wang et al., 2012a) was employed to compare the water quality parameters and log-transformed biological data based on the normality of the data sets. Correlations between different organisms and physiochemical parameters were analyzed by a Spearman test (IBM statistics, version 22). A  $p$ -value of  $<0.05$  was considered statistically significant.



## 2. Results and discussion

### 2.1. Water quality parameters

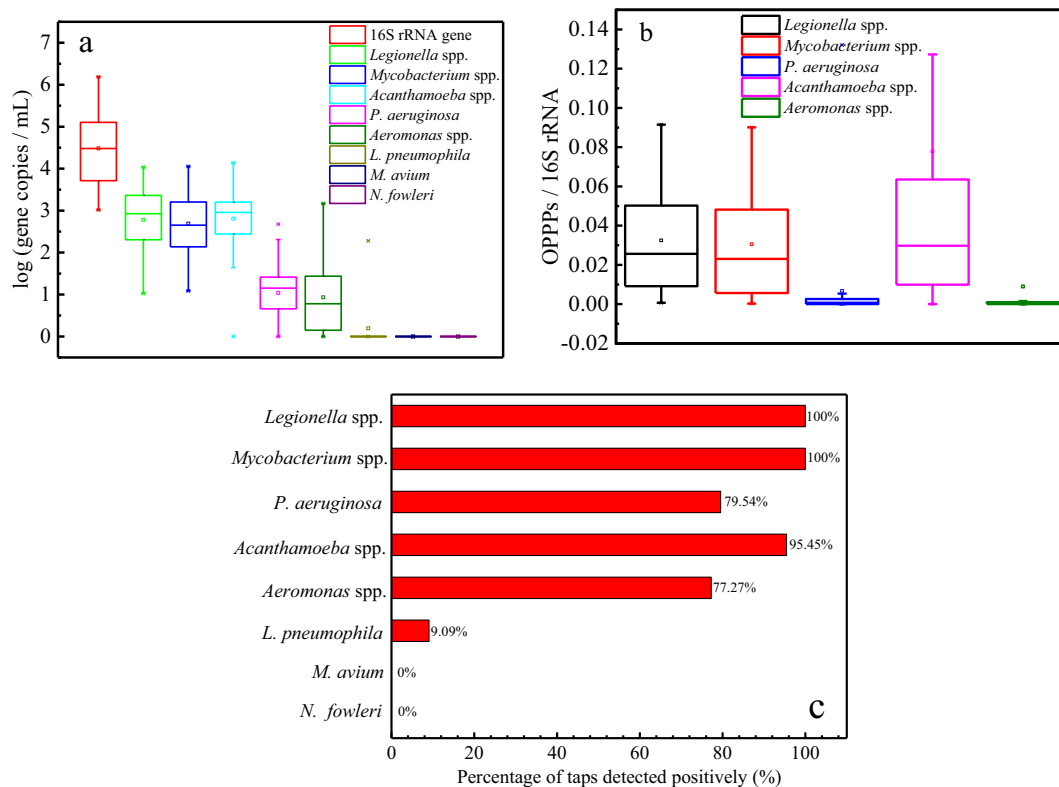
Appendix A Tables S5–8 presented the general water quality parameters during the four sampling events. Briefly, the pH of all the samples for 11 locations varied within a small range (7.44–8.20). However, the average pH in spring and winter was significantly higher than in summer and autumn ( $p < 0.05$ ). Alkalinity varied from 89.77 to 188.43 mg  $\text{CaCO}_3/\text{L}$ . No significant difference was observed for the alkalinity among seasons ( $p = 0.342$ ), which was likely due to the considerable variation in alkalinity among taps. The variation in the proportions between surface water and groundwater could be one reason for the considerable variance in different seasons. Also, the occasional addition of a corrosion inhibitor for the protection of DWDS (such as  $\text{Na}_3\text{PO}_4$ ), causing an artificial increase of alkalinity, the switch of water source between groundwater and surface water, and other unknown factors may cause the considerable variations of alkalinity observed. The average turbidity in the winter was significantly higher than in spring ( $p = 0.008$ ). No significant seasonal difference was observed for average DOC ( $p = 0.730$ ) or BDOC ( $p = 0.534$ ). The average AOC in the summer was lowest among the four seasons, but the difference was not statistically significant ( $p = 0.358$ ). The insignificance of the difference for AOC among the four seasons was possibly due to the high variation in AOC concentrations among the samples. Generally, the AOC content in the bulk water of DWDS could involve a number of factors, such as oxidization by disinfectants (Ramseier et al., 2011), or autotrophic bacteria (such as nitrifying bacteria) producing AOC (Martin, 2012). In addition, a study has shown that some organic nutrients (including AOC) could be released from the corrosion products of DWDS (Morton et al., 2005). These multiple factors could thereby cause high variation in AOC among samples. The average HPC in the summer was highest among the four seasons, but the difference was not statistically significant ( $p = 0.114$ ). The lack of significance for HPC among seasons is likely due to the high variation in HPC number among the sampling sites. The average temperature of tap water in the summer was significantly higher than in other seasons ( $p < 0.05$ , data not shown).

The residual free chlorine concentration varied widely in the range of 0.01–0.51 mg/L during the four sampling events, reflecting the complexity of the DWDS. In the DWDS, water age, pipe materials, distribution distance, water quality, and temperature etc. could influence the decay of chlorine. Among the 44 tap-water samples, the turbidity of all samples was lower than 1 NTU (Chinese national standard, GB/T 5750.4-2006). However, there were 14 tap-water samples (31.82%) whose chlorine concentrations were lower than 0.05 mg/L, the requirement for tap-water in China (Chinese national standard, GB/T 5750.11-2006), suggesting the exhaustion of chlorine in the taps. Twenty-four of the tap water harbored HPC > 100 CFU/mL (Chinese national standard, GB5749-2006), demonstrating that high regrowth potential may exist in the DWDS. These results indicated that some tap water may generate health risk.

### 2.2. The general distribution of OPPPs in the tap water

Fig. 1 shows the OPPP distribution of the tap water during the four sampling events. The 16S rRNA gene copies (indicating total bacteria) varied between 3.02 to 6.19 (mean value  $4.48 \pm 0.82$ ) log gene copies/mL. The gene copy numbers of *Legionella* spp. and *Mycobacterium* spp. ranged from 1.02 to 4.03 (averaged at  $2.78 \pm 0.72$ ) and 1.08 to 4.01 (averaged at  $2.69 \pm 0.73$ ) log gene copies/mL, which were significantly higher than those of *Aeromonas* spp. ( $p < 0.05$ ). The gene copy numbers for *P. aeruginosa* were relatively low and ranged between 0 and 2.67 (averaged at  $1.04 \pm 0.69$ ) log gene copies/mL. Fig. 1b shows the relative abundance of gene copies of OPPPs normalized to 16S rRNA. The relative abundances of *Legionella* spp. and *Mycobacterium* spp. were significantly higher than those of *P. aeruginosa* and *Aeromonas* spp. ( $p < 0.05$ ). Acting as the eukaryotic host for some OPPPs, the gene copies for *Acanthamoeba* spp. varied from 0 to 4.13 (averaged at  $2.81 \pm 0.83$ ) log gene copies/mL. *Legionella* spp. and *Mycobacterium* spp. were 100% positively detected (44/44) (Fig. 1c). The positive detection rates for *P. aeruginosa*, *Acanthamoeba* spp. and *Aeromonas* spp. were 79.54% (35/44), 95.45% (42/44) and 77.27% (34/44), respectively. No *M. avium* or *Naegleria fowleri* were detected during the four sampling events (0/44). *L. pneumophila* were detected in only 4 samples (9.09%, 4/44). These results demonstrated that *Mycobacterium* spp. and *Legionella* spp. were resistant in the tap water.

The number of all target gene copies quantified in this study was comparable to other previous studies (Wang et al., 2013b, 2012b; Yu et al., 2008). One hundred percent positive detection and high gene copy numbers for *Legionella* spp. and *Mycobacterium* spp. suggested their universality and resistance in tap water. Therefore, these two kinds of OPPPs could be classified as resistant groups. Typically, *Legionella* spp. and *Mycobacterium* spp. are the major species of OPPPs colonizing drinking water because of their high resistance. Several factors could increase the resistance of *Mycobacterium* spp. in DWDS. First, the cell membrane of *Mycobacterium* spp. is hydrophobic and lipid-rich (Falkinham, 2009, 2003), which could improve their resistance to the disinfectant. Also, the hydrophobicity of *Mycobacterium* spp. could increase their propensity to live a sessile life rather than planktonic life through particle-adsorption and biofilm-formation (Steed and Falkinham, 2006; Torvinen et al., 2006), enhancing their resistance to adverse conditions. Second, both the *Mycobacterium* spp. and *Legionella* spp. could survive and multiply inside FLA such as *Acanthamoeba* spp. (Wang et al., 2013a). The FLA could serve as the shelter for the *Legionella* spp. and *Mycobacterium* spp. through internalization. Especially when the FLA turned from trophozoites into cysts, their protection for *Mycobacterium* spp. and *Legionella* spp. could be enhanced (Declerck et al., 2010; Greub and Raoult, 2003). In addition, *Mycobacteria* are divided into fast- and slow-growing species (Stahl and Urbance, 1990). The slow-growing *Mycobacteria* are more resistant to disinfection in comparison to fast-growing *Mycobacteria* (Dailloux et al., 1999). *Legionella* spp. shares some similar characteristics with *Mycobacterium* spp. in the DWDS, such as interaction with *Acanthamoeba* spp., formation of biofilms, and resistance to disinfection, resulting in the resistance of *Legionella* spp. For example, *Legionella* spp. could reportedly survive chlorine doses of up to 50 mg/L when living inside protozoan hosts (Lin et al., 1998). Another reason for the preponderance and resistance of *Legionella* spp. is that they could



**Fig. 1 – Distribution of target OPPPs during the four sampling events. (a) the gene copy numbers of the target OPPPs; (b) the relative abundance of OPPPs normalized to 16S rRNA gene copy number; (c) the positive rates of OPPPs detected in tap water. The boxplot was based on 44 water samples collected over 4 sampling events of 11 taps ( $n = 44$ ). The inner box lines and dots represent the medians and means respectively, while the outer box lines represent the 25th and 75th percentiles.**

protect themselves by viable but non-culturable (VBNC) state (Alleron et al., 2008).

*P. aeruginosa* is another common OPPP isolated from the drinking water. *P. aeruginosa* is typically used as a model organism to study biofilms because of its ability to produce extracellular polymeric substances and form biofilms (Xue et al., 2012). Biofilms rather than bulk water are possibly the major habitats for *P. aeruginosa* in DWDS, which could explain its occasional disappearance in this study. Compared with *Legionella* spp. and *Mycobacterium* spp., *Aeromonas* spp. was obviously at a disadvantage in the tap water, because both its positive detection rate and relative abundance of gene copies were lower than those of *Legionella* spp. and *Mycobacterium* spp. *Aeromonas* spp. have been detected in DWDS by previous studies (Yu et al., 2008; Kühn et al., 1997). *Aeromonas* spp. could cause acute gastroenteritis and wound infections in humans (Figueras et al., 2005). In this survey, *Aeromonas* spp. was also generally detected, suggesting its prevalence in the tap water, although their gene copy numbers were relatively lower than those of *Mycobacterium* spp. and *Legionella* spp. No *M. avium* or *N. fowleri* were detected in any of the samples, which was likely because the numbers of gene copies extracted from 2-L water samples were lower than the detection limit of qPCR. Nineteen- or ten-liter water samples were used for DNA extraction in previous studies (Ahmed et al., 2014; Morgan et

al., 2016). Therefore, future studies should sample higher volume water samples.

The high positive detection rate of *Acanthamoeba* spp. demonstrated their ubiquity in the tap water. *Acanthamoeba* spp. in drinking water could cause risks to consumers because *Acanthamoeba* spp. can act as pathogens (such as *A. castellanii* and *A. astronyxis*) for humans as well as reservoirs and protections for some OPPPs (Garcia et al., 2013). The reappearance of OPPPs in the taps suggests the regrowth of OPPPs in DWDS, which has been often reported (Jjemba et al., 2010; van der Wielen and van der Kooij, 2012; Wang et al., 2012b). The regrowth of OPPPs could produce health risks to consumers, especially to susceptible groups (such as the old and sick). To date, many nosocomial infections and community acquired infections caused by OPPPs have been reported (Marston et al., 1997). The persistence of OPPPs in the DWDS could be attributed to several factors, such as resistance to disinfectants, protection by amoebae, biofilms, and corrosion product formation.

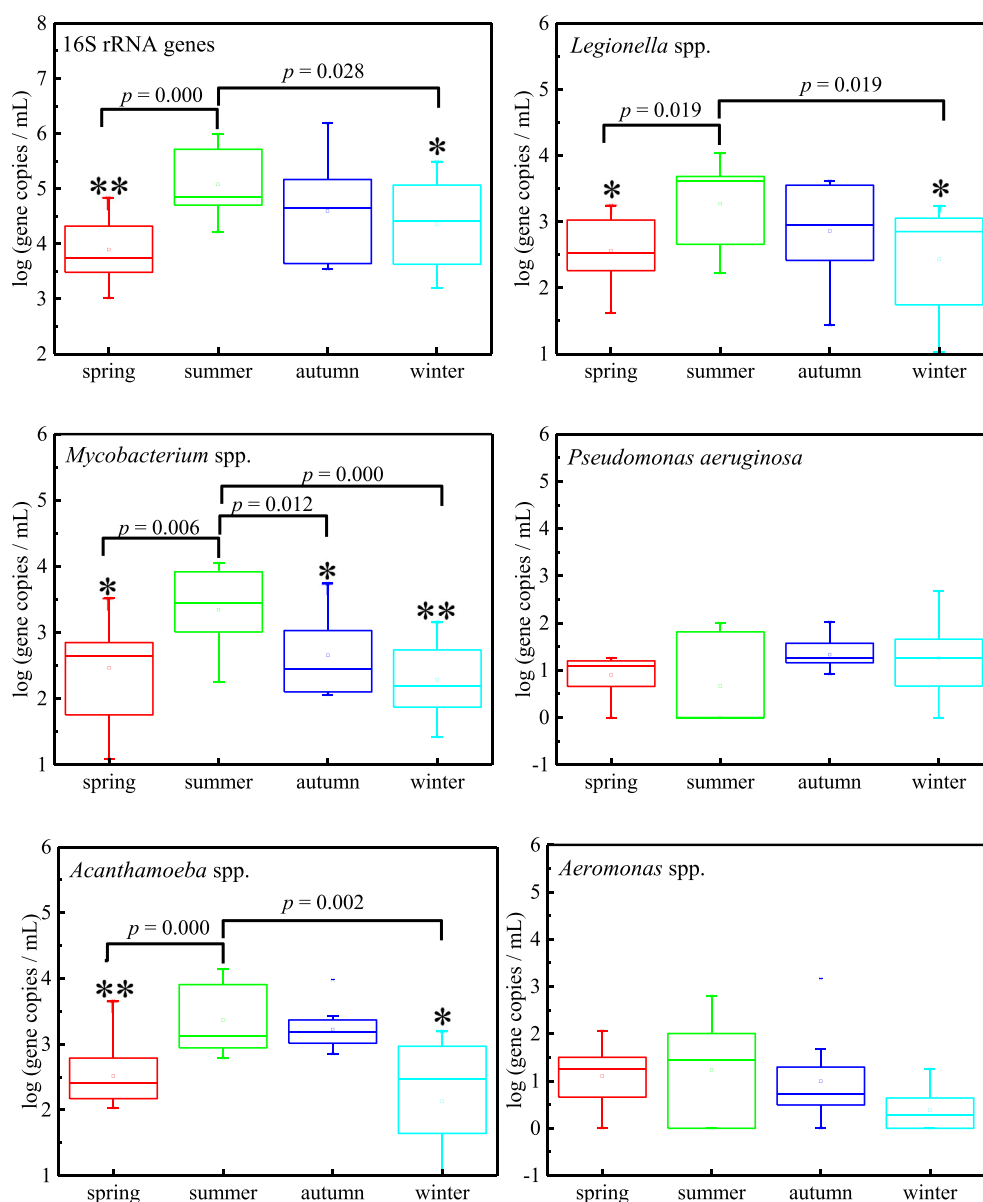
According to the results of this survey, the OPPPs colonizing in tap water could be generally classified into three groups according to their prevalence and characteristics: (1) The resistant group, represented by *Legionella* spp. and *Mycobacterium* spp. These two groups were high in number and widely detected in the drinking water, and they are chlorine-resistant and readily

aerosolized from water (Parker et al., 1983) and tended to live inside biofilms and FLA. (2) The weak group, typified by *Aeromonas* spp. and *P. aeruginosa*. These two groups were occasionally absent and the numbers were relatively small, which may cause sporadic infection. (3) The eukaryotic hosts for OPPPs typified by *Acanthamoeba* spp., which could serve as a “Trojan horse” because of their protection for OPPPs. When a disinfection process is applied to DWDS, these different groups of OPPPs should be considered separately, because their response to disinfection may be different. We should mention that the number of samples for each sampling site was only 1 due to the limited experimental time and resources, which may not be fully satisfactory for statistical analysis. This is one of the shortcomings of this study. In future studies, the number of samples for

each sampling site should be increased to obtain more definitive results.

### 2.3. The distribution of target OPPPs with seasonal changes

Fig. 2 presents the seasonal prevalence of OPPPs. Basically, the gene copy numbers of all targeted microorganisms were the highest in the summer among the four seasons (except *P. aeruginosa*). Taking *Mycobacterium* spp. as an example, the average gene copies in the summer were  $3.35 \pm 0.58$  log gene copies/mL, which was significantly higher than the other three seasons ( $p < 0.05$ ). For 16S rRNA, *Legionella* spp., *Acanthamoeba* spp., and *Aeromonas* spp., the average gene copy numbers in the summer were also the highest,



**Fig. 2 – Seasonal distribution of the target OPPPs.** Nonparametric Kruskal–Wallis rank test or one-way ANOVA test are used based on the normality of the data set. \* and \*\* indicated significant difference compared with summer under  $p < 0.05$  and  $p < 0.001$ , respectively. The boxplots were based on 11 water samples of each season ( $n = 11$ ). The inner box lines and dots represent the medians and means respectively, while the outer box lines represent the 25th and 75th percentiles.

regardless of significance of the difference. However, the average gene copy numbers of *P. aeruginosa* were the lowest in the samples of summer. The seasonal comparisons of each target microorganism are shown in **Appendix A Table S4**. Generally, the average number of target microorganisms is highest in the summer while lowest in the winter, regardless of significance of difference (except *P. aeruginosa*). The differences in the occurrence rate (positive detection ratio) of OPPPs among the four seasons were determined using a Cochran Q test (Cochran, 1954). As shown in **Appendix A Table S3**, there was a significant difference for *P. aeruginosa* ( $Q = 16.04$ ,  $p = 0.001$ ) over the four seasons. No significant difference in seasonal occurrence rate of *Acanthamoeba* spp., *Aeromonas* spp., or *L. pneumophila* was observed ( $p > 0.05$ ).

The results of this study indicated that the OPPPs in the tap water showed seasonal prevalence. The changes of OPPPs with season and water temperature have been studied previously (Thomas et al., 2014; Garcia et al., 2013). The highest numbers of OPPPs (except *P. aeruginosa*) were observed in the summer, which is consistent with a previous study (van der Wielen and van der Kooij, 2012). The high numbers of OPPPs in the summer could be associated with temperature, because temperature is a critical factor affecting the OPPPs, and elevated water temperature could increase the numbers of pathogens (van der Wielen and van der Kooij, 2012; Lasheras et al., 2006; Thomas et al., 2014). Another reason could be the fast chlorine decay occurring in the summer. Surprisingly, the average gene copy numbers of *P. aeruginosa* were lowest in the summer. The reason is unknown. Perhaps this could be the result of microbial interaction. Microbial interactions such as competition and antagonism may exist in the drinking water (Wang et al., 2013a). As discussed above, the tap water was predominated by *Legionella* and *Mycobacteria*, which may produce some suppressive effects on *P. aeruginosa* through competition and/or antagonism. Further study on this should be performed. Based on the results of this study, it could possibly be concluded that the OPPPs in the tap water occurred with seasonal prevalence. More attention to OPPP infection through tap water should be paid in summer.

#### 2.4. The occurrence of OPPPs in sites of different tap use probability

To compare the OPPP distribution characteristics in taps of different-use-frequency, the taps in the 11 sampling places involved in this study were classified into two groups based on daily water consumption and tap-use frequency. The first group was named high-use-frequency taps (daily users are typically more than 100 persons) including 6 locations: 2 public offices, 1 hospital, 2 middle schools, and 1 hotel (Table 1). These places exhibited large water consumption and high tap-use-frequency due to the large numbers of consumers. The second group classified as low-use-frequency taps (daily users are typically fewer than 10 persons) covered 5 locations: 1 residential kitchen, 2 private dining rooms, 1 private residence, and 1 private office. The number of consumers and amount of water consumption in these places were relatively low. However, we should mention that the site numbers of sampling points in this survey were relatively less than in a previous study (Donohue et al., 2014).

As shown in Fig. 3 and **Appendix A Fig. S5b**, the average gene copy numbers of target microorganisms and HPC

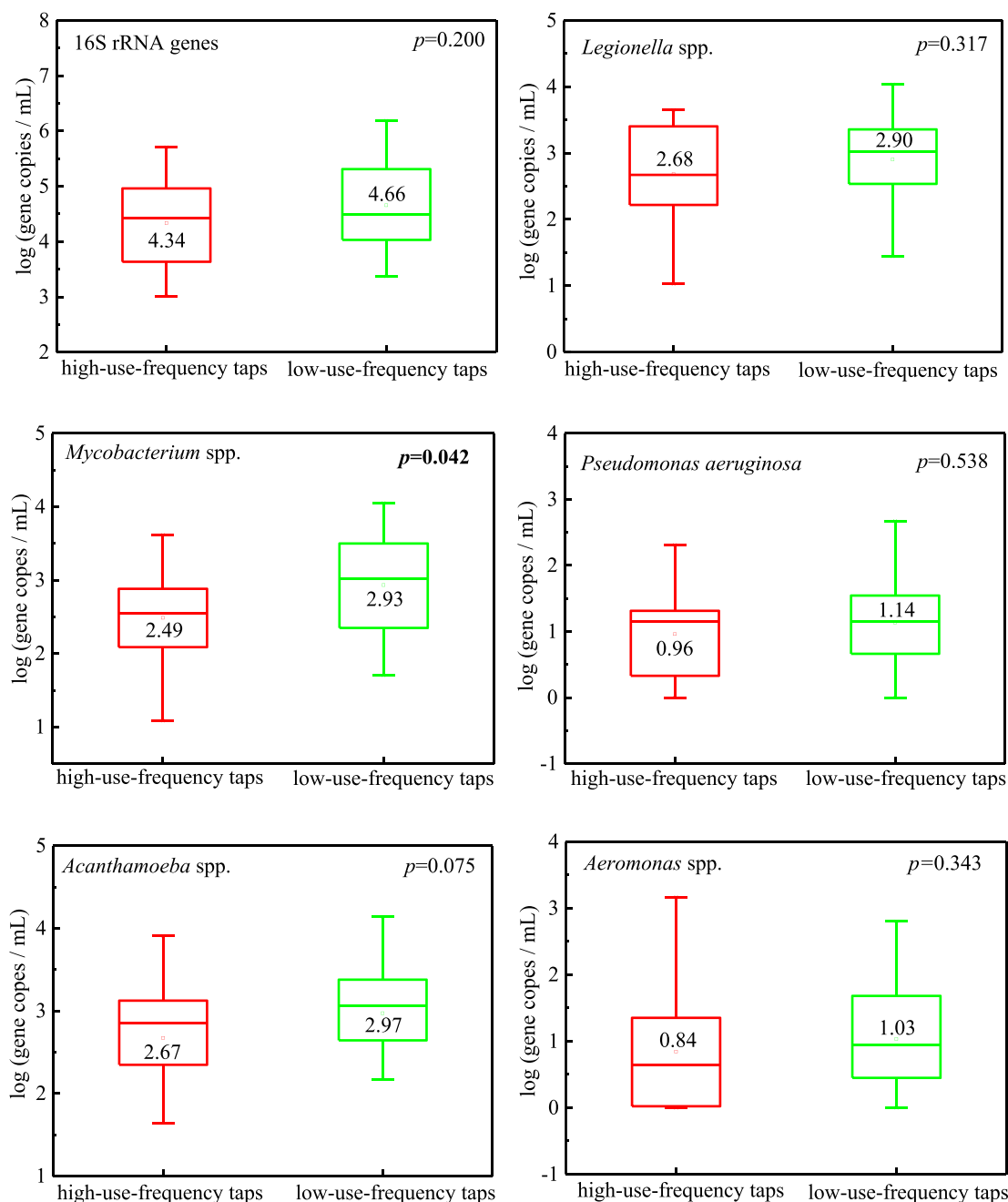
numbers in the low-use-frequency taps were either significantly ( $p < 0.05$ ) or insignificantly ( $p > 0.05$ ) higher than in high-use-frequency taps. Take *Mycobacterium* spp. for example, the gene copy numbers of *Mycobacterium* spp. in the low-use-frequency taps were  $2.93 \pm 0.74$  log gene copies/mL, which were significantly higher ( $p = 0.042$ ) than the values for the high-use-frequency taps ( $2.49 \pm 0.66$  log gene copies/mL). For the 16S rRNA, *Acanthamoeba* spp., *P. aeruginosa*, and *Aeromonas* spp., the gene copy numbers in low-use-frequency taps were also slightly higher than in high-use-frequency taps, but the difference was not significant ( $p > 0.05$ ).

All in all, the results of this study indicated that the number of target OPPPs colonizing the low-use-frequency taps was higher than high-use-frequency taps ( $p < 0.05$ , though it was not statistically significant for some OPPPs), suggesting that the water from low-use-frequency taps may be more likely to produce infection than that from high-use-frequency taps. The lack of significance for some OPPPs was possibly due to the high variance of gene copy numbers among the samples and the small sample size. The higher gene copy number of OPPPs in low-use-frequency taps could be the result of water flushing frequency and water stagnancy time (water age). Typically, a longer stagnant time would cause more disinfectant decay and microbial increase (Wang et al., 2012b). Water consumption in the low-use-frequency taps is typically lower than the high-use-frequency taps due to the lower numbers of consumers. Therefore, the water in the low-use-frequency taps may experience longer stagnant time. A longer stagnant time may be conducive to the decay of chlorine and the growth of OPPPs, resulting in the higher gene copy numbers in the low-use-frequency taps. A similar phenomenon, which the overnight stagnation of water could help increase the microbial cell numbers, was observed by another study (Lautenschlager et al., 2010). A similar previous study showed that the concentration of *Legionella* spp. decreased with increased frequency of use (Collins et al., 2017).

Actually, the taps with low-use-frequency such as family shower sprayers, roof-captured rainwater, garden hoses, and dishwashers have been found as ecological niches for OPPPs (Thomas et al., 2014; Feazel et al., 2009; Dobrowsky et al., 2014; Zalar et al., 2011). The higher numbers of OPPPs detected in low-use-frequency taps demonstrated that OPPP infections may occur in these places during personal daily activities such as taking showers, face-washing, or watering garden flowers. Recently, the colonization of premise plumbing systems by OPPPs has gained attention, since the OPPPs could reside and multiply in premise plumbing systems (Wang et al., 2013a). Likewise, the taps of high-use-frequency such as hospitals and hospital therapy pools are also identified as ideal realms for OPPPs (Mancini et al., 2015; Angenent et al., 2005). Hospitals have been identified as sensitive points for OPPPs since the hospitals are confluence points for the immune-compromised such as sick and injured people, which needs careful attention.

#### 2.5. The relationship between OPPPs and chlorine, AOC, and turbidity

Nonparametric Spearman's rank correlation analysis has often been used to analyze the correlations between different target microorganisms and water quality parameters, based on a



**Fig. 3 – Distribution of target OPPPs in the taps with high-use-frequency and low-use-frequency. The boxplots were based on 6 high-use-frequency taps ( $n = 24$ ) and 5 high-use-frequency taps ( $n = 20$ ) water samples with regard to the 4 sampling events, respectively. The inner box lines and dots represent the medians and means respectively, while the outer box lines represent the 25th and 75th percentiles. Nonparametric Kruskal-Wallis rank test or one-way ANOVA test are used based on the normality of the data set. The numbers in the inner box are the mean values.**

previously published study (Wang et al., 2012b). Therefore, the relationships between OPPPs and chlorine, AOC, BDOC, and turbidity were analyzed by Spearman correlative analysis. As shown in Table 2, 16S rRNA genes ( $r_s = -0.484$ ,  $p = 0.001$ ), *Legionella* spp. ( $r_s = -0.559$ ,  $p = 0.000$ ), *Mycobacterium* spp. ( $r_s = -0.710$ ,  $p = 0.000$ ), *Acanthamoeba* spp. ( $r_s = -0.311$ ,  $p = 0.040$ ) and *Aeromonas* spp. ( $r_s = -0.434$ ,  $p = 0.003$ ) were negatively correlated with chlorine concentration. However, no significant correlated

relationships between OPPPs and AOC, BDOC, or turbidity were found ( $p > 0.05$ ). The relationships between OPPPs and chlorine were also analyzed by linear regression, and were found to be basically consistent with the results of Spearman correlative analysis (Appendix A Fig. S6). For example, the *Legionella* spp. and *Mycobacterium* spp. had a negative linear relationship with chlorine ( $R^2 = 0.2650$  and  $0.4055$ ,  $p < 0.05$ ). Table 3 shows microbial correlations between the target microorganisms by



**Table 2 – Spearman correlation analysis between the microorganisms and chlorine, AOC, BDOC, and turbidity (n = 44).**

Target gene	Chlorine	AOC	BDOC	Turbidity
HPC	–0.281 ( <i>p</i> = 0.064)	0.213 ( <i>p</i> = 0.165)	0.050 ( <i>p</i> = 0.750)	–0.120 ( <i>p</i> = 0.437)
16S rRNA	<b>–0.484</b> ( <i>p</i> = 0.001)	0.138 ( <i>p</i> = 0.370)	0.028 ( <i>p</i> = 0.858)	0.183 ( <i>p</i> = 0.234)
<i>Legionella</i> spp.	<b>–0.559</b> ( <i>p</i> = 0.000)	0.015 ( <i>p</i> = 0.923)	–0.094 ( <i>p</i> = 0.543)	0.039 ( <i>p</i> = 0.803)
<i>Mycobacterium</i> spp.	<b>–0.710</b> ( <i>p</i> = 0.000)	–0.148 ( <i>p</i> = 0.337)	–0.226 ( <i>p</i> = 0.141)	–0.016 ( <i>p</i> = 0.917)
<i>P. aeruginosa</i>	0.115 ( <i>p</i> = 0.456)	–0.186 ( <i>p</i> = 0.227)	–0.062 ( <i>p</i> = 0.691)	–0.145 ( <i>p</i> = 0.347)
<i>Acanthamoeba</i> spp.	<b>–0.311</b> ( <i>p</i> = 0.040)	–0.014 ( <i>p</i> = 0.927)	–0.016 ( <i>p</i> = 0.916)	0.041 ( <i>p</i> = 0.791)
<i>Aeromonas</i> spp.	<b>–0.434</b> ( <i>p</i> = 0.003)	–0.102 ( <i>p</i> = 0.509)	–0.176 ( <i>p</i> = 0.254)	–0.090 ( <i>p</i> = 0.559)

Spearman correlation analysis. Moderate to strong correlation existed among HPC, 16S rRNA genes, *Mycobacterium* spp., and *Legionella* spp. ( $r_s = 0.425$ – $0.785$ ,  $p < 0.05$ ). In particular, moderate correlations existed between *Acanthamoeba* spp. and OPPPs (except *P. aeruginosa*) ( $r_s = 0.319$ – $0.478$ ,  $p < 0.05$ ).

Based on the results of this study, chlorine seemed to have the strongest influence on the OPPPs compared with AOC, BDOC, and turbidity, which was similar to the results of a previous study (Wang et al., 2012a). Although positive relationships between *Mycobacterium* spp. abundance and AOC content were found in previous studies (Falkinham et al., 2001; Torvinen et al., 2004), these relationships were not able to be captured in this study. Several reasons could be proposed. First, a DWDS is typically an oligotrophic environment. For OPPPs in the drinking water, the demand for AOC for survival and growth is quite low compared with traditional fecal-borne pathogens. A previous study showed that OPPPs such as *Mycobacterium* spp. and *P. aeruginosa* could be able to grow at very low carbon levels (Falkinham, 2009). It is reported that an AOC threshold of only 10  $\mu\text{g/L}$  is beneficial for *L. pneumophila* in water (van der Wielen and van der Kooij, 2012). Another study reported that *P. aeruginosa* could even grow in distilled or deionized water (Penna et al., 2002). Obviously, the AOC contents in the water samples of this study were basically in excess of that required to support the growth of OPPPs. Therefore, the relationships between AOC and OPPPs could not be captured. Second, although the OPPPs are heterotrophic, some of them are fastidious about organic matters. For example, *Legionella* spp. is

known to be only capable of using certain amino acids as growth substances (Tesh et al., 1983). *M. avium* could be stimulated by humic acids and fulvic acids (Covert et al., 1999). Therefore, not only the AOC concentration but also the types of organics in AOC may influence the OPPPs. Third, as discussed above, the correlations between the AOC and OPPPs may be overwhelmed by the effects of chlorine, since chlorine had the strongest influence on the OPPPs of bulk water. The lack of significant relationships between turbidity and OPPPs could be because some other factors may also impact the OPPPs, since some OPPPs may be susceptible to environmental factors. In addition, the interactive effects of other abiotic and biotic factors may also mask the relationships between OPPPs and turbidity. An interaction effect of disinfectant type, pipe material, and water age on OPPPs has been observed in a previous study (Wang et al., 2012a).

The positive correlation between *Acanthamoeba* spp. and 16S rRNA is likely due to the fact that *Acanthamoeba* spp. could prey on bacteria for food through phagocytosis (Weekers et al., 1993). The positive correlation between *Acanthamoeba* spp. and OPPPs may suggest a pathogen–host relationship, which is similar to the results of previous studies (Wang et al., 2012a, 2012b). As FLA, *Acanthamoeba* spp. could act as the hosts for OPPPs like *Legionella* spp. and *Mycobacterium* spp. through internalization (Drancourt, 2014), which could offer protection for the OPPPs and improve the resistance of OPPPs to adverse conditions such as disinfection (Lin et al., 1998; Dogruöz et al., 2012). Associations of FLA-mycobacteria, FLA-*legionella*, and FLA-*pseudomonas* have been

**Table 3 – Spearman correlation analysis between the microorganisms during the four sampling events (n = 44).**

	HPC	16S rRNA	Leg	Myco	Ps	Ac	Ae
HPC	1	<b>0.474</b> ( <i>p</i> = 0.001)	<b>0.537</b> ( <i>p</i> = 0.000)	<b>0.425</b> ( <i>p</i> = 0.004)	–0.201 ( <i>p</i> = 0.192)	<b>0.336</b> ( <i>p</i> = 0.026)	0.190 ( <i>p</i> = 0.216)
16S rRNA		1	<b>0.785</b> ( <i>p</i> = 0.000)	<b>0.618</b> ( <i>p</i> = 0.000)	–0.158 ( <i>p</i> = 0.306)	<b>0.494</b> ( <i>p</i> = 0.001)	0.141 ( <i>p</i> = 0.363)
Leg			1	<b>0.620</b> ( <i>p</i> = 0.000)	–0.152 ( <i>p</i> = 0.325)	<b>0.403</b> ( <i>p</i> = 0.007)	0.282 ( <i>p</i> = 0.064)
Myco				1	–0.126 ( <i>p</i> = 0.417)	<b>0.478</b> ( <i>p</i> = 0.001)	<b>0.325</b> ( <i>p</i> = 0.031)
Ps					1	–0.060 ( <i>p</i> = 0.698)	0.048 ( <i>p</i> = 0.759)
Ac						1	<b>0.319</b> ( <i>p</i> = 0.035)
Ae							1

The bold numbers represented significance under  $p < 0.05$ . Leg, Myco, Ps, Ac, Ae represented *Legionella* spp., *Mycobacterium* spp., *P. aeruginosa*, *Acanthamoeba* spp., and *Aeromonas* spp. respectively.

previously reported (Delafont et al., 2014; Thomas et al., 2014; Garcia et al., 2013). Positive correlations between *Mycobacterium* spp. and HPC, *Legionella* spp. and *Mycobacterium* spp. have also been found in previous studies. (Kusnetsov et al., 2003; Wang et al., 2012b), indicating potential symbiotic relationships. *Legionella*'s symbiotic relationships with other microorganisms have already been revealed (Surman et al., 1994; US EPA, 1985). The interaction between OPPPs and other microorganisms may influence the survival of OPPPs. For example, some members in the microbe community (such as *Acanthamoeba* and *H. vermiformis*) (Thomas et al., 2014; Garcia et al., 2013) could boost the OPPPs, while some members (such as *Acidovorax* sp. and *Sphingomonas* sp.) (Gião et al., 2011) could suppress the OPPPs. These results indicated that the interactions between OPPPs and FLA should be considered, as these interactions may influence the persistence and survival of OPPPs in drinking water.

### 3. Conclusions

A one-year survey of the distribution OPPPs in tap water from 11 locations in a northern city of China was conducted in this study by qPCR. The seasonal prevalence, difference between taps of high and low use-frequency, relations with water parameters, and potential microbial interactions of OPPPs were studied. Although the sampling numbers were likely less than in previous studies, some scenarios of OPPPs in the tap water could still be captured. Several conclusions could be drawn. (1) *Legionella* spp. and *Mycobacterium* spp., two resistant genera, were 100% positively detected and exhibited high gene copy numbers in the tap water. *Aeromonas* spp. and *P. aeruginosa* were occasionally undetectable in some samples. As hosts of OPPPs, *Acanthamoeba* spp. widely occurred in the tap water. No *M. avium* or *N. fowleri* were detected in this study. The infectious species *L. pneumophila* were sparsely detected. (2) For each target OPPP, the samples collected in summer typically harbored higher numbers of gene copies than those in other seasons due to the suitable growth temperature and the faster decay of chlorine. (3) Basically, low-frequency-use taps presented higher counts of OPPPs than high-frequency-use taps, but not statistically. (4) Chlorine was the strongest factor influencing the OPPPs in the tap water, which may overwhelm the effects of AOC, BDOC, and turbidity. (5) Some moderate correlations were observed between OPPPs and *Acanthamoeba* spp., which may reflect symbiotic relationships between OPPPs and *Acanthamoeba* spp.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jes.2018.04.020>.

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