

博士論文 (要約)

**Factors Influencing Microbial Regrowth and Occurrence of  
Opportunistic Pathogens in Premise Plumbing**

(水道給配水系における微生物再増殖と日和見病原細菌の存在に及ぼす影響因子)

**IFTITA RAHMATIKA**

イフティタ ラフマティカ

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氏    名    イフティタ ラフマティカ  
                 Iftita Rahmatika

Biological stability is critical for the safety of drinking water. However, microbial water quality gets deteriorated during water distribution, especially in premise plumbing, which can lead to microbial regrowth and occurrence of opportunistic pathogens. Opportunistic pathogens, including *Legionella pneumophila*, *Mycobacterium avium* and *Pseudomonas aeruginosa*, could be linked to waterborne disease, such as pulmonary infection and pneumonia-like symptoms. However, their occurrences in drinking water are not well monitored.

One crucial characteristic of premise plumbing is water stagnation. Water can be stagnant for extended periods, causing the decay of disinfectant, which leads to microbial regrowth. In addition, smaller pipe diameters in premise plumbing cause the higher reaction rates of disinfectant decay. Another critical issue in premise plumbing is the migration of organic matter from polymeric pipe materials (e.g., cross-linked polyethylene [PE], high-density polyethylene [HDPE], polyvinyl chloride [PVC]) that can occur during stagnation. Certain fraction of this organic matter can be biodegradable that can promote microbial regrowth in drinking water. However, little is known about the impacts of migrated organic matter on indigenous microbial communities and occurrence of opportunistic pathogens in drinking water. Moreover, the compositions of the organic matter promoting microbial regrowth are not resolved yet due to the complex nature of organic matter. Hence, a better understanding on the critical factors influencing microbial regrowth and occurrence of opportunistic pathogens in premise plumbing is important in order to efficiently control microbial regrowth in premise plumbing.

The main objective of this study was to understand the critical factors influencing microbial regrowth and occurrence of opportunistic pathogens in premise plumbing, via field investigations and lab-scale experiments. Specific objectives were: (1) to evaluate the influence of stagnation on microbial community structure and occurrence of opportunistic pathogens in premise plumbing, (2) to assess the fate of bacteria growing in premise plumbing and opportunistic pathogens in drinking water supply system and (3) to identify the composition of biodegradable organic matter (BOM) released from polymeric pipe materials that promotes

microbial regrowth by non-target screening using high-resolution Orbitrap mass spectrometry (MS).

Chapter 1 describes the background and objective of this research. Chapter 2 summarizes literature review and studies related with microbial regrowth in drinking water, occurrences and health impact of opportunistic pathogens, and factors influencing microbial regrowth in premise plumbing. Chapter 3 summarizes the materials and methods applied in this study.

In Chapter 4, the influence of water stagnation on microbial regrowth was evaluated. Drinking water samples were collected from nine different faucets at two different buildings in Hongo Campus, the University of Tokyo. The sampling was performed in four sampling events (Summer, June – July 2018; Autumn, Nov 2018; Winter, January 2019; Spring, April 2019). Eight samples were sampled from Building A, while one sample was sampled from Building B. Water samples were collected pre- and post-stagnation. From each faucet, 10 L of pre-stagnation samples were sampled after flushing the water for 5 min. Following 24 h of stagnation, post-stagnation samples were obtained by collecting every 100 mL in the first one liter after stagnation.

The significant decrease of free chlorine to  $<0.02$  mg/L in the first 100 mL after stagnation was observed in all faucets, resulting in the significant increase of Total Cell Counts (TCC) from  $(2.7 \pm 2.5) \times 10^3$  cells/mL to  $(1.2 \pm 0.9) \times 10^5$  cells/mL after stagnation in Building A. Building B also showed similar trend of microbial regrowth, where TCC significantly increased from  $(1.5 \pm 0.4) \times 10^4$  cells/mL to  $(9.3 \pm 1.2) \times 10^4$  cells/mL after stagnation. This indicated that stagnation greatly influences microbial regrowth in the absence of free chlorine. Amplicon sequencing targeting V4 region of 16S rRNA genes revealed that microbial community of Building A samples had distinctive composition between pre- and post-stagnation, indicating microbial community composition was also affected by stagnation. Opportunistic pathogens such as *M. avium*, *L. pneumophila*, *P. aeruginosa* and *Acanthamoeba* spp. were below the quantification limit of real time-PCR in pre- and post-stagnation samples of all faucets. However, other opportunistic pathogens, such as *Mycobacterium gordonae*, *Mycobacterium haemophilum*, *Legionella feeleii*, *Legionella maceachernii*, and *Legionella micdadei* were detected after stagnation using Nanopore sequencing targeting full length of 16S rRNA genes. This result suggested that potential health risk can increase after water stagnation in premise plumbing.

TCC, *Mycobacterium* spp., and *Legionella* spp. were not significantly different between seasons, while microbial community composition showed seasonal trend. Based on community compositions, winter samples were dominated with *Pseudomonas* spp., while *Sphingomonas* spp. were the most dominant bacteria in most of the faucets after stagnation in

other seasons. Faucets with low frequency of use presented the significant higher number of TCC compared with faucets with high frequency of use. Microbial communities of stagnant water between faucets with high and low frequency of use were significantly different, suggesting that microbial regrowth was very site-specific. Significant negative correlation between chlorine concentration in pre-stagnation samples and TCC after stagnation was observed, indicating that low chlorine concentration supports more microbial regrowth after stagnation.

In Chapter 5, the fates of dominant bacteria growing in premise plumbing and opportunistic pathogens were evaluated from treatment process to premise plumbing. Water samples were collected from a drinking water treatment plant in Tokyo Metropolitan Area, which is the main source of drinking water in Building A. Chlorine was added at the dose of 0.5–0.7 mg/L prior to distribution, resulting in the decrease of TCC in finish water down to  $(4.3 \pm 3.9) \times 10^2$  cells/mL. In fresh drinking water samples before stagnation, chlorine was still maintained at 0.17–0.36 mg/L and TCC was  $(2.7 \pm 2.5) \times 10^3$  cells/mL. However, when free chlorine was depleted to <0.02 mg/L after 24 h stagnation, TCC significantly increased to  $(1.2 \pm 0.9) \times 10^5$  cells/mL. The abundance of dominant OTUs in premise plumbing (e.g. OTU-001 *Sphingomonas*) were suppressed in treatment plant, but they increased after stagnation in premise plumbing. The same trend was also observed for *Mycobacterium* spp. and *Legionella* spp. There were also bacteria that was not detected in treatment process, such as OTU-025 *Dechloromonas*, that increased in premise plumbing.

In Chapter 6, the composition of BOM released from polymeric pipe materials and their impact on microbial regrowth were evaluated. First, migration assay was conducted by incubating three new polymeric pipes, including PE, HDPE and PVC, in Milli-Q with and without free chlorine. Migrated dissolved organic matter (DOM) samples were collected after incubation at room temperature for 24 h. Migrated DOM samples from PE without and with free chlorine ( $DOM_{PE}$  and  $DOM_{PE-Cl}$ ) contained 0.17 and 0.36 mg/L of DOC, respectively. Migrated DOM was then concentrated by solid phase extraction using Bond Elut PPL cartridge (Agilent) and analyzed by Orbitrap MS coupled with negative-mode electronic spray ionization (ESI) after liquid chromatographic separation with InertSustain<sup>®</sup> AQ-C18 (GL Science). The results revealed that composition of DOM released from PE, HDPE and PVC were different, indicating that organic matter released from pipes was dependent on pipe material. The addition of chlorine also affected the DOM compositions, especially for  $DOM_{PE-Cl}$  and  $DOM_{HDPE-Cl}$ .

Regrowth potentials of migrated DOM samples from polymeric pipes were evaluated. Migrated DOM samples were incubated with microbial community from a faucet with initial TCC of  $5 \times 10^3$  cells/mL and without any inoculation as a control. The initial free chlorine concentration was set to be below the limit of quantifications (LOQ), and mineral medium was

added in all assays for microbial growth. All sets of samples were incubated in carbon-free glass bottles at 25°C for 4 days. As a comparison, autoclaved tap water (TW) was also prepared for regrowth potential assay in the same manner. Inoculated assay showed the highest growth of microorganisms in DOM<sub>PE and PE-Cl</sub>, with the increase of TCC up to  $>10^6$  cells/mL after 4 days, followed by DOM<sub>PVC and PVC-Cl</sub>, DOM<sub>HDPE and HDPE-Cl</sub> and TW. The higher regrowth potential in migrated DOM sample from pipes compared with TW indicated that additional organic matter released from pipes greatly promoted microbial regrowth. The DOM after regrowth was extracted and the composition was analyzed by LC-Orbitrap MS. Components whose intensities decreased by more than 10% after incubation were screened as BOM candidates promoting microbial regrowth. The results showed that among the total number of DOM components that were assigned for molecular formulae, 4- 27% of them decreased their peak intensity with simultaneous growth of bacteria, indicating that several fractions of organic matter released from pipes could be biodegradable that promoted microbial regrowth.

In Chapter 7, the conclusions and recommendation of this study were summarized. This study demonstrated that stagnation and DOM released from several new polymeric pipe materials could be important factors that greatly promoted microbial regrowth in premise plumbing. Tracking of bacterial community from treatment to end of pipe indicated that bacterial groups with regrowth potential decreased after treatment while they increased again after stagnation in premise plumbing. Further development of guidelines regarding pipe material selection and water usage practice in premise plumbing is also essential to maintain a good microbial water quality prior to the consumption.