Original Article

Impacts of Organic Matter Migrating from Pipe Materials on Microbial Regrowth in Drinking Water

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ABSTRACT

Polymeric materials are widely used in premise plumbing. To assess the potential of pipe materials to promote microbial regrowth in drinking water, we incubated drinking water in a new cross-linked polyethylene pipe (N-PEX), a new steel pipe lined with powdered polyethylene (N-SPE), and an old steel pipe lined with powdered polyethylene (O-SPE). As a reference, we also incubated drinking water in a carbon-free glass bottle (REF). Free chlorine rapidly depleted to below the quantification limit in the pipe incubations. While the original drinking water contained 0.4 mg C/L of dissolved organic carbon (DOC), the DOC concentration in N-PEX, N-SPE and O-SPE increased to 5.2, 3.1 and 1.7 mg C/L, respectively, after 8 days of incubation. The total cell count (TCC) in N-PEX, N-SPE, and O-SPE increased from $<10^3$ cells/mL to 6.0×10^5 , 2.8×10^5 , and 3.6×10^6 cells/mL after 8 days of incubation, respectively. On the other hand, the TCC in REF increased to 1.4×10^5 cells/mL after 14 days of incubation. Dominant bacterial groups were different under the different incubation conditions. These results indicated that organic matter migrating from pipe materials promote microbial regrowth and affect the microbial community composition in drinking water.

Keywords: drinking water, microbial regrowth, polyethylene, premise plumbing

INTRODUCTION

The biological stability of drinking water is critical for the safety of drinking water [1]. However, water quality can get degraded during distribution, causing hygienic, aesthetic, and operational issues [2]. In particular, microbial regrowth can deteriorate the safety of drinking water. To maintain biological stability and limit microbial regrowth during distribution, in several countries, drinking water is distributed with a disinfectant residual. For example, in Japan, the chlorine residual concentration at the tap must be maintained at > 0.1 mg/L in the form of free chlorine or > 0.4 mg/L in the form of combined chlorine. However, during long stagnation of drinking water, chlorine residual is easily diminished. Therefore, limiting biodegradable organic matter (BOM) is another strategy used to prevent microbial regrowth,

especially when drinking water is distributed without a disinfectant residual. The recommended concentration of assimilable organic carbon (AOC), which is part of the BOM that is readily consumed by specific bacterial strains, is < 10 µg acetate-C/L in the distribution system without a disinfectant residual and < 100 µg acetate-C/L in the distribution system with a disinfectant residual [3,4].

Premise plumbing, which is the part of the distribution system located on the consumer's property, often has the issue of microbial regrowth. Critical factors such as temperature fluctuations, long stagnation, and contact with plastic pipes with a large specific area can deteriorate biological stability in the last meter just before consumption [5,6]. Microbial regrowth in premise plumbing has been previously studied. The increase in the total cell count (TCC) from $< 10^3$ to 7.8×10^5 cells/mL was observed after ~6 days of stagnation in

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building plumbing [7]. As a result of excessive microbial regrowth, pathogenic *Legionella pneumophila*, *Mycobacterium avium*, *Pseudomonas aeruginosa*, and *Acanthamoeba* spp. have been frequently detected in tap water in premise plumbing [8,9]. Therefore, microbial regrowth in premise plumbing is a serious issue as pathogens in tap water can increase potential health risks, and it is important to evaluate factors promoting microbial regrowth.

Synthetic polymeric materials (e.g., cross-linked polyethylene [PEX], high-density polyethylene [HDPE], polyvinyl chloride [PVC], and ethylene propylene diene methylene [EPDM]) are widely used in premise plumbing. One of the critical issues with these materials is the migration of organic matter, including additives such as plasticizers and oxidants, and degradation and oxidation products of polymers [10]. A BioMig test was proposed to evaluate the organic matter migration potential of polymeric materials in drinking water [11]. Polymeric materials were immersed in Evian water at 60°C. Results showed that after 24 h of incubation, 0.17, 0.62, and 0.83 mg/L of total organic carbon (TOC) from polyethylene silane cross-linked (PEXb), polybutylene (PB), and EPDM with 2% of plasticizer (EPDM 2%), respectively, were released, indicating that migration of organic matter, depends on polymeric materials. Although migrated organic matter could promote microbial regrowth [12], little is known about the impacts of migrated organic matter on indigenous microbial communities in drinking water.

In this study, we tested three pipes: a new PEX pipe (N-PEX), a new steel pipe lined with polyethylene powder (N-SPE), and an old steel pipe lined with polyethylene powder (O-SPE) to evaluate their potential to promote microbial regrowth and release organic matter during stagnation. In addition, we analyzed the occurrence of pathogens and changes in the microbial community composition in the three pipes.

MATERIALS AND METHODS

Polymeric materials tested

PEXs and SPEs are widely installed in building plumbing, especially in Japan. In this study, the pipe length was 45 cm with a diameter of 2 cm (S/V ratio = 2 cm⁻¹). N-PEX and N-SPE were disinfected with 1 mg/L of sodium hypochlorite for 1–2 h and washed with Milli-Q water to remove possible contamination by bacteria on the surface. The O-SPE selected in this study had been used as premise plumbing for 24 years. Its materials and dimensions were the same as those of N-SPE.

Incubation experiment

Drinking water with a free chlorine concentration of 0.3 mg/L was collected from the tap in the laboratory of the University of Tokyo (Bunkyo-ku, Tokyo, Japan) in October 2018. Prior to collection, the tap water was flushed for 5 min until free chlorine became stable and the temperature became constant. N-PEX, N-SPE, and O-SPE were filled with 150 mL of fresh drinking water and incubated at 25°C for 8 days. Both ends of each pipe were sealed with silicon stoppers. As a reference, the same drinking water (1 L) was also incubated in a carbon-free glass bottle at 25°C for 14 days (REF). Prior to the incubation, a carbon-free glass bottle immersed in HCl (0.2 M) for overnight were baked at 550°C for 6 h in a muffle furnace. Free chlorine and TCC were monitored during incubation. The dissolved organic carbon (DOC) concentration pre- and post-incubation was measured to evaluate the amount of organic matter migrating from the three pipes. Pathogenic microorganisms and the microbial community composition of drinking water pre- and post-incubation in the three pipes were also analyzed.

Water quality analysis

The free chlorine concentration was measured using the *N,N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method (Hach, Loveland, Colorado, USA). The limit of quantification was set at 0.04 mg/L. The TCC was analyzed using flow cytometry. Briefly, 490 µL of each sample was stained with 5 µL of SYBR® Green I (Invitrogen AG, Basel, Switzerland), which was diluted 100-fold with dimethylsulfoxide (DMSO) and 5 µL of 0.5 M ethylenediaminetetraacetic acid (EDTA). The samples were then mixed and incubated at 37°C for 10 min in the dark. Flow cytometry was performed using a BD Accuri C6® flow cytometer (BD Biosciences, San Jose, California, USA). To evaluate the dissolved organic carbon (DOC), water samples were filtered using 0.3 µm pre-combusted glass fiber filters (GF-75, Advantec Toyo, Tokyo, Japan). The DOC concentration was measured as non-purgeable organic carbon with a TOC-L analyzer (Shimadzu, Kyoto, Japan).

Quantitative PCR

For this test, 20 L of original drinking water samples and 100 mL (pipes) and 750 mL (REF) of drinking water samples post-incubation were filtered through 0.2 µm polycarbonate filters (0.20 µm GTTP, Millipore Isopore™, Billerica, Massachusetts, USA) and stored at -20°C prior to DNA extraction. The filters were dissolved in phenol-chloroform-isoamyl alcohol (25:24:1) prior to bead-beating

Fig. 1 Free chlorine concentrations during incubation. Open symbols indicate values below the limit of quantification.

treatment. DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, Ohio, USA) according to the manufacturer's instruction. *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *P. aeruginosa*, and *Achantamoeba* spp. were quantified by quantitative polymerase chain reaction (qPCR) using Taqman assay, as described elsewhere [13–16]. Standards were prepared using tenfold serial dilutions (5.0×10^0 – 5.0×10^6 copies/mL) of plasmid DNA containing target genes. The values below the minimum concentration of the standards were regarded as below the limit of quantification. All reactions were performed using the LightCycler® LC480 system (Roche, Indianapolis, USA).

Microbial community analysis

Extracted DNA was first amplified using the following primers with adapter sequences (515F: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-GGACTACHVGGGTWTCTAAT-3') targeting the V4 regions of 16S rRNA genes [17]. The second PCR and amplicon sequencing was performed on Illumina Miseq platform with 300 bp paired end reads at Bioengineering Lab, Japan. The UCHIME algorithm was applied to eliminate chimeras [18].

Operational taxonomic units (OTUs) of the microbial community were clustered with a 97% similarity using Quantitative Insights Into Microbial Ecology 2 (QIIME 2) [19].

RESULTS AND DISCUSSION

Chlorine consumption by pipe material

As shown in **Fig. 1**, after 1 day, free chlorine in the drinking water incubated in N-PEX, N-SPE, and O-SPE depleted to below the quantification limit of 0.04 mg/L. On the other hand, free chlorine in REF slowly depleted for 9 days. These results indicated that the contact of drinking water with pipe material greatly accelerated the depletion of free chlorine, while organic matter in drinking water gradually reacted with free chlorine. In fact, Mao *et al.* (2018) showed rapid decay of free chlorine in drinking water incubated with new EPDM, PEXb, and polyethylene electron beam cross-linked (PEXc) pipes, with a chlorine decay rate constant of 0.19, 0.20, and 0.05 1/h, respectively [20]. Kowalska *et al.* (2006) reported that chlorine consumption rates depended not only on the pipe materials, but also on the pipe diameters and service age [21].

Migration of organic carbon from pipe materials

DOC concentrations pre- and post-incubation were compared, as shown in **Fig. 2**. While the DOC concentration in

Fig. 2 Changes in DOC concentration pre- and post-incubation samples.

REF decreased from 0.45 to 0.28 mg/L, it increased to 5.2, 3.1, and 1.7 mg/L in N-PEX, N-SPE, and O-SPE, respectively, suggesting that the pipe material has the potential to release organic carbon. In addition, the DOC concentration depended on both pipe material (N-PEX vs. N-SPE) and age (N-SPE vs. O-SPE). It is likely that aging of pipe material decreases the DOC release potential (SPE vs. O-SPE). In fact, a repeated migration assay decreased the DOC release for several polymeric materials, such as polyethylene peroxide cross-linked (PEXa), PEXb, PEXc, and polyvinyl chloride containing 30% of plasticizer (PVC-P) [11].

The apparent specific release potential (measured DOC normalized to the inner specific surface area of the pipe) of N-PEX, N-SPE, and O-SPE was 0.29, 0.16, and 0.07 $\mu\text{g C/cm}^2/\text{day}$, respectively, during 8 days of incubation. A BioMig test showed that after 24 h of incubation, PEXc and PEXb released ~ 0.7 and $\sim 1.9 \mu\text{g C/cm}^2$ of TOC, respectively [20], indicating that the migration potential of polyethylene pipes depends on the type of cross-linking. Since microbial regrowth and organic matter release occurred simultaneously in our assay, it is necessary to consider the consumption by microorganisms to estimate the gross migration potential.

Microbial regrowth after decay of free chlorine residual

Changes in the TCC during incubation are shown in Fig. 3. The TCC in the original drinking water was 9.0×10^2 cells/mL. The TCC increased and reached the maximum at 6.0×10^5 , 2.8×10^5 and 3.6×10^6 cells/mL after 7 days in N-PEX,

N-SPE and O-SPE, respectively. Microbial regrowth in REF became obvious after 8 days when free chlorine was depleted to 0.06 mg/L. After free chlorine depleted, TCC reached the maximum of 1.4×10^5 cells/mL after 14 days. A greater increase in the TCC in N-PEX, N-SPE and O-SPE compared to REF clearly indicated that organic matter supporting microbial regrowth was additionally released from the pipe material. The increase in the TCC in O-SPE was the highest, despite the apparent lower migration of DOC, as shown in Fig. 2, indicating that more BOM was released from O-SPE, which was simultaneously consumed by microorganisms. The BOM consumed during incubation was estimated on the basis of the TCC and the general bacterial growth yield (10^7 cells/ $\mu\text{g C}$) [22]. We estimated that the BOM consumed by microorganisms in REF to be 12 $\mu\text{g C/L}$. On the other hand, BOM in N-PEX, N-SPE and O-SPE was estimated to be 53, 24, and 300 $\mu\text{g C/L}$, respectively, indicating that BOM fraction was probably minor in the migrated DOC. In O-SPE, the properties of the pipe material might change after long operation, resulting in enrichment of the BOM fraction in an aged pipe. In addition, the biofilm developed on the O-SPE surface could be a source of BOM. A BioMig test showed that 10%–65% of the TOC released from PEX pipe was regarded as AOC components [11,20,23]. Migration accelerated at 60°C in the BioMig test, while in our study, we used ambient conditions. Therefore, temperature could affect the composition of migrated organic matter.

Fig. 3 TCCs during incubation.

Occurrence of opportunistic pathogens in the microbial community

Major pathogens that can be found in premise plumbing (*L. pneumophila*, *M. avium*, *P. aeruginosa*, and *Acanthamoeba* spp.) were quantified using qPCR. Their abundance in the drinking water samples pre- and post-incubation was below the quantification limit: 2.5×10^{-1} , 5.0×10^0 and 6.7×10^{-1} gene copies/mL for original drinking water, pipes and REF, respectively. The abundance of *Mycobacterium* spp. and *Legionella* spp. increased in O-SPE (Fig. 4). It was probably because of the biofilm that formed on the O-SPE surface. As biofilm is important source for pathogenic microorganisms, further identification on the microbial abundance and community in biofilm is necessary. Besides *M. avium* and *L. pneumophila*, other species such as *M. gordonae*, *M. haemophilum*, *L. feelei*, *L. micdadei*, and *L. maceachernii* also show pathogenicity [24–27]. It is possible that the biofilm on the O-SPE surface was supported by organic matter that migrating from the pipe material. Further phylogenetic identification of *Mycobacterium* spp. and *Legionella* spp. associated with old pipes is necessary for risk assessment of premise plumbing. On the other hand, *Mycobacterium* spp. and *Legionella* spp. in REF, N-PEX and N-SPE were below the quantification limit: 6.7×10^{-1} and 5.0×10^0 gene copies/mL for REF and the three pipes, respectively (Fig. 4) after incubation. Although *Mycobacterium* spp. and *Legio-*

nella spp. might increase a little bit during incubation, their abundances were below the limit of quantification probably because fast-growing microorganisms in REF, N-PEX, and N-SPE might suppress their growth or organic substrate necessary for their growth was limited under these conditions.

Shift of the microbial community composition

Figure 5 shows the microbial community composition of the original drinking water and the four post-incubation sample. *Phreatobacter* spp. were the dominant microorganisms (31%) in the original drinking water. Studies have also found *Phreatobacter* spp. dominant in drinking water distribution networks in Paris and Belgium [28,29]. Drinking water was delivered from those systems with and without chlorine, respectively. A previous study also showed that *Phreatobacter* spp., referred to as group F0723, were more abundant in waters with a low chlorine concentration [30]. Therefore, the low chlorine concentration in our study (0–0.3 mg/L) probably promoted the regrowth of *Phreatobacter* spp. The relative abundance of *Phreatobacter* spp. increased to 75% in REF postincubation, suggesting that residual BOM in drinking water further promoted the growth of *Phreatobacter* spp.

On the other hand, *Phreatobacter* spp. decreased to < 0.1% in N-PEX, N-SPE, and O-SPE. Different incubation conditions in REF and in N-PEX, N-SPE, and O-SPE caused this

Fig. 4 Gene copy numbers of (a)*Mycobacterium* spp. and (b)*Legionella* spp. pre- and post-incubation.

* Values below the limit of quantification.

shift in the microbial community composition. *Pseudomonas* spp. (61%) and unclassified genera within Comamonadaceae (27%) were dominant in N-PEX. It is possible that these microorganisms specifically respond to organic matter migrating from PEX. On the other hand, *Methylophilus* spp. (49%), *Novosphingobium* spp. (26%), and *Pseudomonas* spp. (11%) were dominant in N-SPE, indicating that the organic matter composition released from N-SPE could be different compared to N-PEX. Previous studies have frequently detected Comamonadaceae, *Novosphingobium* spp., *Methylophilus* spp., and *Pseudomonas* spp. in premise plumbing [7,29,31,32].

In O-SPE, *Novosphingobium* spp. (26%) were dominant, which was consistent with the result of N-SPE. However, the microbial community composition of N-SPE and O-SPE were not identical, probably because of the impact of biofilm formation on the O-SPE surface. We applied general growth yield (10^7 cells/ μgC) to evaluate BOM consumption based on TCC data. However, growth yields of dominant microorganisms under each condition should be checked for accurate estimation of BOM.

Proctor *et al.* (2016) revealed that different pipe materials of shower hoses in a realistic building plumbing simulator

Fig. 5 Changes in microbial community composition.

developed different biofilm community compositions after several months of operation [23]. PEXc, PEXb, and silicone demonstrate a higher relative abundance of *Legionella* spp., *Pseudomonas* spp., *Nocardia* spp., and *Mycobacterium* spp. compared to PVC-P [23]. Although the physical properties of materials could affect biofilm development, different compositions of migrated organic matter could be another important factor determining the microbial community composition.

Our results clearly indicate that pipe incubation not only increases microbial abundance but also alters the microbial community composition. However, the composition of organic matter migrating from pipes remains unclear. Further research is necessary to identify organic matter promoting microbial regrowth.

CONCLUSIONS

Contact of drinking water with new polyethylene materials contributed to the release of organic matter and the decay of free chlorine. The organic fraction migrating from the different pipe materials increased TCC and had a significant impact on the microbial community composition in drinking water. However, major pathogens were below the quantification limit. Old steel pipe lined with polyethylene powder

supported a greater increase of TCC and *Mycobacterium* spp. and *Legionella* spp. which may contain other pathogenic species. These findings suggests that we should focus on the migration of organic matter from polymeric materials in premise plumbing in order to control microbial regrowth in drinking water.

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