Identification of bacteria assimilating formaldehyde in a biological activated carbon filter by means of DNA stable isotope probing and next-generation sequencing
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ABSTRACT
Ozonation followed by a biological activated carbon (BAC) filter is a common process of advanced water purification. Ozone can generate formaldehyde (FA) as a harmful by-product, while the subsequent BAC filter is efficient at removing FA. FA adsorption is limited on activated carbon because of its hydrophilic property and low molecular weight. Thus, biological degradation by biofilms associated with the BAC is regarded as the primary treatment mechanism for FA. However, little is known about the microorganisms involved in the removal of FA. To identify specific microorganisms assimilating FA in a BAC filter, this study applied DNA stable isotope probing (DNA-SIP) combined with next-generation sequencing (NGS) technology. DNA-SIP with NGS clearly revealed that facultative methylotrophs affiliated within the specific taxonomic groups, such as Hyphomicrobium and Methylibium, were suspected to be the key players in FA removal.

Key words | biological activated carbon, DNA stable isotope probing, formaldehyde

INTRODUCTION
Ozonation followed by a biological activated carbon (BAC) filter is widely used as an advanced water purification process (Seredyńska-Sobecka et al. 2006; Simpson 2008). In the BAC filter, organic matter is removed by physicochemical adsorption as well as biodegradation by biofilms associated with the activated carbon (Kim et al. 1997; Chien et al. 2008). Ozonation enhances the biological activity of the BAC filter because partial decomposition of large organic matter increases biodegradability (Volk et al. 1997; Ramseier et al. 2011). Although ozonation can degrade micropollutants and odorous compounds (Broséús et al. 2009; Yuan et al. 2013), some harmful by-products such as formaldehyde (FA) are unintentionally generated (Huang et al. 2005). FA adsorption is limited on activated carbon because of its hydrophilic property and low molecular weight. It has been observed that biological filters were effective for FA removal in a full-scale drinking water purification process (Papageorgiou et al. 2014). Taken together, biological function in the BAC filter is regarded as an essential safety barrier for FA in processes containing ozonation.

Microbial community structures in BAC filters are very complex (Kasuga et al. 2007; White et al. 2012; Lautenschlager et al. 2014). While recent advances in molecular biological technologies have shed some light on the diversity of microorganisms associated with BAC filters, very little is known about the linkage between microbial community structure and function, except for a few functional groups such as ammonia oxidizers (Kasuga et al. 2010; Niu et al. 2013). One of the culture-dependent approaches to address the fundamental question ‘who eats what?’ is DNA stable isotope probing (DNA-SIP) (Neufeld et al. 2007). In the DNA-SIP experiment, microbial communities are incubated with isotopically labelled (e.g., $^{13}$C) compounds to identify specific microorganisms assimilating the target compounds. Since the DNA of active microorganisms is labelled with isotope according to their growth, the target DNA containing heavy isotope can be separated from light DNA derived from inactive
microorganisms. Autotrophic growth competition between ammonia-oxidizing archaea and ammonia-oxidizing bacteria in a BAC filter was characterized by DNA-SIP (Niu et al. 2013). To fully elucidate the biological purification mechanism of BAC filters, DNA-SIP is useful for linking structure and function of the microbial community.

This study aimed to identify the key players assimilating FA in a full-scale BAC filter by means of DNA-SIP. Next-generation sequencing (NGS) technology was combined with DNA-SIP to comprehensively identify important microorganisms.

**MATERIAL AND METHODS**

**Sampling**

BAC in the surface layer was collected from a full-scale drinking water purification plant in Tokyo in February 2014. The plant has a production capacity of 1,700,000 m³/day. The purification process in this plant consists of coagulation followed by sedimentation, primary rapid sand filter, ozonation, BAC filter and secondary rapid sand filter. Chlorination is carried out before coagulation and after the BAC filter. In addition, virgin granular activated carbon (GAC) that was used for the BAC was obtained for reference.

**FA removal potential**

BAC (50 g in wet weight) was statically incubated in an inorganic mineral medium with approximately 500 μg/L of FA (Wako Pure Chemical Industries, Japan) in a closed glass bottle at 20 °C for 8 h in the dark. FA concentration in the supernatant was measured by the 3-methyl-2-benzothiazoline hydrazone method with a DR2800 spectrometer (HACH, USA). As a control experiment, the virgin GAC was prepared in the same manner. An inorganic mineral medium containing FA without BAC or GAC was prepared as an operational blank.

**Isotopic labelling and DNA-SIP**

For the DNA-SIP experiment, 10 g of wet BAC was packed in a glass column (30 mm in diameter and 90 mm in length) and 15C- or 12C-labelled FA (15C-FA and 12C-FA) was continuously supplied as a sole carbon source at 20 °C for 7 days. Methanol-free 13C-FA (99% in water) was purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). FA was prepared at 1,260 μg/L for 13C-FA and 1,248 μg/L for 12C-FA, which were equivalent to 0.5 mg C/L. The upward flow rate was maintained at 1.1 mL/min. The hydraulic residence time in the column was around 60 min. After 7 days of incubation, BAC samples incubated with 13C-FA or 12C-FA were collected and stored at −20 °C until further processing.

Nucleic acids were extracted from BAC by the FastDNA Spin kit for Soil (MP Biomedicals, CA, USA) following the manufacturer’s instructions. DNA concentrations were measured using a NanoDrop 1000 (Thermo Fisher Scientific, DE, USA). Approximately 1 μg of extracted DNA was treated by isopycnic ultracentrifugation in cesium chloride gradients (Neufeld et al. 2007). Initial buoyant density was adjusted to 1.724 g/mL. Ultracentrifugation was conducted at 45,000 rpm (178,000 g) in an NVT65.2 Beckman rotor (Beckman, USA) at 20 °C for 72 h. Twenty fractions of equal volume were obtained using a syringe pump (Microfeeder JP-5, Furue Science, Japan) containing liquid paraffin. The buoyant density was determined with an AR200 digital refractometer (Reichert, NY, USA). DNA in each fraction was purified by polyethylene glycol-assisted ethanol precipitation and dissolved in 30 μL Tris–EDTA buffer.

**Quantitative polymerase chain reaction**

Bacterial 16S rRNA genes in each fraction were determined by quantitative polymerase chain reaction (Q-PCR) with a LightCycler 480 II (Roche, Switzerland) (Nadkarni et al. 2002). A LightCycler 480 Probe Master (Roche, Switzerland) was used for TaqMan PCR assays. All reactions were prepared in triplicate. PCR was conducted at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 50 s and 72 °C for 1 min. The cloned 16S rRNA gene amplicon of *Escherichia coli* IFO3301 was used as an external standard in a 10-fold dilution series from 1.0 × 10³ to 1.0 × 10⁹ copies/reaction. A SYBR Green Q-PCR assay was applied for quantification of archaeal 16S rRNA genes by using the primer set of Ar109f and Ar912rt (Lueders & Friedrich 2002). LightCycler 480 SYBR Green I Master (Roche) was used for the reaction chemistry. Q-PCR was conducted at 95 °C for 5 min followed by 45 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. Melting
curve analysis (65–97 °C with a heating rate of 0.1 °C/s) was subsequently performed. The cloned 16S rRNA gene amplicon of Methanobacterium formicicum ATCC 35274 was used as an external standard ranging from $1.0 \times 10^1$ to $1.0 \times 10^7$ copies/reaction. The quantification of 16S rRNA genes of Hyphomicrobiurn was performed by an SYBR Green Q-PCR assay. The primer set of Hyp298f (5'-AAAggTggATTAATgCCgCATA-3') and Hyp717r (5'-TAAATCCgCCTACgTgCgCTTT-3') was designed for specific amplification of 16S rRNA genes of Hyphomicrobiurn by using the software ARB (Ludwig et al. 2004). The specificity of the designed primers was scrutinized by the probeCheck program (Loy et al. 2008) based on the database of quality checked and aligned 16S/18S rRNA-Silva (version 95) (Pruesse et al. 2007). LightCycler 480 SYBR Green I Master (Roche) was used in the reaction. Q-PCR was conducted at 95 °C for 5 min followed by 50 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. Melting curve analysis was subsequently performed in the same manner as archaeal 16S rRNA genes. The 16S rRNA gene amplicon of the Hyphomicrobiurn-related clone obtained from BAC was used as an external standard ranging from $1.0 \times 10^1$ to $1.0 \times 10^7$ copies/reaction.

NGS

DNA extracts obtained from the $^{13}$C-FA fraction of 1.726 g/mL and the $^{12}$C-FA fraction of 1.726 g/mL were selected for paired-end sequencing on the Illumina MiSeq platform. In addition to these two samples, unfractionated DNA extracted from the original BAC was analyzed by NGS. The V3–V4 region of prokaryotic 16S rRNA genes was amplified using specific primers (forward primer: 5'-CCTACgggNggCWgCAG-3'; reverse primer: 5'-gACTACHVgggTATCTAATCC-3') with Illumina overhang adapter sequences (Illumina, CA, USA). KAPA HiFi HotStart Ready Mix PCR Kit (KAPA Biosystems, USA) was used for PCR reactions. PCR products were purified using Agencourt AMPure Beads XP (Beckman Coulter, IN, USA). The sequences were analyzed using the MacQIIME package ver.1.8.0 (Caporaso et al. 2010) (http://www.wernerlab.org/software/macqiime). Uclust (Edgar 2010) was applied to the qualified sequences for clustering operational taxonomic units. RDP classifier (Wang et al. 2007) was used for taxonomy assignment with the reference database of Greengenes gg_13_8 (http://greengenes.lbl.gov/).

RESULTS

FA removal potential of BAC

The FA removal potential of BAC was compared with that of virgin GAC without biomass. Figure 1 shows FA concentrations in supernatant incubated with BAC or virgin GAC. FA concentration in the mineral medium without BAC or virgin GAC is also presented as the operational blank in Figure 1. The operational blank test showed that FA concentration was maintained between $-2.0\%$ and $+5.7\%$ of the initial concentration, indicating that physical loss of FA from the system was negligible. The FA concentration in the virgin GAC treatment was within the range of $\pm 13\%$ of the initial concentration during incubation. FA concentration in the BAC treatment declined linearly from 2 to 8 h, with 76% of the initial FA concentration removed during the 8 h period. The rate of FA reduction over the period 2–8 h was $2.2 \mu g L^{-1} h^{-1} g^{-1}$ dry weight. These results demonstrated that the primary mechanism of FA removal in the BAC filter was the result of biological activity, not physical adsorption.

Microbial community structure of BAC

Bacterial 16S rRNA gene copy numbers of the original BAC sample were $3.6 \times 10^9 \pm 1.4 \times 10^8$ (average ± standard error) copies/g dry weight, while those of archaeal 16S rRNA genes were $6.2 \times 10^7 \pm 7.3 \times 10^5$ copies/g dry weight. The biofilm associated with the original BAC was dominated by bacteria rather than archaea.

Figure 1 | FA removal potential of BAC compared with virgin GAC.
Illumina paired-end sequencing of prokaryotic 16S rRNA genes revealed the phylum-level classification of the microbial community in the original BAC (Figure 2(a)). The four dominant phyla were Proteobacteria (41.5%), Acidobacteria (17.8%), Planctomycetes (17.2%) and Chloroflexi (7.4%), which together constituted nearly 84% of the total sequences. The class-level analysis of phylum Proteobacteria is shown in Figure 2(b). Alphaproteobacteria were the most abundant, accounting for 59.0% of total Proteobacteria, followed by Betaproteobacteria (24.9%), Deltaproteobacteria (6.7%) and Gammaproteobacteria (4.5%). At the order level, Rhizobiales in the class Alphaproteobacteria was the most abundant group, representing 10.2% of the total biofilm community. The predominance of Proteobacteria, especially Alphaproteobacteria and Betaproteobacteria, in the BAC filter, and the dominance of Rhizobiales, was consistent with previous studies (Pinto et al. 2012; Lautenschlager et al. 2014).

**DNA-SIP to identify FA-assimilating microorganisms**

As shown in Figure 1, FA degradation was observed after 2 h of incubation, and the bacterial community on the BAC had potential to degrade FA without acclimatization. Microbes capable of degrading FA should be in the original BAC. DNA-SIP was applied to explore specific players contributing to FA removal among the complex community. The BAC in glass columns was continuously fed with 0.5 mg C/L of 13C-FA or 12C-FA for 7 days. On day 2, it was observed that 100% and 99% of FA in the influent was removed for 13C-FA and 12C-FA treatments, respectively. After 7 days of incubation, DNA was extracted from both cases for isopycnic ultracentrifugation followed by buoyant density fractionation. Bacterial 16S rRNA gene numbers in each fraction were quantified for both the 12C-FA and 13C-FA treatments. The relative abundances to the maximum in each fraction were plotted in Figure 3 to compare the distribution of bacterial 16S rRNA gene numbers in each fraction of the 13C-FA and 12C-FA treatments. The maximum peaks of these two distributions were observed in the similar buoyant density fractions of 1.715–1.718 g/mL, indicating that the dominant bacterial groups did not assimilate FA. However, bacterial 16S rRNA gene numbers for the 13C-FA treatment were slightly more enriched than those for the 12C-FA treatment in the fractions ranging between approximately 1.725 and 1.740 g/mL. This suggests that some bacteria associated with the BAC proliferated and synthesized DNA by assimilating heavy 13C-FA.

![Figure 2](image1.png)

(a) Relative abundances of prokaryotic phyla of the original BAC sample and (b) class-level composition of the dominant phylum, Proteobacteria.

![Figure 3](image2.png)

Bacterial 16S rRNA gene copy numbers presented as relative to the maximum in fractions obtained from the BAC supplied with 12C-FA and 13C-FA. The fractions marked with asterisks were further analyzed by NGS.
The representative fractions (\(^{13}\text{C-FA}\) fraction and \(^{12}\text{C-FA}\) fraction of 1.726 g/mL, which are marked with asterisks in Figure 3) were selected from among those ranging between approximately 1.725 and 1.740 g/mL, in which bacterial 16S rRNA gene numbers were relatively more enriched in the \(^{13}\text{C-FA}\) treatment than \(^{12}\text{C-FA}\) treatment. They were subjected to Illumina sequencing analysis of 16S rRNA genes. Bacterial genera whose relative abundances to the total in the \(^{13}\text{C-FA}\) fraction were larger by more than 5% than the \(^{12}\text{C-FA}\) fraction are shown in Figure 4. One unassigned genus in the family of Bradyrhizobiaceae, Hyphomicrobium, and Methylibium were selected as FA-assimilating candidates. Each candidate represented more than 10% of the total reads for the \(^{13}\text{C-FA}\) fraction vs less than 6% for the \(^{12}\text{C-FA}\) fraction. In the original BAC, each candidate accounted for less than 5% of the total reads, indicating that the limited functional groups were involved in the removal of FA.

Our results showed that the genera Hyphomicrobium and Methylibium were suspected of assimilating FA in BAC filter treatment. Borodina et al. (2000) revealed that the facultative methylotroph Hyphomicrobium strain S1 assimilated FA as a growth substrate through the serine pathway. Methylibium was also identified as a facultative methylotroph and assimilation of FA was confirmed (Nakatsu et al. 2006). The results in the present study are consistent with the physiological features of Hyphomicrobium and Methylibium which can utilize FA or other C\(_1\) compounds. Low molecular weight carboxylic acids including formate (C\(_1\) compound), aldehydes, and ketones are generated by ozonation, which contribute to the elevation of assimilable organic carbon concentrations (Hammes et al. 2006). BAC filters probably provide a good niche for facultative methylotrophs such as Hyphomicrobium and Methylibium because C\(_1\) compounds such as FA and formate are continuously supplied by ozonation.

In order to verify the results of DNA-SIP, we focused on Hyphomicrobium and designed the primers specific to their 16S rRNA genes. Figure 5 shows Hyphomicrobium 16S rRNA gene copy numbers presented as relative to their maximum in \(^{13}\text{C-FA}\) and \(^{12}\text{C-FA}\) fractions. Compared with the profiles of total bacterial 16S rRNA gene copy numbers in Figure 3, it is clear that the profile for the \(^{13}\text{C-FA}\) treatment significantly shifted in a heavy direction in Figure 5. This supportive result clearly demonstrated the active assimilation of FA by Hyphomicrobium.

DNA-SIP combined with NGS provided valuable information to elucidate the relationship between microbial community structure and function in a BAC filter. The

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**Figure 4** Relative abundances of three FA-assimilating candidates in the \(^{13}\text{C-FA}\) and \(^{12}\text{C-FA}\) fractions at buoyant density of 1.726 g/mL.

**Figure 5** Hyphomicrobium 16S rRNA gene copy numbers presented as relative to the maximum in fractions obtained from the BAC supplied with \(^{13}\text{C-FA}\) and \(^{12}\text{C-FA}\).
reproducibility of the assimilation of FA by the identified bacterial groups and their roles in other BAC samples should be carefully evaluated to expand the knowledge of the present study to full-scale BAC filters. It is possible that the microbial response to the substrate is largely dependent on substrate concentration. The FA concentration used in this study was much higher than the actual levels in water purification plants. Further research is required to check the responses of the candidates such as *Hyphomicrobium* and *Methylibium* to FA at more realistic concentrations.

**CONCLUSIONS**

BAC filters have the potential to remove FA, a hydrophilic and low molecular weight compound. DNA-SIP combined with NGS, which is a promising approach to identify microorganisms assimilating specific compounds, revealed that Rhizobiales such as *Hyphomicrobium* in Alphaproteobacteria and Burkholderiales such as *Methylibium* in Betaproteobacteria played an important role in treating FA in the BAC filter.

**REFERENCES**


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