

## Real time PCR quantification

Quantification of AOA-*amoA* genes and AOB-*amoA* genes were conducted following the protocol described previously (Leininger *et al.* 2006). Copy numbers of AOA-*amoA* genes were determined with primers *amo196F* and *amo277R* and probe *amo247*. The 5' end of *amo247* was labeled with 6-FAM while the 3' end was labeled with carboxytetramethylrhodamine (TAMRA). Reaction mixture was prepared by using iTaq Supermix with ROX (Bio-Rad). On the other hand, AOB-*amoA* genes were quantified with primers *amoA-1F* and *amoA-2R* using QuantiTect SYBR Green PCR Master Mix (Qiagen). All real time PCR reactions were carried out with a LightCycler 2.0 (Roche).

## RESULTS AND DISCUSSION

### Removal of ammonium-nitrogen by pilot-scale plant operation

As water temperature decreased from 25.1°C on 21 September of 2007 down to 6.8°C on 21 February of 2008, ammonium-nitrogen concentration in raw water was increased from less than 0.02 mg N/L on 21 September of

2007 to 0.20 mg N/L on 21 February of 2008, which was in accordance with deterioration of natural nitrification activity in river. On the sampling occasions in September and November of 2007, ammonium-nitrogen was completely treated by BAC filtration in both processes. However, on 21 February of 2008, 0.14 mg N/L and 0.07 mg N/L of ammonium-nitrogen were detected in BAC effluent in Process (A) and Process (B), respectively. Such an incomplete ammonium-nitrogen removal by BAC treatment was frequently observed when water temperature decreased to less than 10°C (data not shown). Ammonium-nitrogen concentrations in BAC influent of both processes were same throughout the investigation period. It indicated that nitrification did not occur in the precedent rapid sand filtration in Process (B). We can assume that the same volume load of ammonium-nitrogen for BAC treatment in both processes.

### Vertical variations of nitrification performance

Figure 2 shows vertical variations of nitrification potential of BAC-A and BAC-B collected on 21 September of 2007. Similar nitrification kinetic profiles were observed for BAC-A and BAC-B at different layers. BAC-A could treat

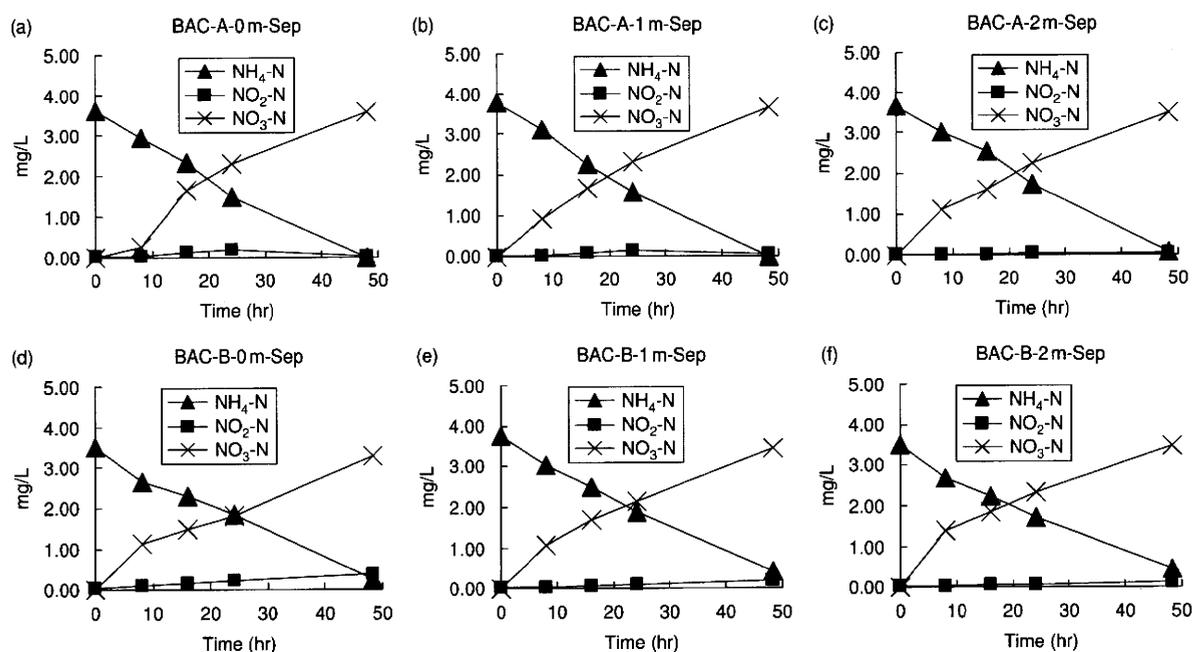


Figure 2 | Nitrification potential of BAC-A and BAC-B on 21 September 2007 collected from surface (0m), middle (1m) and bottom (2m) layers of the BAC beds.

almost all ammonium-nitrogen within 48 hours while 0.27, 0.41, and 0.46 mg N/L of ammonium-nitrogen remained after 48 hours for BAC-B-0 m, BAC-B-1 m, and BAC-B-2 m, respectively. This result demonstrated nitrification potential of BAC-A was slightly better than that of BAC-B.

Figure 3 shows the vertical distribution of AOA-*amoA* and AOB-*amoA* genes in the BAC beds in Process (A) and Process (B) quantified by real time PCR. In both processes, abundances of AOA-*amoA* genes detected at all layers of the BAC beds were in the range of  $10^5 - 10^6$  copies/g-dry, which were one to two orders of magnitude higher than those of AOB-*amoA* genes. The result suggests that AOA rather than AOB could be predominant ammonia-oxidizers in BAC treatment regardless of the position of rapid sand filtration in the treatment process train. AOA-*amoA* and AOB-*amoA* genes were distributed in the BAC beds uniformly in both processes probably due to mixing of BAC beds by regular backwashing. At all layers, abundances of AOA-*amoA* and AOB-*amoA* genes of BAC-A were approximately two times higher than those of BAC-B. The higher nitrification potential of BAC-A in September could probably be due to higher abundances of ammonia-oxidizing microorganisms on BAC. Since AOB-*amoA* genes were not enough in number for the analysis, only AOA-*amoA* genes were characterized by the T-RFLP analysis. As shown in Figure 4, digestion of the PCR products of AOA-*amoA* genes produced two distinctive T-RF peaks of 167 bp and 219 bp in all BAC samples. This composition did not change at all layers in both processes,

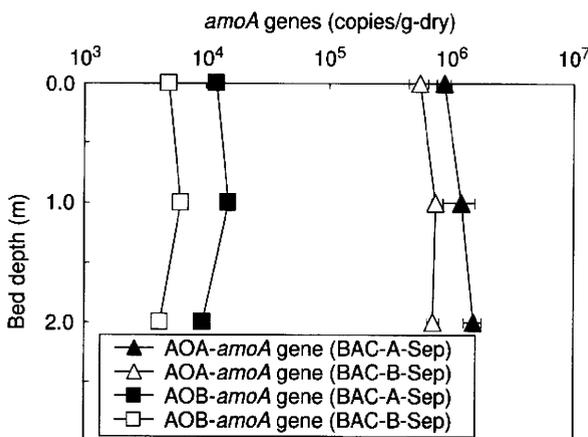


Figure 3 | Vertical distributions of AOA-*amoA* and AOB-*amoA* genes in the BAC beds in Process (A) and Process (B) (21 September, 2007).

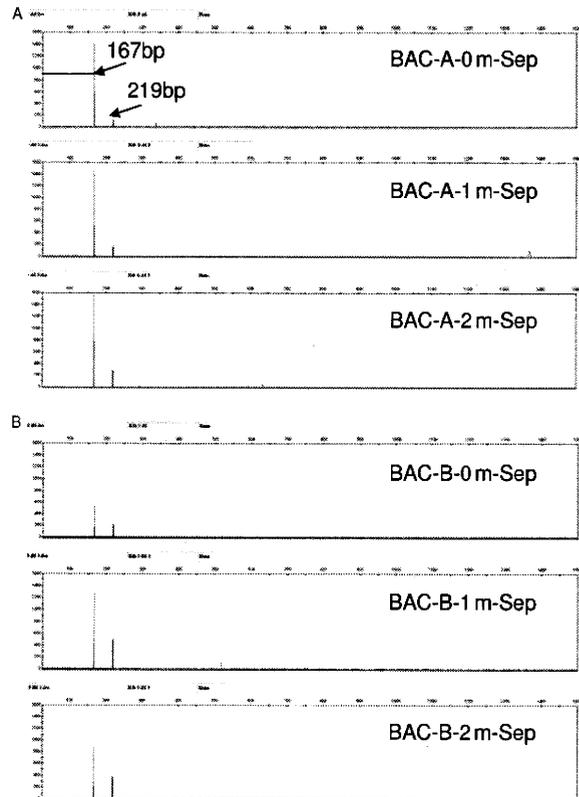


Figure 4 | T-RFLP profiles of AOA-*amoA* genes in the BAC beds in Process (A) and Process (B) (21 September, 2007).

suggesting that the diversity of AOA associated with BAC is not affected by the position of rapid sand filtration.

### Seasonal variations of nitrification performance

In Figure 5, seasonal variations of nitrification potential of BAC-A and BAC-B (surface layer) were compared. The oxidation rates of ammonium-nitrogen for both BAC

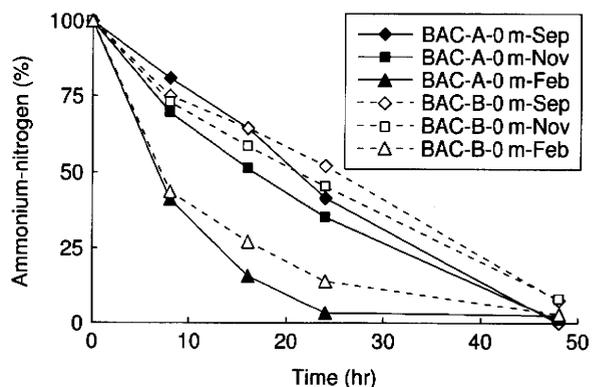
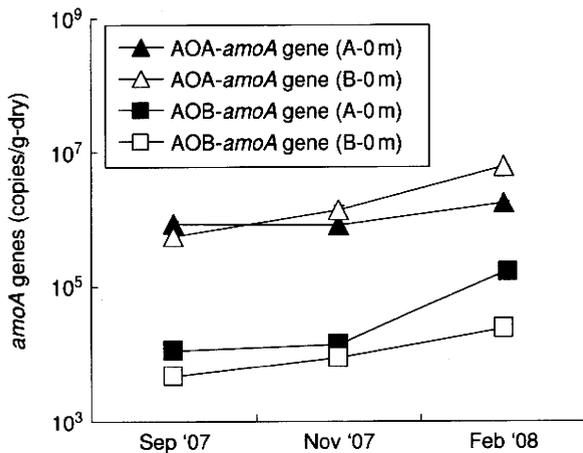


Figure 5 | Seasonal changes of nitrification potential of BAC-A and BAC-B.



**Figure 6** | Seasonal changes in abundances of AOA-*amoA* and AOB-*amoA* genes of BAC-A and BAC-B.

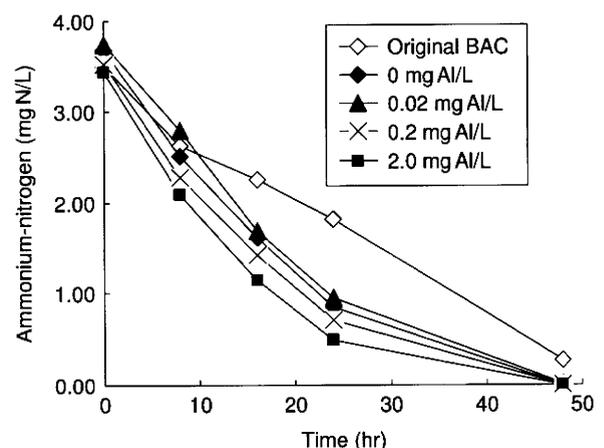
samples were obviously faster in February than those in September and November. BAC-A had slightly higher nitrification potential than BAC-B at every sampling occasion.

Figure 6 shows seasonal changes in abundances of AOA-*amoA* and AOB-*amoA* genes of BAC-A and BAC-B (surface layer). AOA-*amoA* and AOB-*amoA* genes of both BAC samples were gradually increased from September to February. Ammonium-nitrogen concentrations in BAC influent in both processes were increased from less than 0.02 mg N/L in September to 0.20 mg N/L in February. This trend was in accordance with changes in abundances of AOA-*amoA* and AOB-*amoA* genes. The highest nitrification potential of BAC-A and BAC-B in February could be attributed to the highest abundances of ammonia-oxidizing microorganisms on BAC in February. Abundances of AOA-*amoA* genes of BAC-B were lower than those of BAC-A in September, but 1.7 times and 3.6 times higher in November and February, respectively. On the other hand, AOB-*amoA* genes of BAC-A were always approximately 1.5–6.8 times more abundant than those of BAC-B. Some factors of Process (A) could be associated with higher abundances of AOB on BAC-A. The fact that BAC-A demonstrated slightly higher nitrification potential at every sampling occasion might be associated with higher abundances of AOB rather than those of AOA during the investigation period. Diversity of AOA-*amoA* genes observed in BAC-A and BAC-B in November and February was identical to that in September (data not shown), indicating that composition of AOA on BAC was stable in both processes. Though the

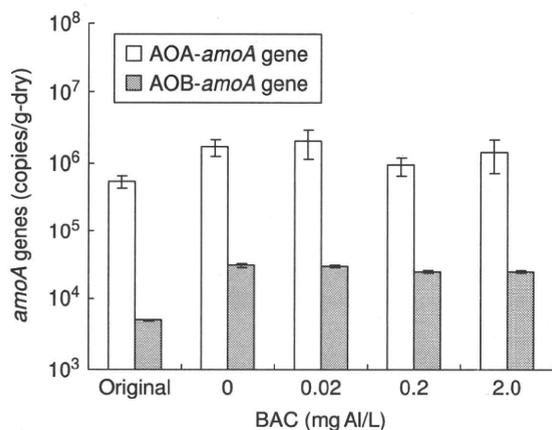
highest nitrification potential and the highest abundances of AOA-*amoA* and AOB-*amoA* genes were observed in February for both BAC samples, the monitoring data of the pilot-scale plant indicated that ammonium-nitrogen was detected in BAC effluent in both processes in February. The actual activity of AOA and AOB were probably suppressed under the low water temperature conditions despite the highest abundances and potential they had in February.

### Effects of aluminum on nitrification activity

Since rapid sand filtration was placed before ozonation in Process (B), suspended solids and residual coagulants such as polyaluminum chloride can be removed before BAC treatment in Process (B). Average concentration of total aluminum after sedimentation was 0.21 mg Al/L. The BAC influent in Process (A) contained almost the same concentration of aluminum. On the other hand, rapid sand filtration preceding ozonation-BAC treatment reduced total aluminum concentration to 0.02 mg Al/L in Process (B). Since aluminum is toxic to bacteria (Wood 1995), effects of aluminum on nitrification activity were evaluated. After incubating BAC samples collected from Process (B) on 21 September of 2007 (original BAC) with 0 (control), 0.02, 0.2 and 2.0 mg Al/L of polyaluminum chloride for one month, nitrification potential was determined (as shown in Figure 7). Compared with nitrification potential of the original BAC sample before one-month incubation, all batches including the control demonstrated faster oxidation



**Figure 7** | Effects of aluminum on nitrification potential. Original BAC indicates BAC sample before one-month incubation.



**Figure 8** | Effects of aluminum on abundances of AOA-*amoA* and AOB-*amoA* genes. Original BAC indicates BAC sample before one-month incubation.

rates after one-month incubation. The first-order reaction rate constants were 0.14, 0.13, 0.14 and 0.16 (1/hr) for the batches with 0, 0.02, 0.2, and 2.0 mg Al/L, respectively. Relationship between aluminum concentration levels and nitrification potential were not observed.

Figure 8 shows abundances of AOA-*amoA* and AOB-*amoA* genes after incubation with aluminum. Compared with the original sample before one-month incubation included in Figure 8, abundances of AOA-*amoA* and AOB-*amoA* genes in all batches after one-month incubation were increased 1.7–3.7 times and 4.8–6.0 times, respectively. Improvement of nitrification potential could be attributed to growth of AOA and AOB during one-month incubation. This result indicates that aluminum concentration levels tested here do not inhibit AOA and AOB associated with BAC.

## CONCLUSIONS

BAC-A collected from Process (A), where rapid sand filtration was placed after ozonation-BAC treatment, demonstrated slightly higher nitrification potential at every sampling occasion. This might be associated with higher abundances of AOB on BAC-A than those on BAC-B, though AOA could be predominant ammonia-oxidizers in BAC treatment regardless of the position of rapid sand filtration. The highest nitrification potential was observed in February for BAC-A and BAC-B when the highest abundances of both AOA-*amoA* and AOB-*amoA* genes were

observed. Diversity of AOA-*amoA* genes was not different between BAC-A and BAC-B during the investigation period. Although higher residual aluminum concentration in BAC influent was observed in Process (A), there were no adverse effects of aluminum on nitrification activity. These results suggest that factors other than aluminum concentration in different treatment processes could possibly have some influence on abundances of ammonia-oxidizing microorganisms on BAC.

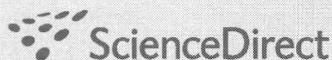
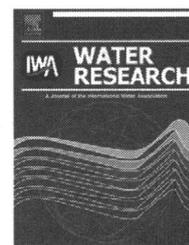
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# Predominance of ammonia-oxidizing archaea on granular activated carbon used in a full-scale advanced drinking water treatment plant

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## ABSTRACT

Ozonation followed by granular activated carbon (GAC) is one of the advanced drinking water treatments. During GAC treatment, ammonia can be oxidized by ammonia-oxidizing microorganisms associated with GAC. However, there is little information on the abundance and diversity of ammonia-oxidizing microorganisms on GAC. In this study, the nitrification activity of GAC and the settlement of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in GAC were monitored at a new full-scale advanced drinking water treatment plant in Japan for 1 year after plant start-up. Prechlorination was implemented at the receiving well for the first 10 months of operation to treat ammonia in raw water. During this prechlorination period, levels of both AOA and AOB associated with GAC were below the quantification limit. After prechlorination was stopped,  $10^5$  copies  $\text{g-dry}^{-1}$  of AOA *amoA* genes were detected within 3 weeks and the quantities ultimately reached  $10^6$ – $10^7$  copies  $\text{g-dry}^{-1}$ , while levels of AOB *amoA* genes still remained below the quantification limit. This observation indicates that AOA can settle in GAC rapidly without prechlorination. The nitrification activity of GAC increased concurrently with the settlement of AOA after prechlorination was stopped. Estimation of *in situ* cell-specific ammonia-oxidation activity for AOA on the assumption that only AOA and AOB determined can contribute to nitrification suggests that AOA may account for most of the ammonia-oxidation. However, further validation on AOB contribution is required.

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## 1. Introduction

Granular activated carbon (GAC) filtration combined with ozonation is widely used in drinking water treatment plants as an advanced treatment process. GAC filtration was originally designed to remove organic matter by adsorption.

However, it has been observed that microorganisms are colonized on the surface of GAC if chemical disinfection does not precede GAC. They can contribute to water treatment through biological oxidation of ammonia and biodegradable organic matter such as assimilable organic carbon (Rittmann and Snoeyink, 1984; Simpson, 2008). This biologically

**Abbreviations:** AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; BAC, biological activated carbon; GAC, granular activated carbon; OTU, Operational Taxonomic Unit; PCR, polymerase chain reaction; Q-PCR, quantitative-polymerase chain reaction; T-RFLP, terminal-restriction fragment length polymorphism.

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enhanced GAC is referred to as biological activated carbon (BAC) (Wilcox et al., 1983). In Japan, this advanced treatment system has been used by large urban water systems since the early 1990s. Tokyo metropolitan government will include ozonation-GAC (BAC) treatment in almost all of its drinking water treatment plants by 2013. One of the specific goals is to remove ammonia by nitrifying microorganisms associated with GAC. Ammonia, usually in the form of ammonium ion in raw water, not only increases chlorine demand in water purification process but also serves as a precursor of trichloramine when chlorine is used. Trichloramine is regarded as a major cause of chlorine odor in tap water. Thus, water utilities are required to control ammonia in order to suppress the unintended production of trichloramine. Although GAC filtration is a possible treatment process for removing ammonia, the nitrification mechanism in GAC filtration is not well understood. Specifically, little information is available on nitrifying microorganisms associated with GAC. Microbiological information, including the abundance, diversity, and activity of ammonia-oxidizers, is helpful when evaluating the nitrification performance of GAC, as well as when achieving rapid development of nitrification activity.

Until recently, it has been known that ammonia oxidation is carried out exclusively by ammonia-oxidizing bacteria (AOB) belonging to *Beta*- and *Gamma*-*proteobacteria*. However, the recent discovery of novel ammonia-oxidizing archaea (AOA) affiliated with non-thermophilic *Crenarchaeota* dramatically changed our understanding of nitrification (Könneke et al., 2005; Schleper et al., 2005; Treusch et al., 2005). AOA are widely distributed and frequently outnumber AOB in various environments (Francis et al., 2005; Leininger et al., 2006; Wuchter et al., 2006). However, only a single isolate of non-thermophilic AOA, *Candidatus Nitrosopumilus maritimus* SCM1, has been obtained to date (Könneke et al., 2005; Martens-Habbena et al., 2009). According to the genomic comparison of *N. maritimus* SCM1 and marine metagenomes, this isolate can be regarded as a representative of AOA found in marine environment (Walker et al., 2010). The contribution of AOA to ammonia oxidation is a controversial issue (Prosser and Nicol, 2008; You et al., 2009). Some researchers argue that AOB could play a central role in ammonia oxidation despite the predominance of AOA (Jia and Conrad, 2009; Wells et al., 2009). Wells et al. (2009) suggested that AOA were minor contributors to ammonia oxidation in highly aerated activated sludge systems. Other studies have demonstrated that AOA are indeed involved in nitrification (Herrmann et al., 2008; Offre et al., 2009; Schauss et al., 2009).

Only two reports describe the occurrence of AOA in drinking water treatment and distribution systems (de Vet et al., 2009; van der Wielen et al., 2009), and they demonstrate that AOA as well as AOB are widely present in groundwater treatment processes and distribution systems in the Netherlands. Because ammonia removal is correlated significantly with the abundance of AOA, van der Wielen et al. (2009) suggested that AOA could be responsible for the removal of ammonia in groundwater treatment plants. However, the contribution of AOA to drinking water treatment processes, including GAC filtration, remains unclear.

In this study, we monitored AOA and AOB associated with GAC at a new full-scale advanced drinking water treatment

plant in Japan from plant start-up. The plant was designed to treat river water with ozonation followed by GAC filtration. Prechlorination was performed during the first 10 months of operation. Abundance and diversity of AOA and AOB on GAC were examined together with nitrification potential in order to better understand the nitrification mechanism.

## 2. Materials and methods

### 2.1. Drinking water treatment plant

Field sampling was conducted at a new advanced drinking water treatment plant in Chiba Prefecture, Japan. The plant, which has a production capacity of 60,000 m<sup>3</sup> day<sup>-1</sup>, started operation on 1 October 2007. River water enters the receiving well and is treated using coagulation/sedimentation followed by ozonation, GAC filtration, disinfection by hypochlorite, and rapid sand filtration. Prechlorination (break-point chlorination) was implemented at the receiving well from plant start-up so that residual free chlorine in the effluent of receiving well would be 0.3 mg l<sup>-1</sup> in order to remove ammonia and algae. However, prechlorination was stopped on 24 July 2008. The GAC used in the plant is made of coal and has an average diameter of 1.2 mm. Ozonated water is introduced from above into the fixed GAC bed, which is 2.0 m deep. The linear velocity of GAC filtration is maintained at 240 m day<sup>-1</sup> so that the average retention time is 12 min. The filters are backwashed every 3–4 days using air and finished water. The data on ammonia concentrations in the treatment process and prechlorination dosage from October 2007 to December 2008 was provided by the drinking water treatment plant.

### 2.2. Sampling of GAC and raw water

Two liters of GAC samples were collected from the surface layer of the same bed approximately every month from October 2007 to December 2008. Two liters of raw water were also obtained at the receiving well. GAC and raw water samples were brought to the laboratory in cold containers within 2 h and treated immediately, as described below.

### 2.3. Nitrification potential test

The nitrification potential of GAC was evaluated by incubating 100 g-wet of GAC with 200 ml of inorganic medium containing 5 mg NH<sub>4</sub><sup>+</sup>-N l<sup>-1</sup> at 20 °C with agitation at 100 rpm. Inorganic medium per 1 L of water was composed of 23.57 mg of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 200 mg of MgSO<sub>4</sub>·7H<sub>2</sub>O, 20 mg of CaCl<sub>2</sub>, 174 mg of K<sub>2</sub>HPO<sub>4</sub>, 156 mg of NaH<sub>2</sub>PO<sub>4</sub>, 1 mg of Fe-EDTA, 200 mg of CaCO<sub>3</sub>, 0.001 mg of Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.002 mg of MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.002 mg of CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.02 mg of CuSO<sub>4</sub>·5H<sub>2</sub>O and 0.1 mg of ZnSO<sub>4</sub>·7H<sub>2</sub>O. After 0, 8, 16, 24 and 48 h of incubation, supernatant was collected and filtered through 0.2-μm pore size polytetrafluoroethylene membranes (Advantec, Japan). The ammonia concentration was determined with a spectrophotometer (U-2000, Hitachi, Japan) following the indophenol-blue colorimetric method. The nitrite and nitrate concentrations were analyzed with a suppresser-type ion chromatograph (761 Compact IC, Metrohm, Switzerland).

## 2.4. Nucleic acid extraction

Three 0.5-g portions of the GAC sample were separately collected into three tubes. Then, nucleic acid was extracted from each portion using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, CA) according to the manufacturer's instructions. The kit can efficiently extract DNA by using bead-beating method. Bead-beating was carried out at a speed 4.0 for 30 s with a FastPrep FP120 (Qbiogene, Irvine, CA). Finally, all three DNA extracts were combined to obtain a composite DNA extract for further analysis. Microorganisms were collected from raw water by filtering a 100-ml sample through 0.2- $\mu\text{m}$  pore size polycarbonate membranes (Isopore track-etched membrane, Millipore, MA). Nucleic acid was extracted from the membranes using the same method.

## 2.5. Quantitative PCR

Quantitative-polymerase chain reaction (Q-PCR) was performed to determine the quantities of 16S rRNA genes of *Bacteria* and *Archaea* as well as *amoA* genes of AOA and beta-proteobacterial AOB. All reactions, including negative control, were performed in triplicate with a LightCycler 480 II (Roche, Switzerland). The crossing point at which the fluorescence of a sample rose above the background fluorescence was calculated following the second derivative maximum method with LightCycler software version 1.5 (Roche). In this study, PCR fragments carrying reference genes prepared from plasmids were used as external standards.

Bacterial 16S rRNA genes were quantified with previously described primers and fluorescence probe (Nadkarni et al., 2002). The PCR reaction mixture (20  $\mu\text{l}$ ) contained 10  $\mu\text{l}$  of LightCycler 480 Probe Master (Roche), 0.50  $\mu\text{M}$  of each primer, 0.20  $\mu\text{M}$  of probe and 1  $\mu\text{l}$  of DNA extract. The amplification program was as follows: 95  $^{\circ}\text{C}$  for 5 min, followed by 45 cycles consisting of 95  $^{\circ}\text{C}$  for 10 s, 60  $^{\circ}\text{C}$  for 50 s and 72  $^{\circ}\text{C}$  for 1 s (detection). Plasmids containing nearly full lengths of 16S rRNA genes of *Escherichia coli* IFO3301 were prepared with a QIAGEN PCR Cloning plus Kit (QIAGEN, Germany). The fragments containing the target were then amplified using the plasmid-specific M13 primer set. The purified M13 products were used as the standard in a 10-fold dilution series from  $5.0 \times 10^1$  to  $5.0 \times 10^8$  copies reaction $^{-1}$ . PCR efficiencies and linearity ( $R^2$ ) for standard curves were 81.5–85.4% and 0.9991–0.9996, respectively.

Archaeal 16S rRNA genes were quantified with the primer set of Ar109f and Ar912rt (Lueders and Friedrich, 2002). The PCR reaction mixture (20  $\mu\text{l}$ ) contained 10  $\mu\text{l}$  of LightCycler 480 SYBR Green I Master (Roche), 0.50  $\mu\text{M}$  of each primer and 1  $\mu\text{l}$  of DNA extract. The amplification program was as follows: 95  $^{\circ}\text{C}$  for 5 min, 45 cycles consisting of 95  $^{\circ}\text{C}$  for 30 s, 52  $^{\circ}\text{C}$  for 30 s and 72  $^{\circ}\text{C}$  for 1 min (detection), followed by melting curve analysis (65  $^{\circ}\text{C}$ –97  $^{\circ}\text{C}$  with a heating rate of 0.1  $^{\circ}\text{C}$  s $^{-1}$ ). Plasmids containing nearly full lengths of 16S rRNA genes of *Methanobacterium formicicum* ATCC 33274 were prepared. The fragments containing the target were then prepared as the standard using the same method as for bacterial 16S rRNA genes. PCR efficiencies and linearity ( $R^2$ ) for standard curves, ranging from  $5.0 \times 10^1$  to  $5.0 \times 10^7$  copies reaction $^{-1}$ , were 86.4–87.0% and 0.9996–0.9997 respectively.

AOA *amoA* genes were quantified with the primer set of Arch-*amoAF* and Arch-*amoAR* (Francis et al., 2005). The PCR reaction chemistry was the same as for archaeal 16S rRNA genes. The amplification program was as follows: 95  $^{\circ}\text{C}$  for 5 min, 45 cycles consisting of 95  $^{\circ}\text{C}$  for 10 s, 53  $^{\circ}\text{C}$  for 20 s and 72  $^{\circ}\text{C}$  for 30 s (detection), followed by melting curve analysis. The AOA *amoA* gene clone "NG-B-081028\_K1" (Accession No. AB550804) retrieved from GAC collected on 28 October 2008 was used as the standard. The 10-fold dilution series, ranging from  $5.0 \times 10^2$  to  $5.0 \times 10^6$  copies reaction $^{-1}$ , demonstrated that PCR efficiencies and linearity ( $R^2$ ) for standard curves were 85.9–88.2% and 0.9982–0.9994, respectively. The lower concentration of  $5.0 \times 10^1$  copies reaction $^{-1}$  was regarded as the detection limit in this study because its response was clearly discriminated from the negative control, although it was removed from quantification.

AOB *amoA* genes were quantified with the primer set of *amoA1F\** and *amoA2R* (Stephen et al., 1999). The PCR reaction chemistry was the same as for archaeal 16S rRNA genes. The amplification program was as follows: 95  $^{\circ}\text{C}$  for 5 min, 45 cycles consisting of 95  $^{\circ}\text{C}$  for 10 s, 54  $^{\circ}\text{C}$  for 20 s, 72  $^{\circ}\text{C}$  for 30 s and 80  $^{\circ}\text{C}$  for 5 s (detection), followed by melting curve analysis. Standards were prepared from plasmids containing *amoA* genes of *Nitrosomonas europaea* ATCC 19718. The 10-fold dilution series, ranging from  $5.0 \times 10^2$  to  $5.0 \times 10^6$  copies reaction $^{-1}$ , guaranteed that PCR efficiencies and linearity ( $R^2$ ) for standard curves were 81.4–82.3% and 0.9982–0.9985, respectively. The lower concentration of  $5.0 \times 10^1$  copies reaction $^{-1}$  was regarded as the detection limit as with AOA *amoA* genes.

## 2.6. T-RFLP

AOA *amoA* genes for T-RFLP analysis were amplified using Arch-*amoAF* and Arch-*amoAR*. In addition, archaeal 16S rRNA genes were amplified using Ar109f and Ar912rt. The 5' ends of Arch-*amoAF* and Ar912rt were labeled with FAM. The PCR mixture (50  $\mu\text{l}$ ) contained 1.25 units of TaKaRa EX Taq HS (TAKARA BIO, Japan), 5  $\mu\text{l}$  of  $10 \times$  Ex Taq Buffer, 4  $\mu\text{l}$  of dNTP mixture (all chemicals were provided by TAKARA BIO), 0.2  $\mu\text{M}$  of each primer and 1  $\mu\text{l}$  of the template. The amplification programs for AOA *amoA* genes and archaeal 16S rRNA genes were as follows: 30 cycles consisting of 94  $^{\circ}\text{C}$  for 45 s, 53  $^{\circ}\text{C}$  for 1 min and 72  $^{\circ}\text{C}$  for 1 min for AOA *amoA* genes; 30 cycles consisting of 94  $^{\circ}\text{C}$  for 30 s, 52  $^{\circ}\text{C}$  for 45 s and 72  $^{\circ}\text{C}$  for 1 min for archaeal 16S rRNA genes. The final extension was carried out at 72  $^{\circ}\text{C}$  for 10 min. The amplified products were checked by electrophoresis on a 1.2% agarose gel.

The fluorescence-labeled PCR products were purified with a MinElute PCR Purification Kit (QIAGEN). The 150 ng of AOA *amoA* gene fragments were digested with 10 U of *HhaI* (TAKARA BIO) at 37  $^{\circ}\text{C}$  for 6 h, while the same amount of archaeal 16S rRNA gene fragments were digested with *TaqI* (TAKARA BIO) at 65  $^{\circ}\text{C}$  for 6 h. The FAM-labeled fragments were separated with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). GeneMapper version 3.5 (Applied Biosystems) was used for fragment analysis.

## 2.7. Cloning and sequencing

The AOA *amoA* genes amplified with the primer set of Arch-*amoAF* and Arch-*amoAR* and archaeal 16S rRNA genes

amplified with the primer set of Ar109f and 1492r (Lane, 1991) were purified using a QIAquick PCR Purification Kit (QIAGEN). They were cloned using a QIAGEN PCR Cloning plus Kit (QIAGEN) according to the manufacturer's instructions. The clones were screened by T-RFLP analysis in the manner shown above in order to find clones that corresponded to each distinctive T-RF. The sequences of the selected clones were determined using a BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Sequence homology searches were performed at the DNA Data Bank of Japan (DDBJ) using the BLAST network service. The alignment and phylogenetic tree construction was performed by the Neighbor-Joining algorithm with CLUSTAL W offered by MEGA version 4.0 (Tamura et al., 2007).

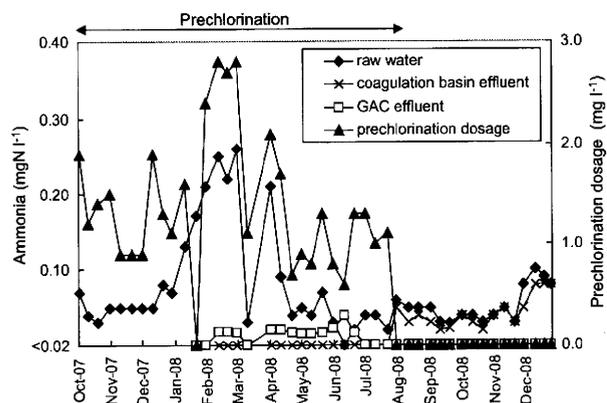
### 2.8. Nucleotide sequence accession numbers

The AOA *amoA* gene and archaeal 16S rRNA gene sequences determined in this study were submitted to the DDBJ/EMBL/GenBank databases under accession numbers AB550804–AB550806 and AB550807–AB550821, respectively.

## 3. Results

### 3.1. Operational performances of the advanced drinking water treatment plant

Fig. 1 shows temporal variations of ammonia concentrations in the treatment process and prechlorination dosage from start-up in October 2007 to December 2008, which were measured by the drinking water treatment plant. The average concentration of ammonia in raw water was  $0.07 \text{ mg N l}^{-1}$ , ranging from below the quantification limit ( $<0.02 \text{ mg N l}^{-1}$ ) to  $0.26 \text{ mg N l}^{-1}$ . Water temperature fluctuated from  $4.0$  to  $28.0^\circ\text{C}$



**Fig. 1 – Temporal variations of ammonia concentrations and prechlorination dosage in actual operation from plant start-up in October 2007 to December 2008. No data is available for coagulation basin effluent and GAC effluent from October 2007 to the middle of January 2008. Prechlorination was implemented from start-up until 24 July 2008.**

during the monitoring period. When the water temperature dropped to less than  $10^\circ\text{C}$  in winter, the ammonia concentration in raw water was frequently above  $0.10 \text{ mg N l}^{-1}$ . Because ammonia was removed by prechlorination from start-up until 24 July 2008, no ammonia was detected in the effluent from coagulation basin. However, trace amounts of ammonia were observed in the GAC effluent until the middle of June 2008. After prechlorination was stopped, ammonia in the coagulation basin effluent increased to the same level as in the raw water, and was significantly higher than that during prechlorination period ( $p < 0.05$ ; t-test). However, ammonia was removed completely by GAC.

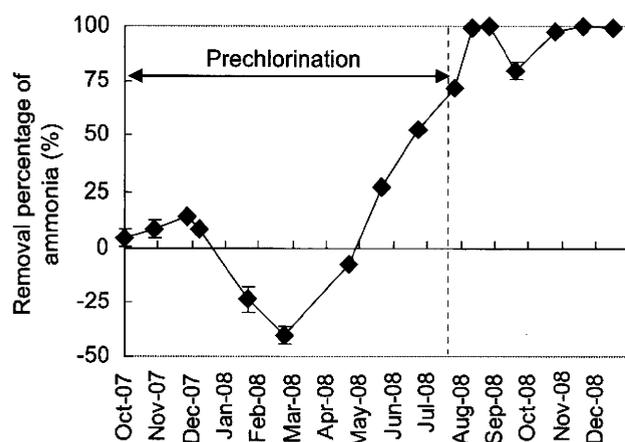
### 3.2. Nitrification potential of GAC

The nitrification potential of GAC was evaluated by incubating GAC with inorganic medium containing  $5 \text{ mg NH}_4^+ \text{ N l}^{-1}$  for 48 h. The removal percentage of ammonia during 48 h incubation period was shown in Fig. 2. From October 2007 to April 2008, ammonia was not well removed and even increased sometimes during incubation. In addition, an increase in nitrate without ammonia oxidation was sometimes observed during this same period. In May and June 2008, the removal percentage of ammonia increased to 50%. However, during this time, ammonia was removed only in the first 16 h and no change in concentration was observed until the end of incubation. On 28 July 2008, 4 days after stopping prechlorination, the removal percentage rose to 72%, and reached almost 100% on 13 August. Thereafter, high potential was maintained until the last sampling on 19 December, except for a tentative drop on 22 September. Ammonia oxidation profiles after stopping prechlorination were well described by the pseudo first-order rate reactions ( $R^2 = 0.924\text{--}0.994$ ), while profiles in May and June 2008 did not follow these reactions.

### 3.3. Temporal changes in the abundance of bacteria and archaea in raw water and associated with GAC

Bacterial and archaeal 16S rRNA genes in raw water ranged from  $3.0 \times 10^5$  to  $4.2 \times 10^6$  (average:  $1.2 \times 10^6$ ) and from  $7.1 \times 10^2$  to  $4.2 \times 10^4$  (average:  $5.6 \times 10^3$ ) copies  $\text{ml}^{-1}$ , respectively. No seasonal variations were observed for both genes.

Fig. 3(a) indicates temporal changes in the quantities of 16S rRNA genes of bacteria and archaea associated with GAC. Quantities of bacterial 16S rRNA genes increased rapidly from  $7.2 \times 10^4$  to  $3.6 \times 10^7$  copies  $\text{g-dry}^{-1}$  (the dry weight indicates the dried GAC weight) in the first 2 months of operation, indicating that bacteria could settle in GAC despite prechlorination. After stopping prechlorination on 24 July 2008, the quantities jumped tenfold in 3 weeks and reached  $1.1 \times 10^9$  to  $2.7 \times 10^9$  copies  $\text{g-dry}^{-1}$ . On the other hand, quantities of archaeal 16S rRNA genes were below the quantification limit until 28 July 2008, 4 days after stopping prechlorination. However, they increased to  $4.8 \times 10^5$  copies  $\text{g-dry}^{-1}$  on 13 August, indicating that certain groups of Archaea could settle in GAC rapidly without prechlorination. Thereafter, they maintained the abundance in the magnitude of  $10^6$  copies  $\text{g-dry}^{-1}$ .



**Fig. 2 – Removal percentage of ammonia during 48 h of incubation in the nitrification potential test. Initial ammonia concentration was set at approximately  $5 \text{ mg N l}^{-1}$ .**

### 3.4. Temporal changes in the abundance of AOA and AOB in raw water and associated with GAC

Quantities of AOA and AOB *amoA* genes in raw water were below the quantification limit throughout the monitoring period, except when AOB *amoA* genes were at the level of  $3.7 \times 10^3 \pm 2.2 \times 10^2 \text{ copies ml}^{-1}$  on 29 August 2008. However, Q-PCR analysis demonstrated positive responses of these genes, which were clearly discriminated from the negative control. Thus, both AOA and AOB *amoA* genes were present at very low concentrations in river water.

As shown in Fig. 3(b), quantities of AOA and AOB associated with GAC showed different trends. Quantities of AOA *amoA* genes were below the detection limit from start-up until 28 July 2008, 4 days after stopping prechlorination. However,  $5.9 \times 10^5 \text{ copies g-dry}^{-1}$  of AOA *amoA* genes were observed on 13 August, 3 weeks after stopping prechlorination. Thereafter, quantities increased to a maximum of  $1.3 \times 10^7 \text{ copies g-dry}^{-1}$  on 28 October and then decreased to  $2.8 \times 10^6 \text{ copies g-dry}^{-1}$  on 19 December. The trend for AOA *amoA* genes clearly indicates that prechlorination inhibited the settlement of AOA in GAC. In contrast to AOA *amoA* genes, *amoA* genes of AOB associated with GAC were always below the quantification limit throughout the monitoring period, even after stopping prechlorination. Although Q-PCR results indicate that AOB *amoA* genes were actually present in quantities above the detection limit from April to December 2008, quantities of AOB associated with GAC were significantly lower than those of AOA.

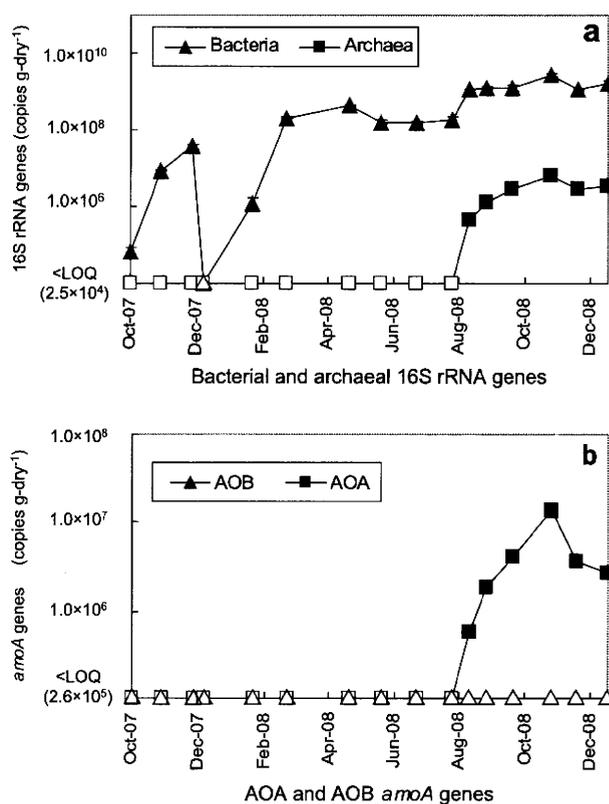
### 3.5. Diversity of *amoA* genes of AOA associated with GAC

PCR products of AOB *amoA* genes were not obtained from all GAC samples. The primer set specific to 16S rRNA genes of betaproteobacterial AOB (Kowalchuk et al., 1997) was used to detect AOB associated with GAC. However, amplification of AOB 16S rRNA genes also failed. In contrast to AOB *amoA* genes, AOA *amoA* genes for T-RFLP analysis were amplified from GAC from 13 August to 19 December 2008. A single-terminal

restriction fragment of 169 bp was detected from GAC after 13 August 2008. The results indicate that the diversity of AOA *amoA* genes assessed by *HhaI* digestion was very simple, primarily consisting of the single OTU (Operational Taxonomic Unit). AOA *amoA* genes corresponding to the fragment of 169 bp were successfully cloned from GAC collected on 28 October 2008. The nucleotide sequence of the target was 100% matched with the *amoA* gene of uncultured *Crenarchaeota* clone DR11 that was retrieved from the Dongjiang River in China. The target also had 81% similarity to *amoA* genes of *N. maritimus* (Könneke et al., 2005). As shown in Fig. 4, AOA *amoA* gene sequences obtained from GAC were affiliated with those of *Crenarchaeota* in the lineage of Group 1.1a, including *N. maritimus*. However, they appeared to form a distinctive cluster which was different from that of *N. maritimus* and clones retrieved from groundwater treatment and distribution systems in the Netherlands (van der Wielen et al., 2009).

### 3.6. Diversity of archaea in raw water and associated with GAC

Archaeal 16S rRNA genes for T-RFLP analysis were amplified from all raw water samples, even though they were obtained



**Fig. 3 – Temporal changes in quantities of 16S rRNA genes of bacteria and archaea (a) and *amoA* genes of AOA and AOB (b) associated with GAC. The quantification limits (LOQ) of both bacterial and archaeal 16S rRNA genes were  $2.5 \times 10^4 \text{ copies g-dry}^{-1}$ , while those of both AOA and AOB *amoA* genes were  $2.6 \times 10^5 \text{ copies g-dry}^{-1}$ . Open symbols show data below the LOQ. Error bars denote standard deviation of the triplicate Q-PCR analysis.**

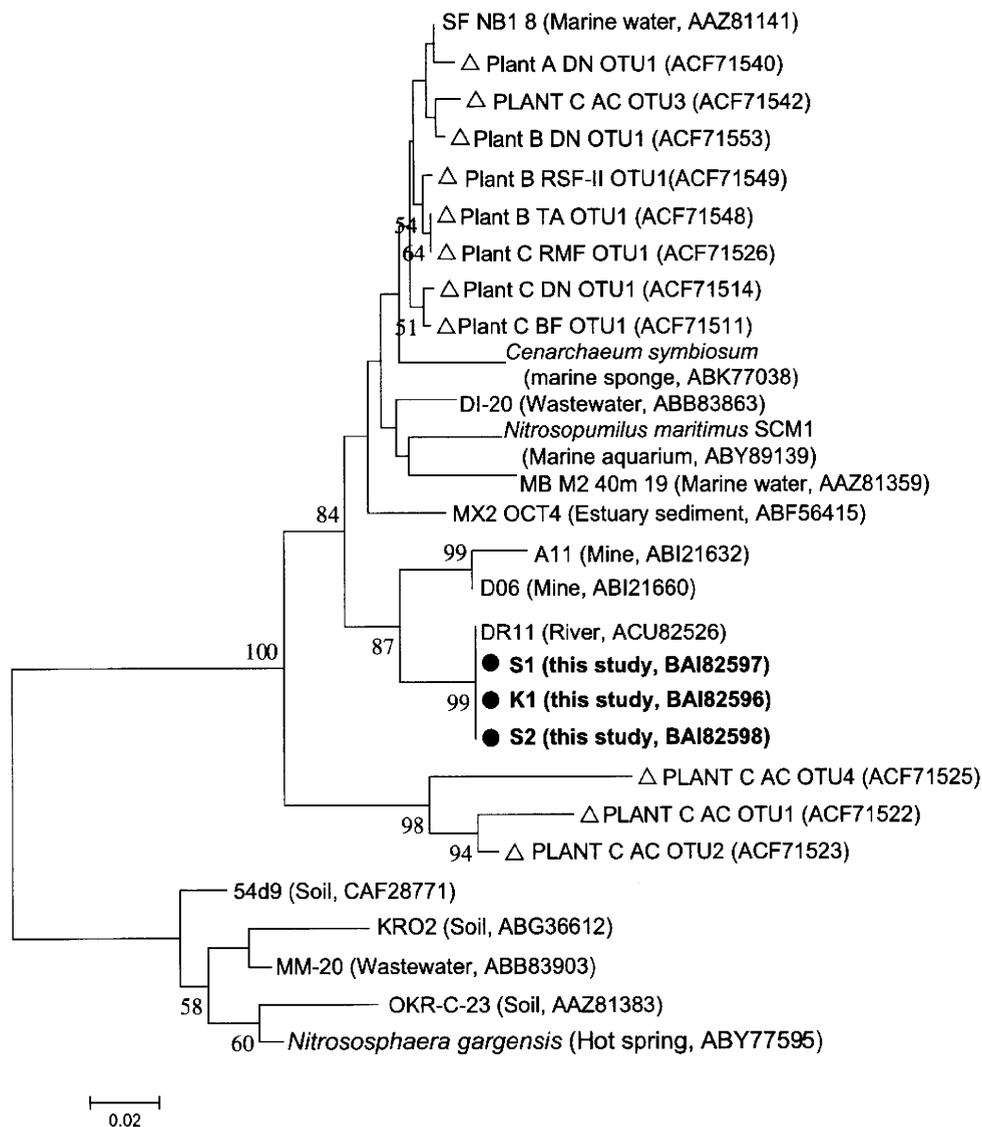
from GAC after 13 August 2008, 3 weeks after prechlorination was stopped. Fig. 5 compares T-RFLP profiles of archaeal 16S rRNA genes in raw water and GAC on 29 August and 28 October 2008. Raw water contained several archaeal 16S rRNA gene fragments. On the other hand, T-RFLP profiles of GAC were dominated by a single OTU of 186 bp. The T-RFLP profiles of the other GAC samples consisted of the same OTU alone. This distinctive fragment was also found in raw water throughout the monitoring period. The clones corresponding to the fragment of 186 bp were successfully retrieved from GAC collected on 28 October 2008. As shown in Table 1, a homology search revealed that they were closely related to 16S rRNA genes of *N. maritimus*, coinciding with the phylogenetic analysis of AOA *amoA* genes. This indicates that the archaeal community associated with GAC was dominated by

only AOA in the Group 1.1a lineage. Archaeal community structures in raw water were stable during the monitoring period, consisting of different members including AOA and methanogens (Table 1). However, the result showed that only AOA could selectively settle in GAC.

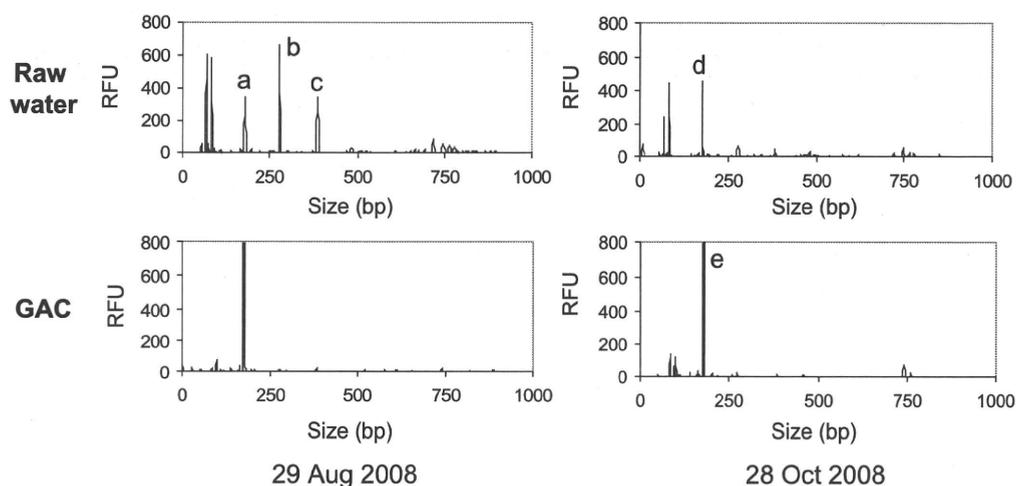
## 4. Discussions

### 4.1. Influence of prechlorination on nitrification activity of GAC

Biological activity of GAC is generally reduced when water is prechlorinated in advance of GAC (Wilcox et al., 1983; Simpson, 2008). From October 2007 to April 2008 when



**Fig. 4 – Phylogenetic tree of deduced amino acid sequences of AOA *amoA* genes.** Neighbor-joining tree was constructed based on the Jones-Taylor-Thornton substitution model. Bootstrap analysis was conducted with 1000 replicates and only bootstrap values above 50% are shown. The bar represents a 2% evolutionary distance. Clones obtained from GAC in this study are marked with closed circles, while those obtained from groundwater treatment processes and distribution systems in the Netherlands (van der Wielen et al., 2009) are marked with open triangles. The tree was rooted with a soil cluster of AOA *amoA* genes.



**Fig. 5 – T-RFLP profiles of archaeal 16S rRNA genes for raw water and GAC collected on 29 August 2008 (left) and 28 October 2008 (right). The restriction enzyme *RsaI* was used for digestion. Phylogenetic affiliations of the designated fragments are shown in Table 1.**

prechlorination was implemented and levels of both AOA and AOB associated with GAC were below the quantification limit, GAC had no nitrification potential and inorganic nitrogen species were sometimes rather released from virgin GAC during the incubation test. It is necessary to find more specific ways to discriminate biological ammonia-oxidation from other factors. In May and June 2008, the increase in nitrification potential up to 53% removal of ammonia was observed. After prechlorination was stopped, ammonia removal percentage in the nitrification potential test reached 100% within 3 weeks. This observation is in close agreement with an earlier report demonstrating that ammonia was completely removed in 2 weeks after start-up of GAC filtration in a full-scale advanced drinking water treatment plant that did not include prechlorination (Muramoto et al., 1995).

Q-PCR clearly revealed that settlement of AOA in GAC was severely inhibited by prechlorination. Quantities of *amoA* genes of AOA associated with GAC were below the detection limit from start-up until 28 July 2008. However, they reached the magnitude of  $10^6$ – $10^7$  copies g-dry<sup>-1</sup> after prechlorination was stopped. This level of AOA is close to or a little bit less than the quantities of AOA reported for soils (Jia and Conrad, 2009; Offre et al., 2009; Schauss et al., 2009), sediments (Bernhard et al., 2010) and sands used for biofiltration in a marine aquarium (Urakawa et al., 2008). Rapid attachment and growth of AOA as well as improvement in nitrification potential were simultaneously observed within 3 weeks after prechlorination was stopped. In addition, ammonia was completely removed by GAC in the actual treatment after prechlorination was stopped as shown in Fig. 1. Therefore, AOA could probably contribute to ammonia-oxidation at least after prechlorination was stopped. We can propose that prechlorination should be avoided in drinking water treatment in order to promote settlement of AOA and establish GAC with high nitrification activity.

However, behavior of AOA alone could not account for partial nitrification potential observed from May 2008 until the stop of prechlorination. It is necessary to consider AOB

contribution though their low abundances were not accurately quantified in this study. AOB *amoA* genes were detected from GAC at levels between the detection limit and the quantification limit from April to December 2008, corresponding to approximately  $2.6 \times 10^4$ – $2.6 \times 10^5$  copies g-dry<sup>-1</sup>, which were in the range of previously reported AOB abundances in soil and so on (Urakawa et al., 2008; Jia and Conrad, 2009; Offre et al., 2009; Schauss et al., 2009; Bernhard et al., 2010). Several researchers pointed out AOB could play a major role in nitrification despite the predominance of AOA (Jia and Conrad, 2009; Wells et al., 2009). Thus, it is possible that low level of AOB associated with GAC might be responsible for moderate nitrification potential observed in May and June 2008 and even after prechlorination was stopped. More sensitive assay for AOB quantification would reveal the correspondence between nitrification potential and AOB abundance in the monitoring period.

#### 4.2. Dominance of AOA on GAC

Although both AOA and AOB were present in raw water at very low concentrations, it is interesting that AOA could selectively settle in GAC after prechlorination was stopped. There are possible explanations for the predominance of AOA on GAC. First, AOB can be more sensitive to environmental perturbation than AOA. Thus, the amounts of AOB which can survive to reach GAC would be less than those of AOA, possibly because AOB are more likely to be removed or inactivated during the precedent treatments such as coagulation and ozonation. In addition, periodic backwashing of GAC filter may have a negative impact on the stable settlement of AOB. Intensive monitoring of the behaviors of AOA and AOB in the overall treatment process and before and after backwashing is necessary. Another explanation is that AOA may have higher affinity for attaching to GAC than AOB. Urakawa et al. (2008) reported that similar amounts of AOA and AOB were associated with sands used for marine aquarium biofiltration. It should be determined whether different surface properties of

**Table 1 – Phylogenetic affiliation of cloned archaeal 16S rRNA genes retrieved from raw water and GAC and their affiliation to major terminal-restriction fragments.**

Sample	Designation <sup>a</sup>	T-RF (bp)	Phylogenetic group	Similarity (%)
Raw water (29 Aug 2008)	a	186	<i>Nitrosopumilus maritimus</i> SCM1 (DQ085097)	92–96
	b	284	<i>Methanothrix soehngenii</i> (X51423)	97–98
	c	393	<i>Methanomicrobiales</i> archaeon SMSP (AB479390)	96
Raw water (28 Oct 2008)	d	186	<i>Nitrosopumilus maritimus</i> SCM1 (DQ085097)	88–96
GAC (28 Oct 2008)	e	186	<i>Nitrosopumilus maritimus</i> SCM1 (DQ085097)	92

a Corresponding to the designated fragments in Fig. 5.

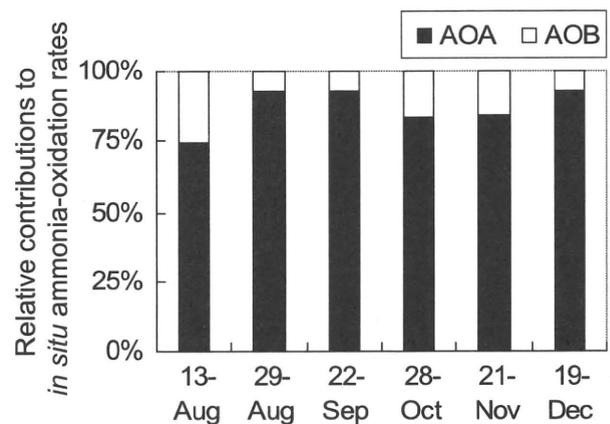
GAC and sand can affect the attachment of AOA and AOB. When substrate level is considered, it is possible that low ammonia concentration in raw water (average: 0.07 mg N l<sup>-1</sup>) could be one reason for giving AOA an advantage over AOB. Ammonia-oxidizers associated with GAC are usually exposed to very low nutrient conditions. In fact, *N. maritimus* SCM1 was stimulated by as little as 0.2 μM (0.003 mg N l<sup>-1</sup>) ammonia, while AOB such as *N. europaea* and *Nitrosococcus oceanii* did not respond to such a low level of ammonia (Martens-Habbena et al., 2009). This suggests that *N. maritimus*-like AOA probably has a much higher affinity to ammonia than AOB under extremely low ammonia concentration. Thus, after attachment to GAC, AOA is likely to use low level of ammonia preferentially and grow better than AOB. Since it is still unclear if ammonia level alone can determine niche of AOA and AOB, further work such as incubation of GAC under different ammonia concentrations could elucidate the mechanism of AOA dominance.

T-RFLP analysis of archaeal 16S rRNA genes for GAC explicitly indicates that the archaeal community associated with GAC consisted of AOA alone. Indeed, temporal changes in the quantities of *Archaea* associated with GAC appeared to be in agreement with those of AOA, as revealed by Q-PCR analysis in Fig. 3. There is a strong correlation ( $R^2 = 0.87$ ) between quantities of archaeal 16S rRNA genes and quantities of AOA *amoA* genes for GAC samples taken after 13 August 2008, 3 weeks after prechlorination was stopped. The slope of the relationship is 2.1, indicating that AOA associated with GAC possess *amoA* genes nearly twice as many copies as 16S rRNA genes though further genome analysis is required. This value is similar to the findings on the relationship between AOA *amoA* genes and crenarchaeotal 16S rRNA genes presented by Beman et al. (2008) and Wuchter et al. (2006), who reported that slope values were 2.5 (the Gulf of California) and 2.8 (North Sea), respectively. On the other hand, *Cenarchaeum symbiosum*, a marine sponge symbiont AOA, and *N. maritimus* SCM1 have only one copy of both *amoA* gene and 16S rRNA gene (Hallam et al., 2006a; Walker et al., 2010). It is likely that freshwater AOA discovered from GAC may have genomic features that differ from those of *C. symbiosum* lineages.

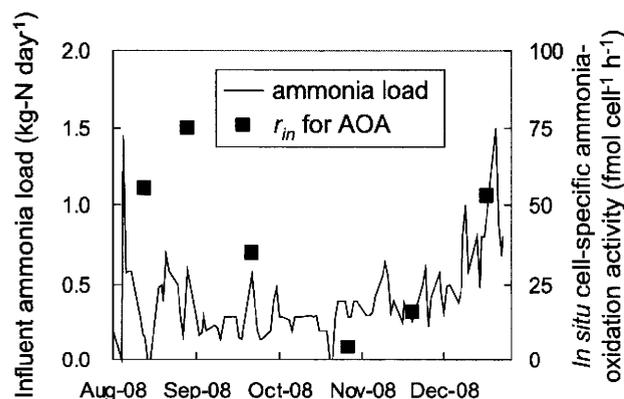
#### 4.3. Contribution of AOA to nitrification

To evaluate the contribution of AOA to actual ammonia removal in GAC treatment after stopping prechlorination, *in situ* cell-specific ammonia-oxidation activity ( $r_{in}$ : fmol cell<sup>-1</sup> h<sup>-1</sup>) for ammonia-oxidizers was estimated by considering removal amounts of ammonia in the GAC filter

and total abundance of AOA and AOB in the filter. The estimation was carried out on the simple assumption that only AOA and AOB determined could take part in ammonia removal. Other possible removal mechanisms such as ammonia assimilation of heterotrophic bacteria were not considered in this study. In activated sludge system where AOB accounted for only a few percent of total biomass and high concentration of ammonia was treated, 10–20% of total ammonia removal was allocated to ammonia assimilation of heterotrophs (Daims et al., 2001; Harms et al., 2003). However, Martens-Habbena et al. (2009) suggested that ammonia turnover per unit biomass of *N. maritimus* SCM1 would be more than 5 times higher than that of heterotrophic bacteria. It means that *N. maritimus*-like AOA can outcompete with ammonia assimilation activity of heterotrophic bacteria under limiting ammonia concentration. In addition, they also indicated that the half-saturation constant ( $K_m$ ) for ammonia of *N. maritimus* SCM1 (0.133 μM total ammonia) was more than 10 times lower than the lowest  $K_m$  for ammonia assimilation of heterotrophic bacteria. These data suggest that ammonia assimilation of heterotrophic bacteria may be insignificant in GAC filter treating low concentration of ammonia. On the other hand, considering that bacterial abundance is approximately two or three orders of magnitude higher than AOA, we may need to take a certain level of ammonia assimilation into account. However, since no data was available for estimating or discriminating actual contribution of ammonia assimilation of heterotrophic bacteria, it was not considered in the following calculation.



**Fig. 6 – Relative contributions of AOA and AOB to *in situ* ammonia-oxidation rates. In the estimation of  $r_{in}$  for AOA,  $r_{in}$  for AOB is set at 50 fmol cell<sup>-1</sup> h<sup>-1</sup>.**



**Fig. 7 – Temporal variations of in situ cell-specific ammonia-oxidation activity for AOA and an influent ammonia load to GAC filter. In situ cell-specific ammonia-oxidation activity for AOA is estimated under the condition that AOB activity is a maximum of  $50 \text{ fmol cell}^{-1} \text{ h}^{-1}$ . Influent ammonia load is calculated by daily amounts of treated water multiplied by ammonia concentration in the coagulation basin effluent.**

Because the ammonia concentration in the GAC influent was not available, the ammonia concentration in the effluent of the coagulation basin was alternatively used. The amounts of ammonia removed in the GAC filter per hour (*in situ* ammonia-oxidation rates) were estimated based on the quantity of water treated hourly multiplied by the difference of ammonia concentrations in the coagulation basin effluent and the GAC effluent. Copy numbers of *amoA* genes were converted to cell numbers based on the simple assumption that an AOA cell carries 2.1 copies of *amoA* genes (this study), while an AOB cell carries 2.5 copies of *amoA* genes (Okano et al., 2004). First, the contribution of AOB to nitrification was estimated assuming that AOB were present on GAC at a maximum of  $2.6 \times 10^5 \text{ copies g-dry}^{-1}$  which corresponds to the quantification limit of Q-PCR. Moreover, we assigned 1 or  $50 \text{ fmol cell}^{-1} \text{ h}^{-1}$  to  $r_{in}$  for AOB by referring to the following studies on *in situ* activity of AOB:  $1.3\text{--}8 \text{ fmol cell}^{-1} \text{ h}^{-1}$  in freshwater sediment (Altmann et al., 2003) and  $0\text{--}49.6 \text{ fmol cell}^{-1} \text{ h}^{-1}$  for activated sludge in municipal wastewater treatment plants (Limpiyakorn et al., 2005). After calculating the AOB contribution,  $r_{in}$  for AOA was estimated by dividing the remaining part of *in situ* ammonia-oxidation rates by AOA abundance. The relative contributions of AOA and AOB to *in situ* ammonia-oxidation rates after 13 August 2008 were compared. When  $r_{in}$  for AOB is  $1 \text{ fmol cell}^{-1} \text{ h}^{-1}$ , AOA can account for almost 100% of the *in situ* ammonia-oxidation rates with  $r_{in}$  of  $4.9\text{--}80.6 \text{ fmol cell}^{-1} \text{ h}^{-1}$ . On the other hand, as shown in Fig. 6, AOA are still responsible for 75–93% of *in situ* ammonia-oxidation rates with  $r_{in}$  of  $4.1\text{--}74.9 \text{ fmol cell}^{-1} \text{ h}^{-1}$  even when AOB have  $50 \text{ fmol cell}^{-1} \text{ h}^{-1}$ , which can be regarded as the highest  $r_{in}$  for AOB. AOB contribution to nitrification is estimated to be at most 7–25% of *in situ* ammonia-oxidation rates. Since the ammonia concentration in the nitrification potential test (initial concentration is  $5 \text{ mg N l}^{-1}$ ) is significantly higher than the actual level in the treatment and AOB have higher  $K_m$  for ammonia than AOA (Martens-Habben

et al., 2009), AOB contribution in the nitrification potential test may be higher than the estimates based on the *in situ* ammonia-oxidation rates. The estimated  $r_{in}$  for AOA is equivalent to or higher than  $r_{in}$  for AOB. However, several studies have shown that the cell-specific ammonia-oxidation activity of AOA was generally lower than that of AOB as follows:  $0.002\text{--}1.2 \text{ fmol cell}^{-1} \text{ h}^{-1}$  in soil (Jia and Conrad, 2009; Schauss et al., 2009),  $0.53 \text{ fmol cell}^{-1} \text{ h}^{-1}$  for *N. maritimus* SCM1 (Martens-Habben et al., 2009) and  $0.5 \text{ fmol cell}^{-1} \text{ h}^{-1}$  in freshwater sediment (Herrmann et al., 2008). It is possible that AOA associated with GAC have physiological features that differ from those of previously known AOA. Although it may be necessary to consider the possibility that the primer sets used in this study fail to cover all AOA or AOB *amoA* genes, further research is required to validate the hypothesis that AOA with higher activity could play an important role in ammonia oxidation in GAC filtration.

In Fig. 7, temporal variations of  $r_{in}$  for AOA estimated under the conditions that  $r_{in}$  for AOB is  $50 \text{ fmol cell}^{-1} \text{ h}^{-1}$  and ammonia assimilation of heterotrophs is not considered are shown with an influent ammonia load to GAC filter. Fig. 7 suggests that  $r_{in}$  for AOA were relatively high in August 2008 ( $55.3\text{--}74.9 \text{ fmol cell}^{-1} \text{ h}^{-1}$ ), likely because fresh AOA was very active during the initial stage of settlement or growth. With an increase in AOA abundance,  $r_{in}$  for AOA remained at low levels ( $4.1\text{--}34.9 \text{ fmol cell}^{-1} \text{ h}^{-1}$ ) from September to November 2008. However,  $r_{in}$  for AOA again increased to  $52.8 \text{ fmol cell}^{-1} \text{ h}^{-1}$  probably in accordance with an increase in the influent ammonia load. A similar relationship between  $r_{in}$  or the maximum cell-specific ammonia-oxidation rates and influent ammonia load has been presented for AOB in wastewater treatment (Fujita et al., 2007, 2010). Thus, it is likely that AOA also change their activity depending on their abundance and the influent ammonia load.

When calculating  $r_{in}$ , we assumed that all AOA and AOB were equally active enough to contribute to nitrification. A more accurate estimation of  $r_{in}$  should be based on only active players. For example; quantification of *amoA* gene transcripts would demonstrate more direct activity of AOA and AOB. In addition, it is possible that AOA associated with GAC are capable of using organic matter as an energy source instead of autotrophic metabolism. Such a mixotrophic feature of AOA has been suggested by several researchers (Hallam et al., 2006b; Jia and Conrad, 2009; Walker et al., 2010). Due to insufficient availability of ammonia, AOA could possibly utilize organic matter to maintain their abundance. Only *in situ* autotrophic activity should be considered when assessing  $r_{in}$  for AOA associated with GAC.

## 5. Conclusions

- AOA, rather than AOB, could settle in GAC rapidly after stopping prechlorination. In accordance with AOA settlement, the nitrification potential of GAC increased. Prechlorination should be avoided to achieve GAC with high nitrification activity.
- Among archaeal community members in raw water, only AOA could selectively settle in GAC. Thus, the archaeal community associated with GAC was dominated by AOA.

- *In situ* cell-specific ammonia-oxidation activity for AOA associated with GAC was estimated to be equivalent to or higher than that of AOB. Considering the predominance of AOA over AOB, AOA may be major contributors to ammonia-oxidation in GAC filtration.
- Though AOB abundance was not accurately quantified, AOB contribution to ammonia-oxidation was suggested. Further validation on AOB activity is required.

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[報 文]

## 定量的感染リスク評価の感度分析における 非加熱飲料水消費量データの影響

Effects of Water Consumption Data on Sensitivity Analysis  
in Quantitative Microbial Risk Assessment

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オランダの浄水場を取り上げたケーススタディにおいて、*Campylobacter*の年間感染リスクを評価した。処理前後の微生物濃度データのペアリング方法としてはランク法を使用し、モンテカルロシミュレーションによって4つの処理ステップの総合除去・不活化能を評価したところ、中央値 $7.46\log_{10}$ 、平均値 $6.22\log_{10}$ を得た。年間感染確率の平均値は $1.68 \times 10^{-3}$ /人/yearと評価された。感度分析の結果、処理水中*E. coli*濃度に対してはオゾン処理が最も大きく影響することがわかった。一方、*E. coli*摂取量に対する感度分析結果からは、非加熱飲料水消費量(以下、飲水量)が最も大きく影響することがわかった。これは飲水量データが、48%の人がまったく水道水を飲まない統計データであることによる。飲水量データの統計的扱いを適切な方法で行なわないと、感度分析に混乱を招くおそれがあり、注意すべきである。これを回避するためには、少なくとも離散モデルであるポアソンモデルよりも、指数モデルのような連続モデルを用いるほうが好ましい。以上の結果、年間感染確率を低下するためには、オゾン処理を適切に管理し、微生物を確実に不活化することが最も有効であると指摘した。不確実性分析の結果、浄水処理における除去能に対する水温の影響、病原微生物数と指標微生物数の比の影響、処理前後の微生物濃度データのペアリング方法の影響が大きいことを示した。これらをもとに、定量的感染リスク評価(QMRA)の評価値の精度を向上させるために必要な事項、および収集する必要性が高いデータを指摘した。

**Key words** : 定量的感染リスク評価, *Campylobacter*, 残留塩素, 塩素消毒, オランダ

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## 1. 緒言

1980年代以来、飲料水の微生物的安全性を定量化するため定量的感染リスク評価(Quantitative Microbial Risk Assessment; QMRA)が適用されてきた<sup>1)2)</sup>。感染リスクは、飲料水中に含まれる病原微生物の摂取量と、その摂取によって感染が起きる確率から計算される。多くの研究では、浄水処理ステップの除去・不活化能のような各要素の変動は確率密度関数(PDF)によって記述され、年間感染確率はモンテカルロシミュレーションによって定量的に評価される。これまで行なわれたQMRAの研究によって、その手法は発展し改善されてきた。

オランダでは現在、水道水は塩素をまったく使用せずに配水されている。従来から塩素を使用しない浄水場は多かったが、最後に塩素消毒が停止されたのは2005年である。塩素を二酸化塩素等に置き換えた例もあるが、代替消毒剤を使用するというよりは、浄水処理の各ステップを微生物の除去・不活化のために必要なプロセスととらえ、浄水処理プロセス全体として、要求される除去・不活化能を確保するようにしている。また、浄水場で微生物的に安全な水道水を生産することに加えて、さまざまな安全確保策を設けている。それらは、微生物的に安定な水の配水、配水管材質の選定を含む配水管内面での生物膜生成の制御、配水管網の維持管理などであり、実務上必要と考えられる方策を重ね合わせることによって、はじめて塩素を使用しない水道水の配水を実現しているとみることができる<sup>3)</sup>。

このオランダの水道水質管理において特筆されるのは、水道水の微生物的安全性を確保するため、QMRAの手法を実務に導入していることである。2001年から施行されている現行水質基準には、「QMRAによって、腸管系ウイルス、*Cryptosporidium*, *Giardia*などの年間感染リスクが $10^{-4}$ 以下と評価されること」という項目が存在する<sup>4)</sup>。すなわち各水道会社は、法律によって、配水する水道水の微生物感染リスクを定量評価し、提示することが義務付けられている。

*Campylobacter*は上記要件として明記されてい

ないものの、多くのヨーロッパ諸国において、細菌のなかでは水系感染症を引き起こす最も重要な微生物と考えられている<sup>5)</sup>。また、オランダで評価された例<sup>6)</sup>をみると、原水中の微生物濃度と許容感染確率( $10^{-4}$ /人/year)から求めた浄水処理に必要な除去・不活化 $\log_{10}$ 数が最も大きいのは、上記4種類のうちでは*Campylobacter*のようである。本研究では、その感染リスク評価を精緻化および高度化するため、*Campylobacter*を取り上げる。なお、近年のわが国における飲料水等を介した水系感染症をみると、*Campylobacter*は病原大腸菌に次いで発生事例が多い<sup>7)</sup>。1982年から1996年までに発生した飲料水による健康被害事例でも、病原大腸菌が最も多いが、*Campylobacter*は第2位で21%を占めている<sup>8)</sup>。

本研究では、まず、これまでのQMRA研究で開発されてきた手法を取り込み、ケーススタディのなかで*Campylobacter*の年間感染リスクを評価した。次いで、評価結果に対して感度分析と不確実性分析を行ない、QMRAに関する全体の作業を完結させることを目標とする。感度分析は、4つの処理ステップの総合除去・不活化能、水道水中大腸菌(*E. coli*)濃度、*E. coli*摂取量、年間感染確率に対して行なった。一方、不確実性分析で検討した項目は、取水する原水の種類の影響、浄水処理における除去能に対する水温の影響、病原微生物数と指標微生物数の比の影響、用量反応モデルの影響、処理前後の微生物濃度データのペアリング方法の影響である。この結果、年間感染確率を低下させるために有効な個所、およびQMRAの評価値の精度を向上させるために必要な事項を指摘した。また、とくに、非加熱飲料水消費量(以下、飲水量と記す)データの統計的取り扱い方法が、感度分析結果に大きな影響を及ぼすことを見出した。

## 2. 方法

### 2.1 対象浄水場とその概要

オランダ西部に位置するA浄水場をケーススタディとして用いた。浄水量は平均 $115,000\text{m}^3/\text{d}$ であり、水道水は主としてアムステルダム東部地域に配水されている。水源地は、農牧業を可能にす

るために造られた干拓地であるボルダー（面積5.4km<sup>2</sup>）である。原水は、ボルダーに隣接する水路から表流水として取水しているが、ボルダーの地下層を浸透してきた水を多量に含んでいる<sup>4)</sup>。この原水に加えて、夏季に需要水量が増大する時期には、アムステルダム－ライン運河の水が追加取水され、その取水量は年間の全水量の5%である。運河はライン川の水を引いており、ボルダーからの水よりも汚染されている<sup>9)</sup>。

原水はまず、凝集－貯水－急速砂ろ過によって前処理される。貯水池（123ha, 6.9×10<sup>6</sup>m<sup>3</sup>）における平均滞留時間は89日である。前処理された水はおもな処理プロセスをもつA浄水場に送られ、オゾン処理－軟化－粒状活性炭－緩速砂ろ過によって処理される。塩素はいずれの処理ステップでも使用されていない。

## 2.2 対象微生物と使用データ

一般に、病原微生物を直接測定するのは容易ではなく、測定値の数は多くないのが普通である。これに対して、*E. coli*は日常的に測定されている微生物であり測定数が多い。このため、原水濃度や除去性の変動幅についても議論できる可能性が高い。配水過程での汚染による感染リスクを評価する場合も、病原微生物と*E. coli*または耐熱性大腸菌との比を用いて、*E. coli*または耐熱性大腸菌が検出されたときの感染リスクを推定する試みが行なわれている<sup>10)11)</sup>。本研究でも、まず*E. coli*を取り上げ、浄水プロセスの除去・不活化能を評価する。その後、表流水中で測定された*E. coli*に対する*Campylobacter*の比率を用いて*Campylobacter*数に換算する。*E. coli*と*Campylobacter*は、急速砂ろ過、緩速砂ろ過、オゾン処理で、同様に除去・不活化されることが認められている<sup>12)13)</sup>。

ボルダーで取水された原水中の*E. coli*濃度のデータはA浄水場から提供されたもので、2002年4月から2004年12月までに46回測定された値を用いた。

## 2.3 除去・不活化能の評価方法<sup>14)15)</sup>

QMRAでは、原水中微生物濃度や各処理ステップにおける除去・不活化率などに分布を設定し、モンテカルロシミュレーションを行なう。この場合、ある流入水中微生物濃度に対して、生成

乱数に対応した除去・不活化率を乗じて処理水中微生物濃度を求めることになる。すなわち、除去・不活化率としていかなるデータセットをもっておくかが重要となる。このため、はじめに浄水処理プロセスの除去・不活化能を適切に評価する方法について検討した。

浄水処理プロセスの除去・不活化能は、通常デイト法によって評価される。この方法では同じ日（または同時刻）に採取された流入水と流出水のサンプルの測定値をペアリングし、除去・不活化率を計算する。この場合、浄水処理施設内での滞留時間（通常数時間）の影響は無視されることが多い。この取り扱いは、滞留時間が短い場合はよいが、滞留時間が長くなる（たとえば数日、数週間、数カ月など）と、その取り扱いを吟味する必要があるであろう。

デイト法に対し、ランク法とよばれる方法では、流入水濃度と流出水濃度との間に相関を仮定する。つまり、流入水濃度が高ければ流出水濃度も高くなり、流入水濃度が低ければ流出水濃度も低くなるという仮定である。はじめに流入水濃度と流出水濃度のデータセットを降順にソートしておき、その後、ペアリングを行なって除去・不活化率を計算する。この他、ランダム法という方法もある。この方法ではランクや日付による相関を仮定せず、流入水濃度と流出水濃度のデータセットから値をランダムに取り出してペアリングする。

ペアリングによって得られた除去率を、流入水濃度に乗じて流出水濃度を計算する。この計算された流出水濃度と実際に測定された流出水濃度を比較した。上記3つのペアリング方法を試行した結果、デイト法は除去能を低く評価する傾向にあること、ランク法のほうが測定値をよく再現しており望ましいこと、ランダム法はデイト法とよく似た結果を与えることを示した<sup>15)</sup>。以下の解析ではランク法を用いた。

## 2.4 除去・不活化能に対する分布形の当てはめ

対象とした浄水場の処理プロセスでは、おもな微生物のバリアと考えられるステップは、凝集－貯水、急速砂ろ過、オゾン処理、緩速砂ろ過であり、ここでの評価対象はこれらの4段階とする。

*E. coli*濃度は、凝集-貯水後の水では、2002年1月から2005年8月までに91回測定された値を用いた。急速砂ろ過の流出水では、2003年1月から2004年12月までに556回測定された値を用いた。オゾン処理の流出水では、2003年1月から2004年12月までに326回測定された値を用いた。326回のうち30回の測定値は、10l から100l の大容量のサンプルを測定した結果である。

凝集-貯水、急速砂ろ過、オゾン処理の各ステップにおいて、それぞれの流入・流出水濃度をランク法によってペアリングし、除去・不活化能を求めた。この除去・不活化能の分布に対して、最も当てはまるPDFを選択した。

緩速砂ろ過後の水は、配水される水道水を意味する。2003年1月から2004年12月まで毎日測定されたデータがある。2系列について合計1,393回の*E. coli*の測定値は、1回だけ検出例(0.2*E. coli*/100ml)がある他はすべて0であった。このため、このデータを用いて緩速砂ろ過の除去能を評価することができない。そこで、除去能を評価するために、パイロットスケールで行なわれた*E. coli*の除去実験の結果を用いた<sup>16)</sup>。水温13℃以下の条件下で測定された6回の除去能測定値から、最小値2.0log<sub>10</sub>、平均値2.4log<sub>10</sub>、最大値4.2 log<sub>10</sub>を得、これらをパラメーターとする三角分布を設定した。なお、水温13℃以上の条件下での3回の測定値があるが、この影響については不確実性分析で取り扱う。

除去・不活化能に対して与えられたPDFをもとにモンテカルロシミュレーションを行ない、4段階の処理ステップの総合除去・不活化能を計算した。シミュレーション回数は安定した結果が得られる100,000回とした。

以上の分布形の適合およびモンテカルロシミュレーションには、Crystal Ball 7<sup>®17)</sup> (Decisioneering社製)を用いた。

## 2.5 感染確率の計算

*E. coli*の1日当たり摂取量(*E. coli*/d)は、水道水中*E. coli*濃度と加熱処理していない水道水の摂取量(飲水量)を乗じて求めた。

オランダにおける飲水量調査は、1997年から1998年にかけて、6,250人を対象とし、連続しな

い2日間の飲水量を日記形式で記録することを依頼して行なわれた。そのデータを統計解析した結果、QMRAを行なうためには、飲水量データそのものを用いるか、または、分布形を当てはめるならば平均値(比率)0.706グラス/dをもつポアソンモデルを使用するのが適当と評価された<sup>18)</sup>。ポアソンモデルの場合、飲水量は1日当たりのグラス数という離散値で表わされる。1グラスは250mlに相当すると仮定されているので、平均値は177ml/dに相当する。

つぎに、表流水中の*E. coli*に対する*Campylobacter*の比率(C/E値)を用いて、*E. coli*摂取量(*E. coli*/d)を*Campylobacter*摂取量(*Campylobacter*/d)に換算した。1994年に1年間にわたって、マース川で*E. coli*と*Campylobacter*の濃度を22回測定した例<sup>10)</sup>があるのでこのデータを使用することとし、C/E値の分布に対して適切なPDFを選択した。

*Campylobacter*に関する用量反応モデルを用いて、1日当たりの感染確率 $P_d$ (/人/d)を計算した。*Campylobacter jejuni*の用量反応モデルとしては、Teunisら<sup>19)</sup>によって提示された $\alpha = 0.024$ 、 $\beta = 0.011$ をもつベータ-ポアソンモデルがある。ここで、ベータ-ポアソンモデルの $\alpha$ 、 $\beta$ の値は $\beta \geq 1$ 、 $\alpha \leq \beta$ を満たす必要があるが、上記の値はこれらを満たさない。実際、このベータ-ポアソンモデルを適用すると、感染確率が低用量域では最大感染確率曲線よりも大きな値となる問題が指摘されている<sup>20)</sup>。したがって、この場合ベータ-ポアソンモデルの使用は適切ではない。ベータ-ポアソンモデルは、低用量域では $\gamma = \alpha / (\alpha + \beta)$ とおくことにより指数モデルで近似できる。 $\alpha = 0.024$ 、 $\beta = 0.011$ を代入して $\gamma = 0.686$ を得る。

本研究では、この指数モデル( $P_d = 1 - \exp(-0.686 \times D)$ 、 $D$ :用量)を使用することとした。最大感染確率曲線やベータ-ポアソンモデルを用いたケースは、不確実性分析で検討する。

1日当たりの感染確率を $P_d$ とすると、1年間に1回以上感染が起きる確率 $P_y$ (/人/year)は式(1)で計算される。

$$P_y = 1 - (1 - P_d)^{365} \dots\dots\dots (1)$$

原水中*E. coli*濃度、4段階の処理ステップ、飲