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

Ikuro Kasuga [a,*], Hirotaka Nakagaki [a], Futoshi Kurisu [b], Hiroaki Furumai [b]

[a] Department of Urban Engineering, School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo, Tokyo 113-8656, Japan
[b] Research Center for Water Environment Technology, School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo, Tokyo 113-8656, Japan

ARTICLE INFO

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 g-dry^{-1} of AOA $amoA$ genes were detected within 3 weeks and the quantities ultimately reached 10^6–10^7 copies 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.

© 2010 Elsevier Ltd. All rights reserved.

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

---

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^3 day^{-1}, 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^{-1} 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^{-1} 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_4^+-N l^{-1} at 20 °C with agitation at 100 rpm. Inorganic medium per 1 L of water was composed of 23.57 mg of $(NH_4)_2SO_4$, 200 mg of $MgSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2$, 174 mg of K_2HPO_4, 156 mg of NaH_2PO_4, 1 mg of Fe-EDTA, 200 mg of $CaCO_3$, 0.001 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.002 mg of $MnCl_2 \cdot 4H_2O$, 0.002 mg of $CoCl_2 \cdot 6H_2O$, 0.02 mg of $CuSO_4 \cdot 5H_2O$ and 0.1 mg of $ZnSO_4 \cdot 7H_2O$. 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-μm 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 μl) contained 10 μl of LightCycler 480 Probe Master (Roche), 0.50 μM of each primer, 0.20 μM of probe and 1 μl of DNA extract. The amplification program was as follows: 95 °C for 5 min, followed by 45 cycles consisting of 95 °C for 10 s, 60 °C for 50 s and 72 °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 5.0×10^1 to 5.0×10^8 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 μl) contained 10 μl of LightCycler 480 SYBR Green I Master (Roche), 0.50 μM of each primer and 1 μl of DNA extract. The amplification program was as follows: 95 °C for 5 min, 45 cycles consisting of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min (detection), followed by melting curve analysis (65 °C–97 °C with a heating rate of 0.1 °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 5.0×10^1 to 5.0×10^7 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 °C for 5 min, 45 cycles consisting of 95 °C for 10 s, 53 °C for 20 s and 72 °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 5.0×10^2 to 5.0×10^6 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 5.0×10^1 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 °C for 5 min, 45 cycles consisting of 95 °C for 10 s, 54 °C for 20 s, 72 °C for 30 s and 80 °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 5.0×10^2 to 5.0×10^6 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 5.0×10^1 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 μl) contained 1.25 units of TaKaRa EX Taq HS (TAKARA BIO, Japan), 5 μl of 10 × Ex Taq Buffer, 4 μl of dNTP mixture (all chemicals were provided by TAKARA BIO), 0.2 μM of each primer and 1 μl of the template. The amplification programs for AOA *amoA* genes and archaeal 16S rRNA genes were as follows: 30 cycles consisting of 94 °C for 45 s, 53 °C for 1 min and 72 °C for 1 min for AOA *amoA* genes; 30 cycles consisting of 94 °C for 30 s, 52 °C for 45 s and 72 °C for 1 min for archaeal 16S rRNA genes. The final extension was carried out at 72 °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 *Hha*I (TAKARA BIO) at 37 °C for 6 h, while the same amount of archaeal 16S rRNA gene fragments were digested with *Taq*I (TAKARA BIO) at 65 °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 mg N l^{-1}, ranging from below the quantification limit (<0.02 mg N l^{-1}) to 0.26 mg N l^{-1}. Water temperature fluctuated from 4.0 to 28.0 °C
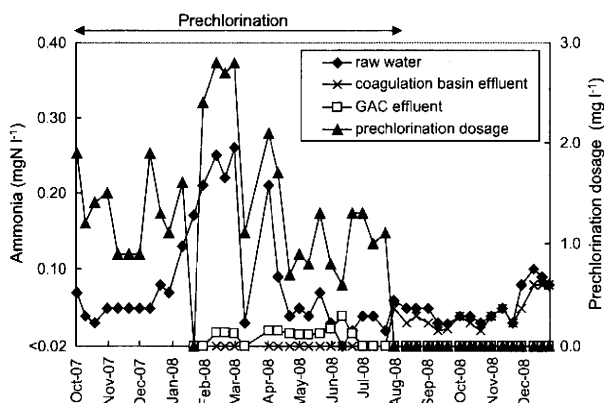

Fig. 1 – Temporal variations of ammonia concentrations and prechlorination dosage in actual operation from plant start-up in October 2007 to December 2008. No data is available for coagulation basin effluent and GAC effluent from October 2007 to the middle of January 2008. Prechlorination was implemented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

during the monitoring period. When the water temperature dropped to less than 10 °C in winter, the ammonia concentration in raw water was frequently above 0.10 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 mg NH_4^+-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 ($R^2 = 0.924-0.994$), 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 3.0×10^5 to 4.2×10^6 (average: 1.2×10^6) and from 7.1×10^2 to 4.2×10^4 (average: 5.6×10^3) copies 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 7.2×10^4 to 3.6×10^7 copies 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 1.1×10^9 to 2.7×10^9 copies 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 4.8×10^5 copies 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 g-dry^{-1}.
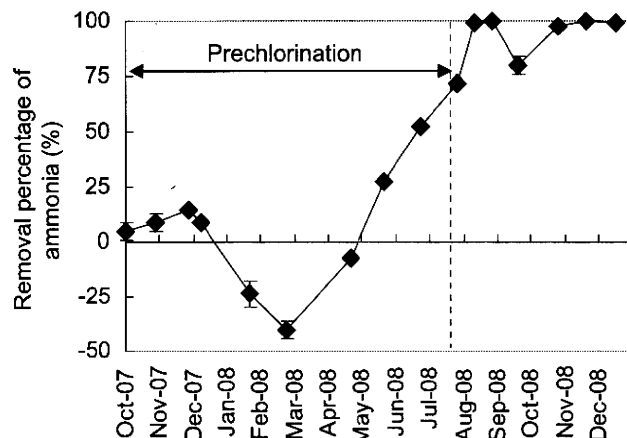
Fig. 2 — Removal percentage of ammonia during 48 h of incubation in the nitrification potential test. Initial ammonia concentration was set at approximately 5 mg N l^{-1}.

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 $3.7 \times 10^3 \pm 2.2 \times 10^2$ 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, 5.9×10^5 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 1.3×10^7 copies g-dry^{-1} on 28 October and then decreased to 2.8×10^6 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

Bacterial and archaeal 16S rRNA genes

AOA and AOB amoA genes
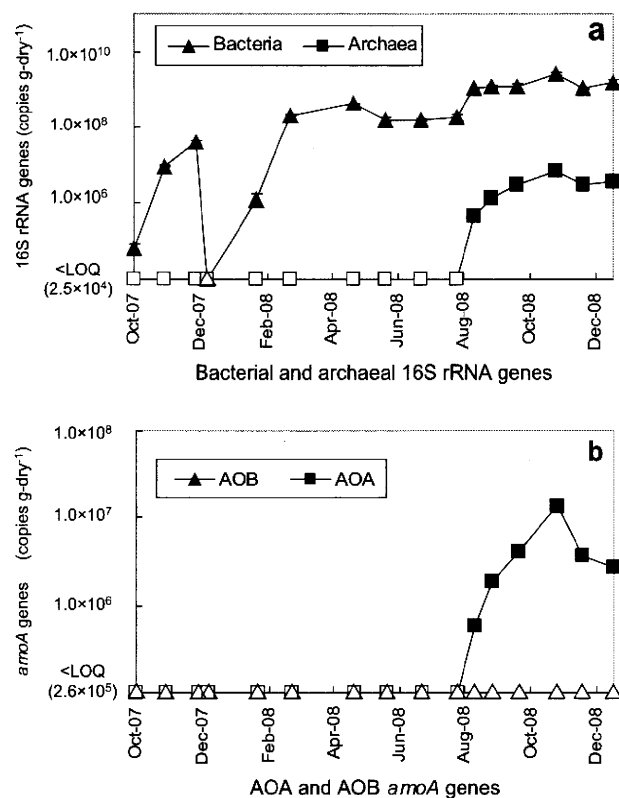
Fig. 3 — Temporal changes in quantities of 16S rRNA genes of bacteria and archaea (a) and amoA genes of AOA and AOB (b) associated with GAC. The quantification limits (LOQ) of both bacterial and archaeal 16S rRNA genes were 2.5×10^4 copies g-dry^{-1}, while those of both AOA and AOB amoA genes were 2.6×10^5 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
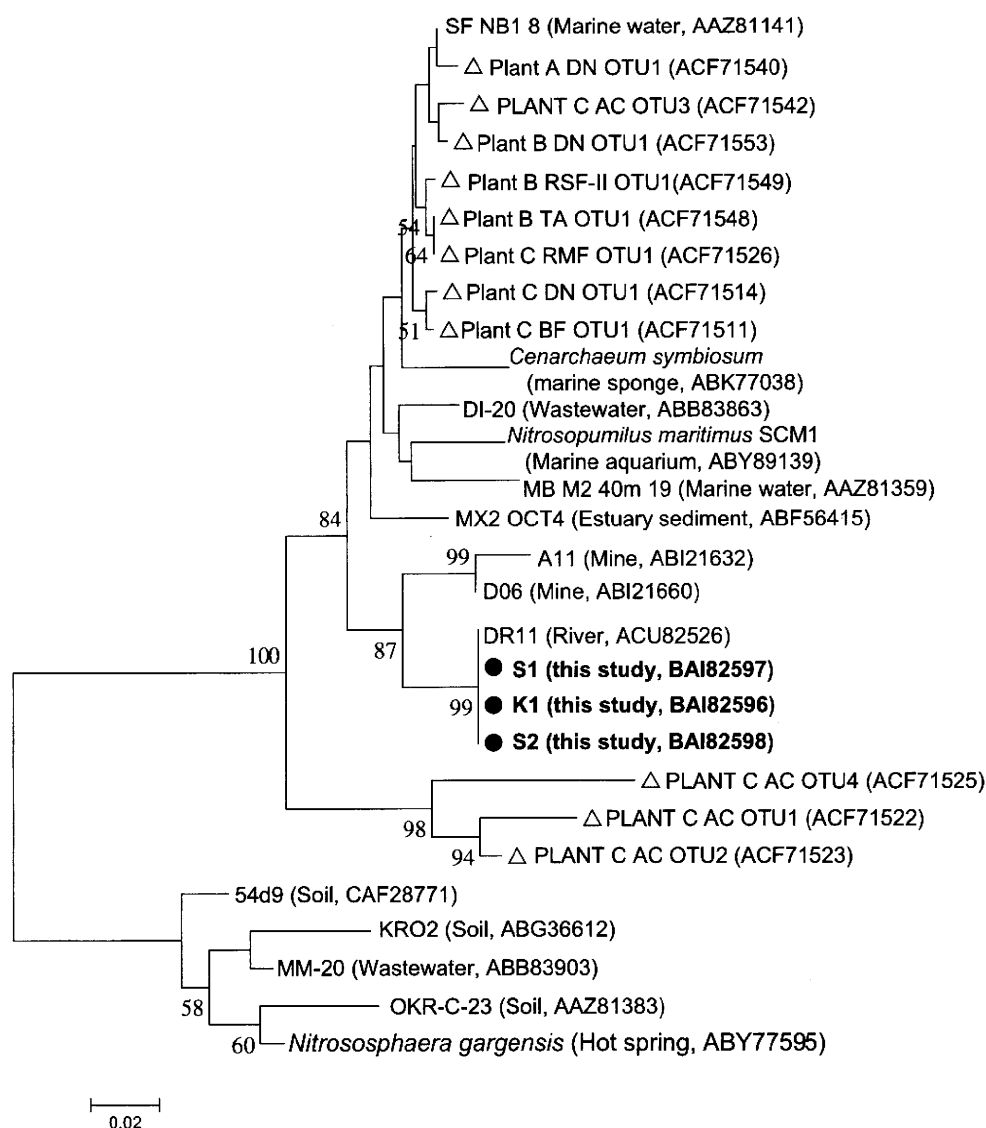

Fig. 4 – **Phylogenetic tree of deduced amino acid sequences of AOA *amoA* genes. Neighbor-joining tree was constructed based on the Jones-Taylor-Thornton substitution model. Bootstrap analysis was conducted with 1000 replicates and only bootstrap values above 50% are shown. The bar represents a 2% evolutionary 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.**
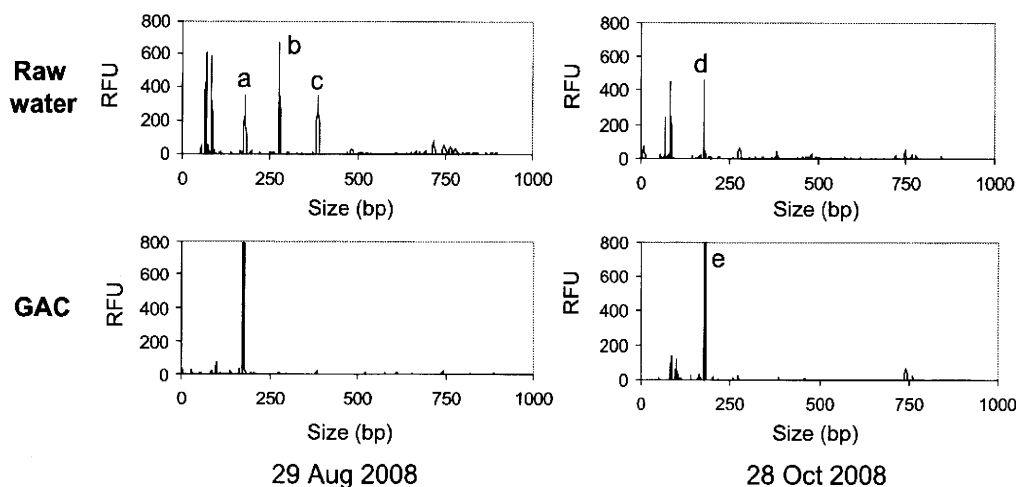
Fig. 5 — T-RFLP profiles of archaeal 16S rRNA genes for raw water and GAC collected on 29 August 2008 (left) and 28 October 2008 (right). The restriction enzyme Rsal was used for digestion. Phylogenetic affiliations of the designated fragments are shown in Table 1.

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^{-1} 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 2.6×10^4–2.6×10^5 copies g-dry^{-1}, 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[a]	T-RF (bp)	Phylogenetic group	Similarity (%)
Raw water (29 Aug 2008)	a	186	*Nitrosopumilus maritimus* SCM1 (DQ085097)	92–96
	b	284	*Methanothrix soehngenii* (X51423)	97–98
	c	393	*Methanomicrobiales* archaeon SMSP (AB479390)	96
Raw water (28 Oct 2008)	d	186	*Nitrosopumilus maritimus* SCM1 (DQ085097)	88–96
GAC (28 Oct 2008)	e	186	*Nitrosopumilus maritimus* 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^{-1}) 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^{-1}) ammonia, while AOB such as N. europaea and Nitrosococcus oceani 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 $cell^{-1}$ h^{-1}) 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.
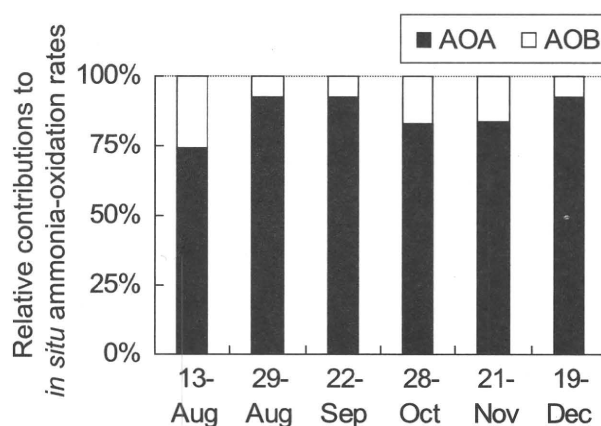

Fig. 6 – Relative contributions of AOA and AOB to in situ ammonia-oxidation rates. In the estimation of r_{in} for AOA, r_{in} for AOB is set at 50 fmol $cell^{-1}$ h^{-1}.
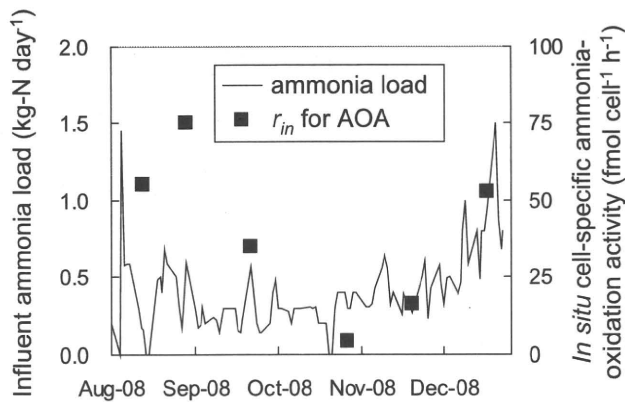
Fig. 7 – Temporal variations of in situ cell-specific ammonia-oxidation activity for AOA and an influent ammonia load to GAC filter. In situ cell-specific ammonia-oxidation activity for AOA is estimated under the condition that AOB activity is a maximum of 50 fmol cell^{-1} h^{-1}. Influent ammonia load 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 2.6×10^5 copies g-dry^{-1} which corresponds to the quantification limit of Q-PCR. Moreover, we assigned 1 or 50 fmol cell^{-1} h^{-1} to r_{in} for AOB by referring to the following studies on in situ activity of AOB: 1.3–8 fmol cell^{-1} h^{-1} in freshwater sediment (Altmann et al., 2003) and 0–49.6 fmol cell^{-1} 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 fmol cell^{-1} h^{-1}, AOA can account for almost 100% of the in situ ammonia-oxidation rates with r_{in} of 4.9–80.6 fmol cell^{-1} 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 4.1–74.9 fmol cell^{-1} h^{-1} even when AOB have 50 fmol cell^{-1} 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 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-Habbena

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: 0.002–1.2 fmol cell^{-1} h^{-1} in soil (Jia and Conrad, 2009; Schauss et al., 2009), 0.53 fmol cell^{-1} h^{-1} for N. maritimus SCM1 (Martens-Habbena et al., 2009) and 0.5 fmol cell^{-1} 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 fmol cell^{-1} 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 (55.3–74.9 fmol cell^{-1} 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 (4.1–34.9 fmol cell^{-1} h^{-1}) from September to November 2008. However, r_{in} for AOA again increased to 52.8 fmol cell^{-1} 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.

Acknowledgements

REFERENCES

Altmann, D., Stief, P., Amann, R., de Berr, D., Schramm, A., 2003. In situ distribution and activity of nitrifying bacteria in freshwater sediment. Environmental Microbiology 5 (9), 798–803.

Beman, J.M., Popp, B.N., Francis, C.A., 2008. Molecular and biogeochemical evidence for ammonia oxidation by marine Crenarchaeota in the Gulf of California. The ISME Journal 2, 429–441.

Bernhard, A.E., Landry, Z.C., Blevins, A., de la Torre, J.R., Giblin, A. E., Stahl, D.A., 2010. Abundance of ammonia-oxidizing archaea and bacteria along an estuarine salinity gradient in relation to potential nitrification rates. Applied and Environmental Microbiology 76 (4), 1285–1289.

Daims, H., Ramsing, N.B., Schleifer, K.H., Wagner, M., 2001. Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence in situ hybridization. Applied and Environmental Microbiology 67 (12), 5810–5818.

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., Oakley, B.B., 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proceedings of the National Academy of Sciences USA 102 (41), 14683–14688.

Fujita, M., Tsuji, K., Takeda, N., Minakawa, M., Nakamura, M., Mino, T., Akashi, A., 2007. Temporal variation in maximum cell-specific nitrification rates in municipal wastewater treatment plant and controlling factors. Journal of Japan Society on Water Environment 30 (12), 723–729 (in Japanese).

Fujita, M., Tsuji, K., Akashi, A., 2010. Temporal variation in maximum cell-specific nitrification rate. Water Science and Technology 61 (8), 2069–2073.

Hallam, S.J., Konstantinidis, K.T., Putnam, N., Schleper, C., Watanabe, Y., Sugahara, J., Preston, C., de la Torre, J., Richardson, P.M., Delong, E.F., 2006a. Genomic analysis of the uncultivated marine crenarchaeote *Cenarchaeum symbiosum*. Proceedings of the National Academy of Sciences 103 (48), 18296–18301.

Hallam, S.J., Mincer, T.J., Schleper, C., Preston, C.M., Roberts, K., Richardson, P.M., DeLong, E.F., 2006b. Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analysis of marine Crenarchaeota. PLOS Biology 4 (4), 520–536.

Harms, G., Layton, A.C., Dionisi, H.M., Gregory, I.R., Garrett, V.M., Hawkins, S.A., Robinson, K.G., Sayler, G.S., 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environmental Science and Technology 37 (2), 343–351.

Herrmann, M., Saunders, A.M., Schramm, A., 2008. Archaea dominate the ammonia-oxidizing community in the rhizosphere of the freshwater macrophyte *Littorella uniflora*. Applied and Environmental Microbiology 74 (10), 3279–3283.

Jia, Z., Conrad, R., 2009. Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. Environmental Microbiology 11 (7), 1658–1671.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543–546.

Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M., Woldendorp, J.W., 1997. Analysis of ammonia-oxidizing bacteria of β-subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Applied and Environmental Microbiology 63 (4), 1489–1497.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. Wiley, Chichester, UK, pp. 115–175.

Leininger, S., Urich, T., Schloter, S., Shwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809.

Limpiyakorn, T., Shinohara, Y., Kurisu, F., Yagi, O., 2005. Communities of ammonia-oxidizing bacteria in activated sludge of various sewage treatment plants in Tokyo. FEMS Microbiology Ecology 54 (2), 205–217.

Lueders, T., Friedrich, M.W., 2002. Effects of amendment with ferrihydrite and gypsum on the structure and activity of methanogenic populations in rice field soil. Applied and Environmental Microbiology 68 (5), 2484–2494.

Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., Stahl, D.A., 2009. Ammonia oxidation kinetics determine niche separation of nitrifying archaea and bacteria. Nature 461, 976–979.

Muramoto, T., Udagawa, T., Okamura, T., 1995. Effective removal of musty odor in the Kamachi purification plant. Water Science and Technology 31 (11), 219–222.

Nadkarni, M.A., Martin, F.E., Jacques, N.A., Hunter, N., 2002. Determination of bacterial load by real-time PCR using a broad range (universal) probe and primer set. Microbiology 148, 257–266.

Offre, P., Prosser, J.I., Nicol, G.W., 2009. Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene. FEMS Microbiology Ecology 70 (1), 99–108.

Okano, Y., Hristova, K.R., Leutenegger, C.M., Jackson, L.E., Denison, R.F., Gebreyesus, B., Lebauer, D., Scow, K.M., 2004. Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Applied and Environmental Microbiology 70 (2), 1008–1016.

Prosser, J.I., Nicol, G.W., 2008. Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. Environmental Microbiology 10 (11), 2931–2941.

Rittmann, B.E., Snoeyink, V.L., 1984. Achieving biologically stable drinking water. Journal of American Water Work Association 76 (10), 106–114.

Schauss, K., Focks, A., Leininger, S., Kotzerke, A., Heuer, H., Thiele-Bruhn, S., Sharma, S., Wilke, B.M., Matthies, M., Smalla, K., Munch, J.C., Amelung, W., Kaupenjohann, M., Schloter, M., Schleper, C., 2009. Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. Environmental Microbiology 11 (2), 446–456.

Schleper, C., Jurgens, G., Jonuscheit, M., 2005. Genomic studies of uncultivated archaea. Nature Reviews Microbiology 3 (6), 479–488.

Simpson, D.R., 2008. Biofilm processes in biologically active carbon water purification. Water Research 42 (12), 2839–2848.

Stephen, J.R., Chang, Y.J., Macnaughton, S.J., Kowalchuk, G.A., Leung, K.T., Flemming, C.A., White, D.C., 1999. Effect of toxic metals on indigenous soil β-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. Applied and Environmental Microbiology 65 (1), 95—101.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4; Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24 (8), 1596—1599.

Treusch, A.H., Leininger, S., Kletzin, A., Schuster, S.C., Klenk, H.P., Schleper, C., 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesotrophilic crenarchaeota in nitrogen cycling. Environmental Microbiology 7 (12), 1985—1995.

Urakawa, H., Tajima, Y., Numata, Y., Tsuneda, S., 2008. Low temperature decreases the phylogenetic diversity of ammonia-oxidizing archaea and bacteria in aquarium biofiltration systems. Applied and Environmental Microbiology 74 (3), 894—900.

de Vet, W.W.J.M., Dinkla, I.J.T., Muyzer, G., Rietveld, L.C., van Loosdrecht, M.C.M., 2009. Molecular characterization of microbial populations in groundwater sources and sand filters for drinking water production. Water Research 43 (1), 182—194.

Walker, C.B., de la Torre, J.R., Klotz, M.G., Urakawa, H., Pinela, N., Arp, D.J., Brochier-Armanete, C., Chain, P.S.G., Chan, P.P., Gollabgirj, A., Hemp, J., Hügler, M., Karr, E.A., Könneke, M., Shin, M., Lawton, T.J., Lowe, T., Martens-Habbenaa, W.,

Sayavedra-Soto, L.A., Lang, D., Sievert, S.M., Rosenzweig, A.C., Manning, G., Stahl, D.A., 2010. Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proceedings of the National Academy of Sciences USA 107 (19), 8818—8823.

Wells, G.F., Park, H.D., Yeung, C.H., Eggleston, B., Francis, C.A., Criddle, C.S., 2009. Ammonia-oxidizing communities in a highly aerated full-scale activated sludge bioreactor: betaproteobacterial dynamics and low relative abundance of Crenarchaea. Environmental Microbiology 11 (9), 2310—2328.

van der Wielen, P.W.J.J., Voost, S., van der Kooij, D., 2009. Ammonia-oxidizing bacteria and archaea in groundwater treatment and drinking water distribution systems. Applied and Environmental Microbiology 75 (14), 4687—4695.

Wilcox, D.P., Chang, E., Dickson, K.L., Johansson, K.R., 1983. Microbial growth associated with granular activated carbon in a pilot water treatment facility. Applied and Environmental Microbiology 46 (2), 406—416.

Wuchter, C., Abbas, B., Coolen, M.J.L., Herfort, L., van Bleijswijk, J., Timmers, P., Strous, M., Teira, E., Herndl, G.J., Middelburg, J.J., Schouten, S., Damsté, J.S.S., 2006. Archaeal nitrification in the ocean. Proceedings of the National Academy of Sciences 103 (33), 12317—12322.

You, J., Das, A., Dolan, E.M., Hu, Z., 2009. Ammonia-oxidizing archaea involved in nitrogen removal. Water Research 43 (7), 1801—1809.

[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

伊藤　禎彦*

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1.68×10^{-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

* Sadahiko ITOH, 京都大学大学院工学研究科都市環境工学専攻　博士(工学)　〒615-8540 京都市西京区京都大学桂
C-1, Dr. Eng., Department of Environmental Engineering, Graduate School of Engineering, Kyoto University, C-1
Kyotodaigaku Katsura, Nishikyo-ku, Kyoto-shi 615-8540 Japan(E-mail : itoh@urban.env.kyoto-u.ac.jp)

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[1][2]。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[3]。

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[4]。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[5]。また，オランダで評価された例[6]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で必要な除去・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[7]。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[8]。

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115,000m³/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ポルダー（面積5.4km²）である。原水は，ポ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，ポルダーの地下層を浸透してきた水を多量に含んでいる[4]。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，ポルダーからの水よりも汚染されている[9]。

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，$6.9 \times 10^6 m^3$）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[10)11]。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[12)13]。

ポ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[14)15]

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生じるであろう。

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[15]。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。

650

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(0.2E. 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[16]。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$_{10}$，平均値2.4log$_{10}$，最大値4.2 log$_{10}$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[17]（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[18]。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[10]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[19]によって提示されたa =0.024，β =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a，βの値は$\beta \geqq$ 1，$a \leqq \beta$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[20]。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$\gamma = a/(a+\beta)$とおくことにより指数モデルで近似できる。a =0.024，β =0.011を代入してγ =0.686を得る。

本研究では，この指数モデル(P_d = 1 － exp($-$0.686×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とするとき，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。

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

原水中E. coli濃度，4段階の処理ステップ，飲

表1 各要素に当てはめた確率密度関数

要素		分布形	パラメーターとその推算値
原水中E. coli濃度(E. coli/100ml)		ガンマ分布	$\mu = -2.50$;$\lambda = 383$;$p = 0.674$
E. coliの除去・不活化能(log₁₀数)	凝集―貯水	ロジスティック分布	$\mu = 1.48$;$\lambda = 0.15$
	急速砂ろ過	ワイブル分布	$\mu = 1.74$;$\lambda = 0.59$;$p = 2.38$
	オゾン処理	正規分布	$\mu = 1.91$;$\sigma = 0.88$
	緩速砂ろ過	三角分布	最小値=2.00;平均値=2.40;最大値=4.20
C/E値		対数正規分布	$\mu = 0.0415$;$\sigma = 0.104$

水量，C/E値に対してモンテカルロシミュレーションを行ない，年間感染確率P_yを計算した。なお，シミュレーションにおいて各項目の間の相関関係は仮定していない。シミュレーションの回数は，安定した結果を得るために必要な100,000回とした。

2.6 感度分析

4つの処理ステップの総合除去・不活化能，水道水中E. coli濃度，E. coli摂取量，年間感染確率に対して感度分析を行なった。感度分析のためには，まず，予測される項目と条件設定された各要素の間でスピアマンの順位相関係数を算出する。つぎに，この順位相関係数を2乗した後，それらの値を要素全体で100%となるように正規化することによって寄与率を算出する。寄与率は，予測対象の不確実性に対して各要素が何%寄与しているかを表わす。

2.7 不確実性分析

QMRAにおける各予測項目および最終的に評価された年間感染確率は，当然，大きな不確実性をもつ。ここでは結果に大きく影響すると予想される項目を取り上げ，不確実性分析を行なった。検討する項目は，アムステルダム―ライン運河水の取水の影響，緩速砂ろ過における高水温期の除去率の影響，C/E値の影響，用量反応モデルの影響，ランク法とデイト法の比較である。

3. 結果と考察

3.1 分布形の当てはめ

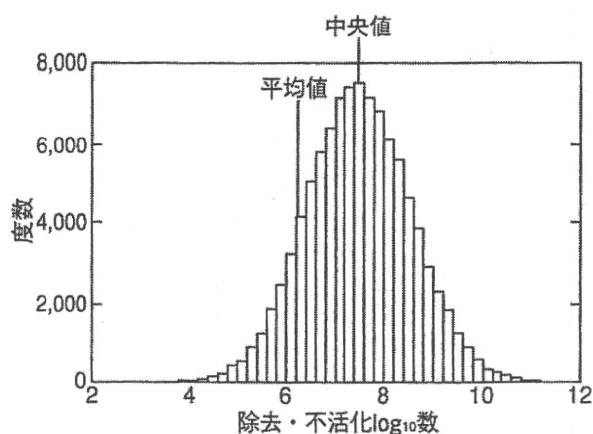原水のE. coli，凝集－貯水，急速砂ろ過，オゾン処理，緩速砂ろ過の各ステップでの除去・不活化能，およびC/E値について分布形を当てはめた。分布形の選定に当たっては，感染確率に大きな影

図1 総合除去・不活化能の分布

響を及ぼす低頻度ケースへの適合性を重視して，カイ2乗(χ^2)検定やコルモゴロフ・スミルノフ検定よりも，アンダーソン・ダーリング検定の結果を重視した。選定したPDFを表1に示した。

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[10]があり，この表流水の測定値をC/E値の評価に用いた。E. coliとCampylobacterの比率を求めた後，それに分布形を当てはめたところ，対数正規分布が最も適合した(表1に併記)。ただし，これは原水であるポルダー水そのもののC/E値ではない。C/E値は大きな不確実度をもつと考えられるので，不確実性分析で取り上げる。

3.2 総合除去・不活化能と年間感染確率

4段階の処理ステップの総合除去・不活化能の分布を，図1に示した。中央値7.46log₁₀，平均値6.22log₁₀が得られた。処理水中のE. coli濃度がほぼすべて0であるにもかかわらず，除去率を100%とは考えず，7.46log₁₀（中央値）の除去能を有すると評価している点が重要である。

表2　QMRAによる推算値

	P2.5	中央値	平均値	P97.5
総合除去・不活化 $\log_{10}$ 数	5.41	7.46	6.22	9.58
水道水中 E. coli（E. coli/100ml）	1.07×10^{-8}	4.35×10^{-6}	1.64×10^{-4}	9.25×10^{-4}
E. coli 摂取量（E. coli/d）	0	1.24×10^{-8}	2.99×10^{-4}	1.36×10^{-3}
Campylobacter 摂取量（Campylobacter/d）	0	1.35×10^{-10}	9.52×10^{-6}	3.36×10^{-5}
1日当たり感染確率（/人/d）	0	9.24×10^{-11}	6.51×10^{-6}	2.30×10^{-5}
年間感染確率（/人/year）	0	3.37×10^{-8}	1.68×10^{-3}	9.06×10^{-3}

　原水中 E. coli 濃度にこの除去率を適用するので，表2に示したように処理水中 E. coli 濃度は0にはならない。結果として，平均値は1.64×10^{-4} E. coli/100ml，中央値は4.35×10^{-6} E. coli/100ml と計算された。

　E. coli 摂取量は，水道水中 E. coli 濃度に飲水量を乗じて求められる。平均値は2.99×10^{-4} E. coli/d と求められた。C/E値を用いて E. coli 摂取量を Campylobacter 摂取量に換算した後，Campylobacter に関する用量反応関係を適用した。その結果，Campylobacter による1日当たりの感染確率は，平均値6.51×10^{-6}/人/d，中央値9.24×10^{-11}/人/d と推算された。

　1日当たりの感染確率から式(1)によって年間感染確率を計算した結果，平均値1.68×10^{-3}/人/year，中央値3.37×10^{-8}/人/year が得られた。以下，この評価結果をベースケースとよぶ。オランダでは，QMRAによって年間感染確率が1×10^{-4}/人/year以下であることが求められているが，平均値はこれより大きな値となった。

3.3　感度分析と飲水量の影響

　処理水中 E. coli 濃度に対する感度分析結果を，図2に示す。オゾン処理が最も大きく，ついで原水中 E. coli 濃度となっている。凝集－貯水，急速砂ろ過，オゾン処理，緩速砂ろ過の4段階の処理ステップのなかではオゾン処理の影響が最も大きい。これはすなわち，オゾン処理による E. coli 不活化率に差が大きく0 $\log_{10}$ から4 $\log_{10}$ まで分布しており，ここでいかに不活化されるかが処理水中 E. coli 濃度に大きく影響するのである。処理水中 E. coli 濃度を低下させるためには，オゾン処理の段階で E. coli を確実に不活化することが最も重要
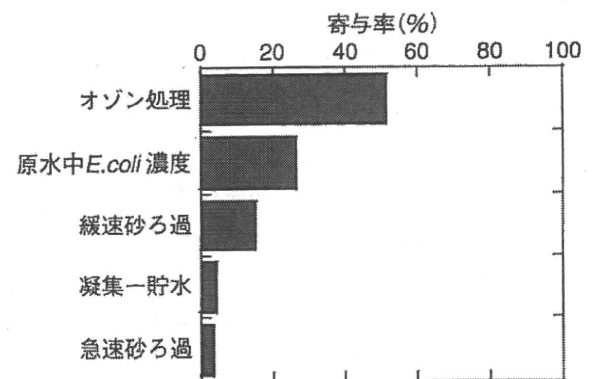
図2　処理水中 E. coli 濃度に対する感度分析結果
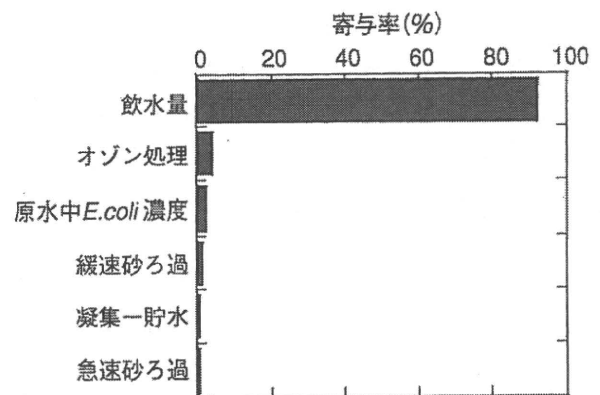
図3　E. coli 摂取量に対する感度分析結果

であると指摘できる。

　E. coli 摂取量に対する感度分析結果を，図3に示した。E. coli 摂取量に最も大きく影響するのは飲水量で，これに比べて他の項目は非常に小さい寄与と評価された。E. coli 摂取量は，処理水中 E. coli 濃度に飲水量を乗じて求めるだけだが，感度分析結果は図2と大きく異なる結果となった。なお，年間感染確率に対する感度分析結果は，図3

とほぼ同じ結果であった(C/E値の寄与はわずか)。

これは，飲水量のデータ集計の方法に理由がある。飲水量は連続分布ではなくポアソン分布という離散分布で与えられており，そのパラメーターは平均値0.706である。この結果，48%の人々がまったく水道水を飲まない統計となっている。表2で*E. coli*摂取量，*Campylobacter*による1日当たりの感染確率，年間感染確率の2.5パーセンタイル(P2.5)がいずれも0なのはこのためである。すなわち，48%の人々はまったく水道水を飲まないので，*E. coli*摂取量は0であり，感染確率も0である。この飲水量が0であるか否かが，*E. coli*摂取量および年間感染確率に最も大きく影響すると考えられる。

*E. coli*摂取量と年間感染確率に対する飲水量データの統計的取り扱い方法の影響について，さらに検討した。

飲水量データについては，2.5に記したように，統計的取り扱い方法が検討された結果，分布形としてはポアソンモデルを使うことが推奨された[18]。しかし，感度分析の結果，*E. coli*摂取量および年間感染確率に対して飲水量の寄与が最も大きいという意外な結果を得た。

そこで飲水量データに指数モデルを適用した。指数分布のパラメーターである比率は$6.05×10^{-3}$と推定された。年間感染確率は平均値$1.64×10^{-3}$/人/yearと計算され，ポアソンモデルを適用した場合(表2に示した$1.68×10^{-3}$/人/year)とほとんど変化はなかった。わずかに小さくなったのは，ポアソンモデルの場合，飲水量の平均値が177mlであるのに対して，指数モデルでは165mlであるためと考えられる。また，ポアソンモデルの場合では，48%の人は水道水を飲まないので彼らの感染確率も0であったが，指数モデルでは，*E. coli*摂取量および年間感染確率に0ではない下限値が存在する。この結果，年間感染確率の2.5パーセンタイルは$1.64×10^{-8}$/人/yearであった。年間感染確率に対する感度分析の結果を，図4に示す。図3とは大きく異なる結果であることが明らかで，最も寄与するのはオゾン処理であり，飲水量は第3位である。このように，飲水量データの統計的取り扱い方法が感度に大きく影響するので注意を要する。

さらに，飲水量データの影響について考察を行なった。一般に日常生活においては，シャワー・入浴，歯磨き時の誤飲などがあり，水道水の飲水量が完全に0とは考えにくい。ここでは歯磨き時の誤飲を想定する。誤飲量については，河川や海域での水泳，ダイビング，ゴルフ(下水処理水による灌漑後の芝の上のゴルフボールへの接触)などの活動に対して設定されてきた[21]。これらを参考に，歯磨き時の誤飲量として1mlおよび10mlを設定する。ポアソンモデルにおいて，これらの量を摂取水量として上積みした。つまり，飲水量が0グラス/dである人も1ml/dあるいは10ml/dを飲用しているとみなす。

年間感染確率は，誤飲量1mlの場合，平均値$1.67×10^{-3}$/人/yearとなり，ベースケースと比較してほとんど変化はなかった。しかし，*E. coli*摂取量および年間感染確率に下限値が存在するため，2.5パーセンタイルには0ではなく$9.21×10^{-10}$/人/yearという値が表われた。誤飲量10mlの場合では，平均値は$1.76×10^{-3}$/人/yearとなり，わずかに増大した。2.5パーセンタイルも$7.96×10^{-9}$/人/yearと増大した。年間感染確率に対する感度分析の結果では，誤飲量1mlの場合，最も大きく寄与するのは図3と同様に飲水量であるが，その寄与率は91.9%から50.5%に大きく低減した。誤飲量10mlの場合では，オゾン処理(寄与率32.0%)と飲水量(寄与率25.7%)の大小関係が逆転し，オゾン処理が最も影響する結果となった。や
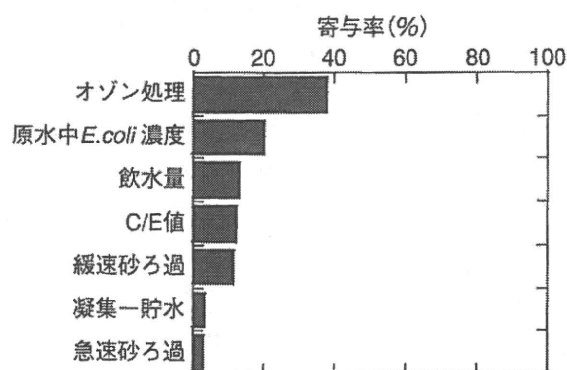

図4　年間感染確率に対する感度分析結果(飲水量データに指数モデルを適用した場合)

はり，ポアソンモデルにおいて飲水量0が出現することが大きく影響しているとわかる。

このように，飲水量データの統計的取り扱い方法が感度分析の結果に大きく影響することがわかった。感度分析は，重要管理点を抽出したり，データ収集を重点的に行なうべきポイントを指摘するのが目的である。飲水量のデータ集計の方法が混乱を招くおそれがあるので注意すべきである。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と考えられる。

3.4 不確実性分析

(1) アムステルダム－ライン運河水の取水

アムステルダム－ライン運河水の取水量は年間の全水量の5%であるが，ポルダー水よりも汚染されている。しかし，日々の取水量や微生物濃度の変動などに関する詳しいデータは不足している。そこで，ベースケースではポルダー水に含まれる*E. coli*を解析対象とし，アムステルダム－ライン運河水の取水は，これに影響を与える因子として不確実性分析の対象とした。

アムステルダム－ライン運河水を取水するのは，夏季の主として6月～8月の3カ月間である。したがって，この間ポルダーからの水にアムステルダム－ライン運河水が20%加わるものと考える。アムステルダム－ライン運河水の*E. coli*濃度は，ポルダー水の1.75倍に設定した[9]。

年間感染確率の計算結果を表3に示す。平均値は1.72×10^{-3}/人/yearとわずかに増大したが，大きな影響はみられなかった。

(2) 緩速砂ろ過における高水温期の除去率の影響

水温13℃以下での測定結果6例に対し，13℃以上での測定結果が3例ある[16]。これを用いて，緩速砂ろ過の年間の除去能が一定ではなく変化するものとして組み込むことも可能である。しかし，他の3つの処理ステップにおいて除去能に対する水温の影響は調べられていない。したがって，ここでは緩速砂ろ過における高水温期の除去能の影響については不確実性分析の対象とした。

3回の除去能測定値から，最小値$3.1\log_{10}$，平均値$3.6\log_{10}$，最大値$5.6\log_{10}$を求め，これらをパラメーターとする三角分布を設定した。水温13℃以下での除去能（平均値$2.4\log_{10}$）と比較して，高水温期のほうが除去能は向上することがわかる。水温13℃以上となるのは，5月～9月のおおむね5カ月間である。この期間の緩速砂ろ過の除去能は上記の値に従うものとした。

年間感染確率の計算結果を表3に示した。平均値は1.01×10^{-3}/人/yearとやや低減した。1年のうち5カ月間にわたって緩速砂ろ過の除去能を1$\log_{10}$以上高めた効果が表われているといえる。

(3) C/E値

ベースケースに用いたC/E値としては，マース川での測定値に対して平均値0.042，標準偏差0.10をもつ対数正規分布を設定した。これに対して，WHO飲料水水質ガイドライン第3版[22]には，科学文献をレビューし，各種の水源における腸管系病原微生物とその指標微生物の存在濃度の範囲が記載されている。このデータから，C/Eの最小値としては0.001（0.1%）を設定する。一方，平均滞留時間89日の貯水池においては水鳥等による再汚染も観察されている。オランダ西部のライダイン浄水場では，原水である河川水はまず前処理されるが，その後砂丘で浸透ろ過された後，さらに池に貯水される。この池でも，やはり再汚染があるという。この池の水の*E. coli*

表3 年間感染確率に対する不確実性分析結果

		年間感染確率(/人/year)		
		P2.5	平均値	P97.5
ベースケース		0	1.68×10^{-3}	9.06×10^{-3}
アムステルダム－ライン運河水の取水		0	1.72×10^{-3}	8.60×10^{-3}
緩速砂ろ過における高水温期の除去		0	1.01×10^{-3}	3.72×10^{-3}
C/E値	0.001(0.1%)	0	6.59×10^{-5}	3.54×10^{-4}
	1(100%)	0	2.53×10^{-2}	2.84×10^{-1}
用量反応モデル	最大感染確率曲線	0	2.30×10^{-3}	1.27×10^{-2}
	ベータ-ポアソン	0	4.24×10^{-3}	2.77×10^{-2}
デイト法		0	3.18×10^{-2}	4.72×10^{-1}

と*Campylobacter*の濃度の調査結果によれば，*Campylobacter*濃度の平均値は*E. coli*濃度の平均値の約50%であった[23]。これを参考にして，ここではC/Eの最大値として1（100%）を設定する。

年間感染確率の計算結果を，表3に示した。0.001に設定した場合では，平均値は6.59×10^{-5}/人/yearときわめて低い値となった。一方，1とした場合では，平均値は2.53×10^{-2}/人/yearと増大した。C/Eの値が感染確率の評価値に大きく影響することがわかる。ベースケースに用いたC/E値はマース川で測定された値であり，ポルダー水やこれにアムステルダム－ライン運河水が加わった水の値ではない。本浄水場の原水でのC/E値，または*Campylobacter*濃度そのものの調査を進める必要性が高いと指摘できる。

（4）　用量反応モデルの影響

指数モデルの代わりに最大感染確率曲線を適用した。これは，摂取されたどんな微生物も感染を起こし得ると想定した場合の感染確率を表わす。年間感染確率の計算結果を，表3に示した。平均値は2.30×10^{-3}/人/yearとやや増大した。最大感染確率曲線の場合，感染確率P_d（/人/d）は$P_d = 1 - \exp(-D)$と記述される。これは，Dが小さいとき（低用量域）では$P_d \approx D$と近似できる。これに対して，指数モデルは低用量域では$P_d = 1 - \exp(-\gamma D) \approx \gamma D = 0.686 \times D$と近似される。ベースケースの平均値$1.68 \times 10^{-3}$/人/yearと最大感染確率曲線での平均値$2.30 \times 10^{-3}$/人/yearとは，ほぼこの比率になっていることが確認できる。

ベータ－ポアソンモデル[19]を使用することは適切でない，と記した。ここでは，試しにベータ－ポアソンモデルを使用して感染確率を計算してみた。平均値は4.24×10^{-3}/人/yearとなり，最大感染確率曲線での平均値2.30×10^{-3}/人/yearよりも大きな値となった。やはり，ここでベータ－ポアソンモデルを使用するのは適切ではないことが確認される。

（5）　ランク法とデイト法の比較

処理前後の微生物濃度データをペアリングする方法としては，デイト法やランダム法よりもランク法を使用するのが適切であると述べた。ここでは参考のため，凝集－貯水，急速砂ろ過，オゾン処理の3段階の除去・不活化能を，デイト法を用いて評価した。不確実性分析の項目として取り上げたのは，データのペアリング方法を吟味しない場合，通常，デイト法を採用するからである。

まず，総合除去・不活化能の中央値は$6.18\log_{10}$とランク法の中央値$7.46\log_{10}$より$1.28\log_{10}$小さい値となった。また，平均値は$2.16\log_{10}$であり，ランク法の平均値$6.22\log_{10}$よりも非常に小さな値であった。これは，デイト法では，しばしば低除去率がみられるためで，負の除去率が出現することさえある。これは通常あり得ない。年間感染確率の計算結果を，表3に示した。平均値3.18×10^{-2}/人/yearとランク法（ベースケース）と比較して19倍も大きな確率となった。QMRAを行なう前に，データの適切なペアリング方法を選択する必要があるといえる。

3.5　感度分析と不確実性分析結果に関する総合的考察

飲水量のデータ集計の方法やあてはめ分布の種類は，感染確率の大きさにはあまり影響しないが感度分析の結果には大きく影響する。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。

本浄水場では，年間感染確率の大きさに最も寄与するのはオゾン処理である。この処理施設では年間感染確率の平均値が1.68×10^{-3}/人/yearとなり，10^{-4}/人/yearより大きいと評価された。この感染確率を低下させるためには，オゾン処理で微生物を確実に不活化することが最も有効と指摘できる。滞留水等をなくし，オゾン槽全体でCt値（溶存オゾン濃度×接触時間）を保持することなどが有効な手段であろう。

緩速砂ろ過において高水温期の除去能が高まるが，このデータを組み込んだ場合，年間感染確率はやや低減した。水温が除去・不活化能に大きく影響すると予想される処理ステップでは，除去・不活化能に対する水温の影響を調べることが望ましい。

C/E値が感染確率の評価値に大きく影響するこ