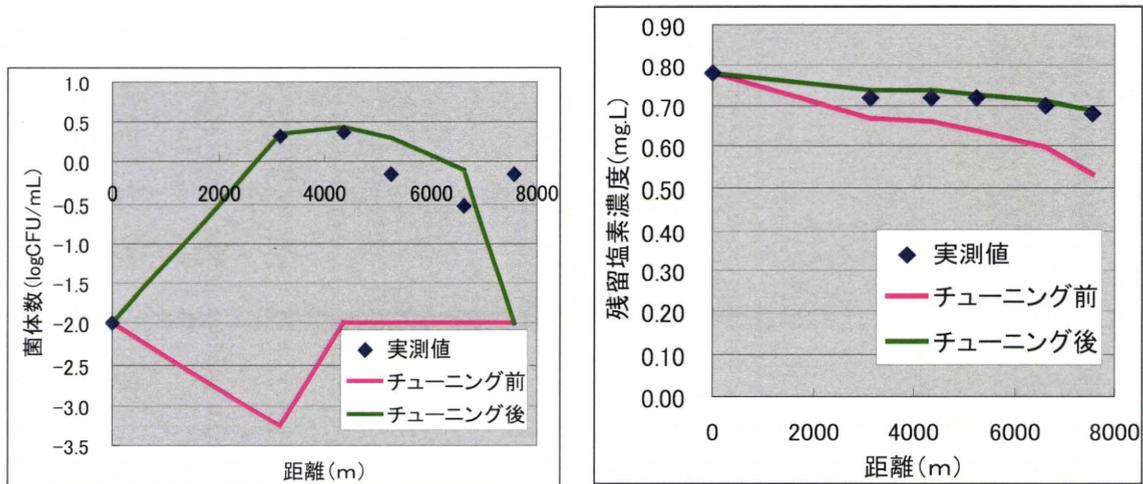


い、適切なパラメータ値の設定とシミュレーションモデルの妥当性の検討を行い、実際の配水管網についても妥当な計算を行えることを示した。



(1)従属栄養細菌

(2)残留塩素濃度

図7 実測値と計算結果の比較

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F健康危険情報

該当なし

G研究発表

(1) 論文発表

該当なし

(2) 学会発表

該当なし

H知的財産権の出願・登録状況（予定を含む）

該当なし

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

Full Length Research Paper

Detection of active *Escherichia coli* after irradiation by pulsed UV light using a Q β phage

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Accepted 19 May, 2010

The infectivity rates of Q β phage is used as a bio-indicator of the physiological state of host cells and for the detection of active *Escherichia coli* irradiated by an increasing number of pulsed ultra-violet (UV) light. Indeed, the combination of a conventional method used to measure colony-forming ability of tested bacteria after exposure to an increase number of pulsed UV light, and the simulation of phage adsorption kinetic, can reveal the existence of active bacteria which lose the cultivability in usual culture media, but keep viability and phage susceptibility.

Keys words: Ultra-violet inactivation, viable but non-culturable bacteria, Q β phage, active bacteria.

INTRODUCTION

Disinfection, as applied in water and wastewater treatment, is a process by which pathogenic micro-organisms are inactivated to provide public health protection. Chlorination has been used for most water disinfection operation for many years. However, it is no longer the disinfection method automatically chosen for either water or wastewater treatment because of potential problems with disinfection by products and associated toxicity in treated water. Among the alternatives to conventional chlorination, ultra-violet (UV) irradiation is chosen the most frequently (Bolton et al., 2003). The effectiveness of UV light in biological inactivation arises primarily from the fact that DNA molecules absorb UV photons between 200 and 300 nm, with peak absorption at 260 nm (Byrd et al., 1990). This absorption creates damage in the DNA by altering nucleotide base pairing, thereby creating new linkages between adjacent nucleotides on the same DNA strand. If the damage goes unrepaired, the accumulation of DNA photoproducts can be lethal to cells through the blockage of DNA replication

and RNA transcription, which ultimately result in reproductive cell death (Zimmer and Slawson, 2002). However, UV disinfection is noted to have some problems, one of them is reactivation. Most bacteria repair these lesions in two ways; light dependent photoreactivation catalyzed by an enzyme name photolyase and light-independent restore mechanisms such as nucleotide excision repair (Lilved and Landfalde, 1996). The goal is the production of save water, thereby, after disinfection of water, health protection programs require to estimate the level of contamination of treated water (Byrd et al., 1990).

The methodologies used to evaluate the performance of disinfection system, is based on the determination of colony-forming ability of indicator bacteria after treatment. However, the information given from the simple viable and cultivable count of microorganisms is incomplete. Indeed, the investigations of bacterial survival in natural environments have indicated that some of them lose notion of cultivability on appropriate growth media under certain conditions and yet still exhibit signs of metabolic activity and thus viability (Armisen and Servais, 2004).

For years, total coliforms and fecal coliform were the most widely used indicators for water quality but, more

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recently, the abundance of *Escherichia coli* has been shown to be more related to the sanitary risk than that of coliforms (Armisen and Servais, 2004). Accurate enumeration of *E. coli* is thus important to assess microbiological water quality. Classical methods for enumerating *E. coli* are based on culture in liquid (most probable number, MPN) or solid (plate counts) media (Byrd et al., 1990). These methods are not allowed to detect all the target bacteria in natural environments. Indeed, when released in natural waters, fecal bacteria were shown to lose their ability to growth on culture media while preserving their viability.

The presence of these viable but non-culturable (VBNC) bacteria in the environment could be important from a sanitary point of view as some authors (Colwell et al., 1996; Pommepuy et al., 1996) suggested that pathogenic VBNC bacteria could maintain their virulence being thus a potential reservoir of disease. The public health risk is thus not an only function of the abundance of the microorganism's contaminants in water, but also of their capacity to survive in the receiving environments and to maintain their virulence (Chedad and Assobhei, 2007).

As a consequence, alternative methods were developed during the last 10 years to detect and enumerate *E. coli* in waters. Those are direct enzymatic methods, immunological methods, quantitative polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) (Armisen and Servais, 2004).

The purpose of this study is to determine the relationship between indicator of fecal indicator bacteria (*E. coli*) and phage (Q β phage) in order to detect the presence of active bacteria undetected by culture in usual media.

MATERIALS AND METHODS

Bacterial strain and bacteriophage

The RNA F-specific coliphage, Q β phage and its cell host, *E. coli*, were obtained from the American type culture collection (23631-B1 and 13965, respectively).

Q β phage was commonly recommended for modelling viral behaviour in water (Espinosa et al., 2009) and also was used in biological actinometry (Biodosimetry) (Fallon et al., 2007). In this study, we used Q β phage as an indicator of active VBNC bacteria after UV irradiation.

Pulsed UV radiation

The pulsed UV system is developed by the combination with power and flash UV lamp technology. Pulsed UV light was differed from the traditional continuous UV light by much higher irradiance of UV illumination and reduction of exposure time. Indeed a flash lamps commonly use in operating with pulse lengths ranging from a few tens of milliseconds to over milliseconds.

UV irradiation for polychromatic UV source (UV pulse lamp) was measured using a Potassium iodide/iodate actinometry (KI/KIO₃) according to Rahn et al. (2003). For this study, UV dose determined by chemical actinometry was equal to 5.72 mJ/cm² per UV-pulse. Noting that, in order to reduce the photo-thermal effect of pulsed

UV light due to visible light and infra-red, the pulsed UV system was equipped by a ventilator.

UV-irradiated bacteria

For dose/response relationship and reactivation experiments, the strain of *E. coli* was cultured in Luria-Bertani broth (LB). Bacterial suspension was diluted in saline Phosphate buffers (PBS) in order to obtain a concentration ranged from 1 x 10⁵ to 1 x 10⁶ bacteria per ml. Then, the bacterial suspensions were used for irradiation experiments. A volume of 20 ml of the prepared suspensions was transferred into a standard Petri dish for the eventual exposure to an increasing number of pulsed UV-light (0, 8, 12, 18, 25 and 30 UV pulses).

Viable cell counts

Viable cell counts were taken before and immediately after UV exposure. A 100 μ l portion of each irradiated samples was removed in order to prepare serial dilutions in PBS buffer. A volume equal to 100 μ l of the appropriate serial dilutions was spread in duplicate onto LB agar. The number of colony-forming unit (CFU/ml) or a number of viable and cultivable bacteria was determined after 24 h of incubation at 37°C. The fraction of viable and cultivable bacteria was calculated by dividing the number of CFU in the UV-treated sample (*N*) by the number of CFU determined at time zero before UV irradiation (*N*₀).

Q β phage replication experiments

To investigate the presence of active bacteria in the UV-irradiated sample, Q β phage adsorption experiments were performed with a modification of the procedure of Woody and Cliver (1995). UV-irradiated suspensions of host cells were infected with Q β phage. After infection and at the indicated time intervals (each 5 min after infection), 100 μ l was removed from different suspensions and after a cumulative dilution, a volume of 100 μ l of each dilution was periodically assayed to determine the phage adsorption's kinetic to the host cell. The phage titration was determined using the double-layer agar plate method. Q β phage replication experiments were determined at time zero, before UV irradiation; using initial host cells and phage densities to know the optimal timing of phage adsorption.

In this work, we have repeated all experiments more than 3 times and we have choice to work with average values. This choice was based on the use of different mathematical models to simulate the Q β phage's replication and to determine different kinetic parameters related to the phage adsorption to the host cells under different UV irradiation conditions.

Simulating Q β phage's growth

To simulate Q β phage adsorption to the host cells, using the model employed by Levin et al. (1977) and reported by Abedon (2001). The modelling of Q β phage adsorption was applied to investigate the impact of host cell after irradiation by an increasing number of pulsed lights, in the phage adsorption kinetic. The adsorption constant (*k*) and the density of uninfected host cells (*N*) were determined and compared to control test determined at time zero using initial cell and phage densities. In addition, the adsorption time (*t*_A) was determined according to Wang et al. (1996).

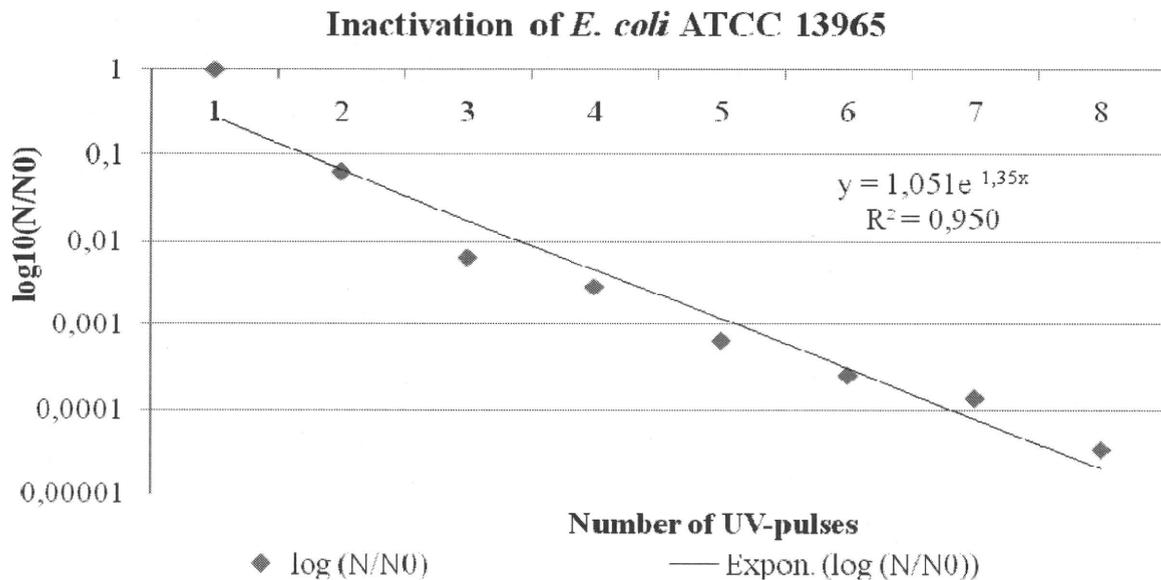


Figure 1. The Kinetic of *E. coli* ATCC13965 inactivation following exposure to UV-C irradiation according to the model of Chick-Watson, y : Reduction = N/N_0 with N_0 : Number of viable and cultivable cell before exposure to UV light; N : Number of viable and cultivable cell after exposure to UV-C irradiation, $x = I^*t$ with I : UV intensity (mW/cm^2), t : exposition time (s), n : Threshold level of series-event model, $n = 1$ for the first order Chick-Watson model, where error bar are not shown, differences between duplicates were not detected.

Titration of Q β phage

The irradiated host cell respectively by 0, 8, 12, 18, 25 and 30 UV pulses infected Q β phage were incubated at 37°C. After 18 h, all cell-phage suspensions were filter sterilized using a 0.45 μm syringe filter to obtain free phage in the filtrate. After that, the supernatants were diluted in order to determine the titration of phage. The phage titre was determined by using a standard double-layer agar plate. After appropriate dilution with PBS buffer, a volume equal to 100 μl of free phage was added to a 100 μl of active growing culture of *E. coli* (10^6 CFU/ml) and the mix were added to 3 ml of soft agar (maintained at 48°C). The mixture was overlaid on TSA agar plates and incubated 18 h at 37°C to enumerate plaques.

The phage titration experiment was determined at time zero using initial unirradiated cells and phage densities. All phage adsorption experiments were done more than three times, to verify the sensibility and the reproducibility of this method.

RESULTS AND DISCUSSION

The inactivation kinetic of *E. coli* ATCC13965: Pulsed UV dose/response

The inactivation kinetic of *E. coli* ATCC 13965 was function of UV-C dose. The germicidal dose was expressed as the product of UV radiation intensity (I) and number of pulsed UV light (T) (Figure 1). In order to study the behavior or the response of tested bacteria to an increasing UV dose (Dose/response), we used the mathematical model of Chick-Watson according to Hassen et al. (2000).

$$N/N_0 = A \exp(-kI^n T)$$

Where, N_0 is Number of viable cultivable bacteria before exposure to UV light; N is Number of viable cultivable bacteria after exposure to pulsed UV light, A is constant corresponding to bacteria retaining viability following UV irradiation, k is Coefficient of lethality, I is the UV-C intensity expressed in mW/cm^2 , T is number of UV pulse and n is threshold level of series-event model, $n = 1$ for the first order Chick-Watson model. The constants k and A were determined by linear regression.

The inactivation kinetic (dose/response) according to the model of Chick-Watson shows that the irradiation of *E. coli* by 8 UV pulses is sufficient to inactivation 99.99% of colony-forming ability thus, after exposure to a UV dose equal to 45.76 mJ/cm^2 . This UV dose is nearest of the UV fluency used usually in Europe and USA for the disinfection of drinking water. Indeed, according to the literature, 40 mJ/cm^2 is enough to inactivate 4 U- \log_{10} of pathogenic bacteria as *Legionella*, enteric viruses, *Cryptosporidium* oocysts and *Giardia* cysts (US-EPA, 2003).

Exploitation of VBNC bacteria after UV irradiation

According to the inactivation kinetic, *E. coli* loss the colony forming ability after irradiation by 12 pulses of UV light (non detected CFU/ml; ND). But it is well known that the loss of bacterial cultivability is not synonyms of

bacterial death. The question is how we can detect the presence of active but non cultivable bacteria in irradiated samples?

Detection of active post-UV-irradiated *E. coli* ATCC 13965: Study of Q β phage replication in presence of VBNC host cell

Our propose is to detect the presence of VBNC *E. coli* after UV irradiation and then measured its production of Q β phage during a single cycle of infection.

The lytic Q β phage life cycle involves free-phage diffusion, host cell adsorption, an eclipse period, a period of progeny maturation and host cell lysis (Abedon et al., 2003). Lysis ends the phage latent period but initiates the extracellular diffusion of phage progeny to new host cells. The aim of this study was to examine the potential replication of Q β phage in relation with UV irradiated *E. coli* (host cell) not detected in usual growth media. Based on UV-inactivation's kinetic curve of *E. coli*, we are exposed the tested bacteria to 8, 12 and 18 UV pulses.

These doses allowed respectively the inactivation of 99.99% of bacteria; the loss of cultivability of *E. coli* with and without subsequent reactivation. Moreover, exposure of the host bacteria to a higher number of UV pulse (25 and 30 UV pulses) in order to verify the sensibility of lytic phage to detect active bacteria persist in the irradiated suspensions. This series of experiments were conducted to determine in part, the detection of active bacteria present in the UV irradiated suspensions and on the another hand, to reveal the capability of VBNC bacteria to support phage replication.

Study of phage adsorption kinetic

Data exposed in Figure 2, do not show an inhibitory effects on the first step of phage replication in relationship with an irradiated bacteria. This result demonstrates the presence of enough bacteria in good physiological condition despite, the UV irradiation conditions and the decrease of viable and cultivable bacteria density. The modeling of phage adsorption was determined according the model employed by Levin et al (1977):

$$P(t) = P_0 e^{-k'N't} = P_0 \cdot (1 - k' \cdot N' \cdot t)$$

Where; P_0 is the initiated free-phage concentration at time Zero, (t_0); $P(t)$ is the free-phage concentration at time (t); k' is the phage adsorption constant, and N' is the density of uninfected host cells.

The adsorption constant K' , determined by the analysis of the adsorption curves (Figure 2), showed that the level of phage adsorption to host cells decrease with the increase number of pulsed UV light. The changes in the phage growth parameters (adsorption phage, elongation of latent period) were probably due of the changes of host

quality (stressed and damaged bacteria by UV light in poorer growth environment, PBS buffer).

For instance, after inactivation of 99.99% of host bacteria, the constants of Q β phage replication's kinetic, do not affect compared with the control test (phage in the presence of unirradiated host cell). Although, the phage adsorption rate, determined after irradiation of host cell by 25 and 30 UV pulses is lower than 6.2 to 8 fold respectively compared to the adsorption constant determined at time zero, before UV irradiation. In this case, the decrease of phage adsorption rate can be explained by the fact that, the exposure of host bacteria to a sub lethal dose can conduct to a modification of a physiological and metabolic state of irradiated bacteria cell caused by the bactericidal effect of UV light (Makarova et al., 2000).

Furthermore, it is well known that the phage adsorption rates are proportional to host cell surface area (Woody and Cliver, 1996); thereby, after UV irradiation, bacteria can change its morphological shape as a strategy of bio-protection against stress and to survive in a hostile environment (Langer and Hengge-Aronis, 1991). In our case, to escape or to minimize the germicidal effect of UV irradiation, bacteria has changed its shape consequently; this change can have an effect on the phage adsorption by stopping or prolonging the timing of phage adsorption to the host cell.

Determination of uninfected bacteria

After fixing the timing of optimal phage adsorption at 45 min based on the interaction of initial cell density and Q β phage (at time zero before UV radiation), it was possible to achieve reasonably simulation of the number of uninfected host cells after each irradiated by an increase UV dose samples according to the model of Levin et al (1977). Time adsorption (t_A) was defined as the mean free time (MFT) according to Wang et al (1996): $t_A = (k'N')^{-1}$

The MFT represents the average length of time a cohort of free phages requires to adsorb to host cells expressed by min/host cell (Table 1). Noted that, 15 min was chosen to determine the time of the initial uninfected host cells density and this host cells density was fixed as an initial density of viable but non cultivable bacteria (VBNC) infected by a constant amount of phage.

The uninfected cell density can reflect directly the level of inactivated bacteria by a germicidal effect of UV light compared with time zero before UV irradiation (maximum level of phage infectivity). In fact, when we increase the UV exposure dose, the density of uninfected bacteria increases.

The results exposed in Table 1 shown, that the time required for the Q β phage to be attached in the host cell increase, with the increase of UV dose. This result, support the previous results concerning the decrease of adsorption kinetic constant (k') and the increase of

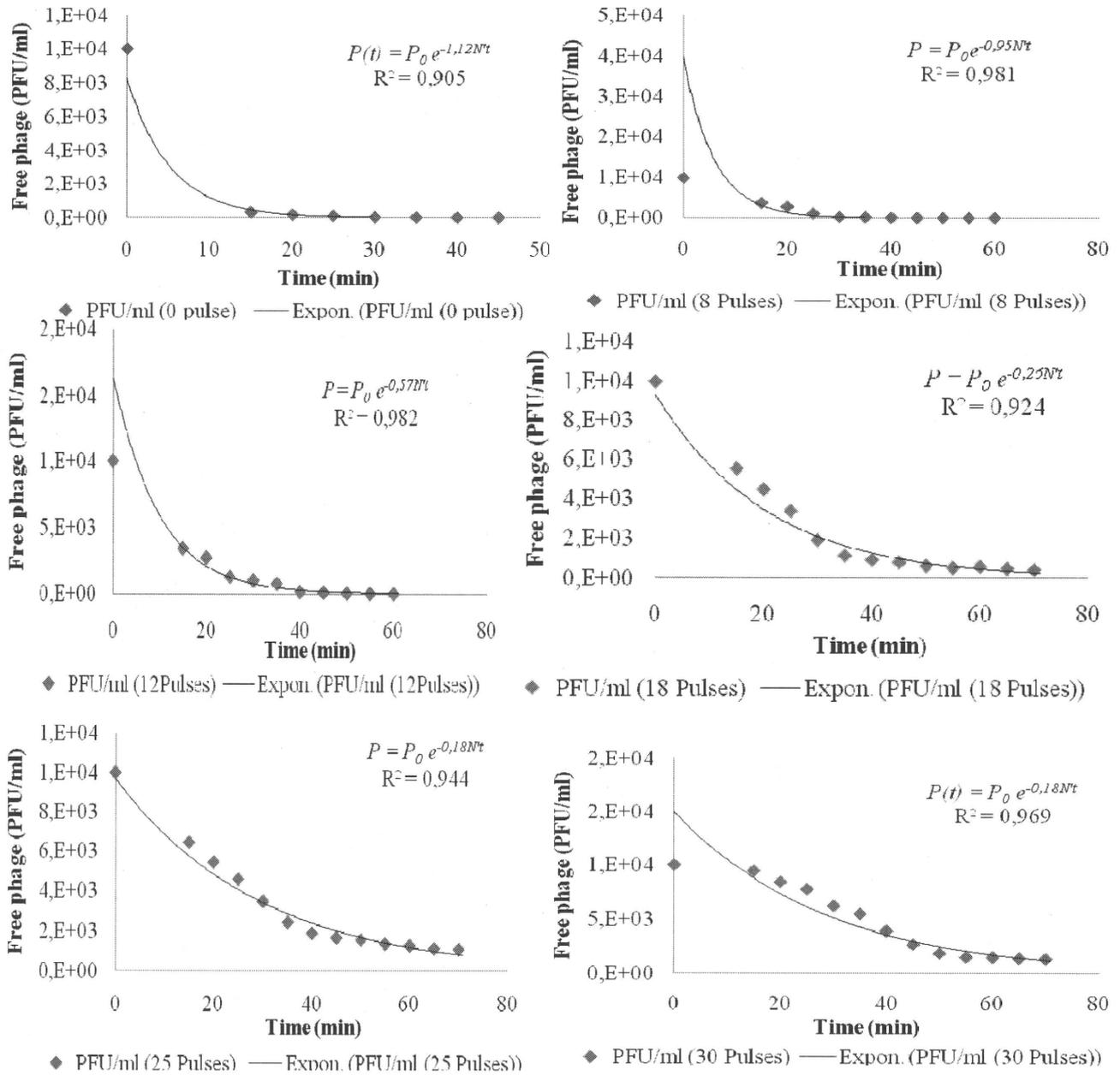


Figure 2. Qβ phage adsorption kinetics dependence on host UV irradiation state (UV irradiation by 0, 8, 12, 18, 25 and 30 UV pulses), P_0 : the initiated free-phage concentration at time zero (t_0), $P(t)$: the free-phage concentration at time (t), K : the phage adsorption constant and N : the density of uninfected host cells.

Table 1. Determination of different parameters related to Qβ phage growth kinetic

	Number of UV Pulses					
	0	8	12	18	25	30
Qβ Phage adsorption constant (K)	1.12	0.95	0.57	0.26	0.18	0.14
Density of uninfected host cells (N)	0.019	0.02	0.04	0.09	0.12	0.13
Adsorption time (t_A)	0.04	0.07	0.20	0.98	2.04	2.57

Legend: K : Phage adsorption constant (ml/min); N : Number of uninfected cells at time 15 min (cell/ml); t_A : Adsorption time (min/host cell).

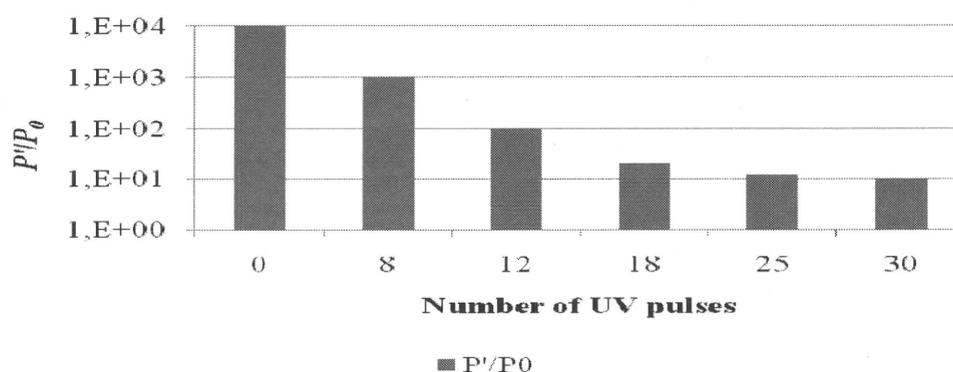


Figure 3. Q β phage titers dependence on host UV irradiation state after 18 h of incubation at 37°C, P_0 : the Q β phage titer after incubation in the presence of unirradiated bacteria, P' : the Q β phage titers after 18 h of incubation at 37°C in the presence of irradiated *E. coli* by 8, 12, 18, 25 and 30 pulsed UV light .

uninfected hosts cells. In fact, there changes in the phage growth parameters were caused by the change in host quality and therefore by the pressure of selection of good quality of host cells by phage (Wang et al., 1996). Conclusively UV-irradiation of host cells have a negatively affect phage growth, by lengthening the phage latent period, or reducing the phage adsorption constants.

The titration of Q β phage, after 18 h of incubation at 37°C in the present of susceptible host cells after UV irradiation by increasing number of pulsed UV light; showed an increase of phage density. Compared to the control test (infection of non irradiated host cell by an initial concentration of Q β phage; P_0), different level of progeny phage was release in relation with UV *E. coli* irradiation conditions.

The amount of phages released by irradiated host cell by 8 pulsed UV light is higher than the amount of phages released by *E. coli* irradiated respectively by 12, 18, 25 and 30 pulsed UV light (Figure 3). The decrease in the level of phage infectivity and release of infectious Q β progeny phage is directly related by the decrease of active bacteria's density in relation with the increase of exposure of UV dose. Figure 3 shown, that the infection of irradiated *E. coli* by Q β phage allowed the detection of active cells. In fact, despite their loss of bacteria's cultivability in usual growth media and the affection of phage replication cycle (by extension of latent period, decrease in the adsorption constant, etc.); portion of VBNC bacteria or in the correct term, active bacteria can let a replication of phage manifested by the enhance of the phage' titers after 18 h of incubation at 37°C.

The increase in bacteriophage titer and the maintenance of infectivity of VBNC bacteria or active but non cultivable bacteria can be related by the sigma factor RpoS. In fact, RpoS was shown to have a key role in survival of bacterial cells exposed to starvation, freezing, desiccation and UV irradiation, in addition to its

established role in oxidative and osmotic stress response (Saint-Ruf et al., 2004; Hengge-Aronis., 2002).

The activation of *rpoS*-encoded σ^S subunit of RNA polymerase in *E. coli* is a global regulatory factor involved in several stress responses. HF-I protein is a component that, is essential for *rpoS* translation. This factor is encoded by the *hfq* gene (Muffler et al., 1996). This gene, *hfq*-encoded RNA-binding protein HF-I, which has been known previously only as a host factor for the replication of phage Q β RNA; as an essential factor for *rpoS* translation (Muffler et al., 1996).

HF-I constitute a part of the Q β replicase and was required for the synthesis of the minus strand from the original viral RNA (Barrera et al., 1993). Based on this information, hypothesis was establish as follows: the enhancement of Q β phage titer was related probably by the induction of *hfq* gene and consequently, the increase of the intracellular concentration in HF-I factor in relation with the induction of alternative sigma factor (RspoS) involved in bacteria stress response.

In the end of this study, conclusion have be made that different phage growth constants defining phage-host cell interaction (extension of latent period, change in the timing of phage adsorption to the host cell, etc.) were directly related by the host cell quality (damaged cells by UV light, accumulation of photoproduct, enter in a VBNC state, etc).

The infectivity rates of Q β phage is used as a bio-captor or bio-indicator of the physiological state of irradiated host cell mainly, the active but non cultivable bacteria. Thus, we can use this propriety to evaluate water after disinfection step by physical or chemical process and also to control food safety.

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Abundance and diversity of ammonia-oxidizing archaea and bacteria on biological activated carbon in a pilot-scale drinking water treatment plant with different treatment processes

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ABSTRACT

The effects of different placements of rapid sand filtration on nitrification performance of BAC treatment in a pilot-scale plant were evaluated. In this plant, rapid sand filtration was placed after ozonation-BAC treatment in Process (A), while it preceded ozonation-BAC treatment in Process (B). Analysis of *amoA* genes of ammonia-oxidizing archaea (AOA) and bacteria (AOB) combined with nitrification potential test was conducted. BAC from Process (A) demonstrated slightly higher nitrification potential at every sampling occasion. This might be due to higher abundances of AOB on BAC from Process (A) than those on BAC from Process (B). However, AOA rather than AOB could be predominant ammonia-oxidizers in BAC treatment regardless of the position of rapid sand filtration. The highest nitrification potential was observed for BAC from both processes in February when the highest abundances of AOA-*amoA* and AOB-*amoA* genes were detected. Since rapid sand filtration was placed after BAC treatment in Process (A), residual aluminum concentration in BAC influent was higher in Process (A). However, adverse effects of aluminum on nitrification activity were not observed. 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.

Key words | ammonia-oxidizing archaea, ammonia-oxidizing bacteria, biological activated carbon

INTRODUCTION

Ozonation followed by biological activated carbon (BAC) is one of the advanced drinking water treatment technologies. Some organic matters are degraded by ozone oxidation and then adsorbed by granular activated carbon. In addition to the physicochemical removal, microorganisms associated with granular activated carbon are known to be involved in the treatment. One of the biological contributions is removal of ammonium-nitrogen through nitrification (Andersson *et al.* 2001; Simpson 2008; Wert *et al.* 2008). When ammonium-nitrogen in raw water reacts with chlorine, trichloramine which causes smell of chlorine could be produced as a by-product. Since consumers are

doi: 10.2166/wst.2010.204

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sensitive to smell or odor of drinking water, waterworks are required to suppress production of trichloramine. Biological nitrification in BAC treatment can efficiently treat ammonium-nitrogen which resulted in the reduction of the precursor of trichloramine. Though trichloramine is not included in the Japanese water quality standard for drinking water, Tokyo metropolitan government has established the original drinking water standard in which trichloramine concentration should be controlled at 0 mg/L. To achieve this standard, ozonation-BAC treatment will be introduced to almost all major drinking water treatment plants in Tokyo by 2013.

Though some studies investigated bacterial community associated with BAC by using culture-dependent (Burlingame *et al.* 1986) and culture-independent techniques (Kasuga *et al.* 2007, 2009), the mechanisms of biological treatment including diversity, abundance and activity of attached microorganisms remain unknown. Little information is available especially for nitrifying microorganisms on BAC. So far, ammonia-oxidizing bacteria (AOB) have been regarded as only players for oxidation of ammonium-nitrogen. However, the recent discovery of ammonia-oxidizing archaea (AOA) capable of oxidizing ammonium-nitrogen has dramatically changed our understanding (Könneke *et al.* 2005; Treusch *et al.* 2005; Nicol & Schleper 2006). AOA have been found in various environments including ocean, soil, and water treatment processes (Francis *et al.* 2005; Leininger *et al.* 2006; Park *et al.* 2006; Wuchter *et al.* 2006; de Vet *et al.* 2009). Contribution of AOA to nitrogen cycle needs to be considered, though ecology and physiology of AOA have not been elucidated yet. Thus, microbiological diagnosis of AOA as well as AOB is required to evaluate nitrification performance of BAC treatment.

In this study, nitrification performance of BAC treatment in a pilot-scale plant was investigated by analyzing abundance and diversity of AOA and AOB associated with BAC. This pilot-scale plant compared two treatment processes in which rapid sand filtration was placed before or after ozonation-BAC treatment. In Japan, both treatment processes have been put in practical use in advanced drinking water treatment plants. However, effects of different placements of rapid sand filtration on treatment performance especially for biological nitrification have not been investigated. Here, we discussed vertical and seasonal variations of nitrification performance of BAC treatments in both processes. In addition, since rapid sand filtration preceding BAC treatment can efficiently remove residual polyaluminum chloride used for coagulation, effects of aluminum on nitrification activity were evaluated.

MATERIALS AND METHODS

Pilot-scale plant for advanced drinking water treatment

A pilot-scale plant capable of treating 200 m³/day with two different processes is shown in Figure 1. After river water

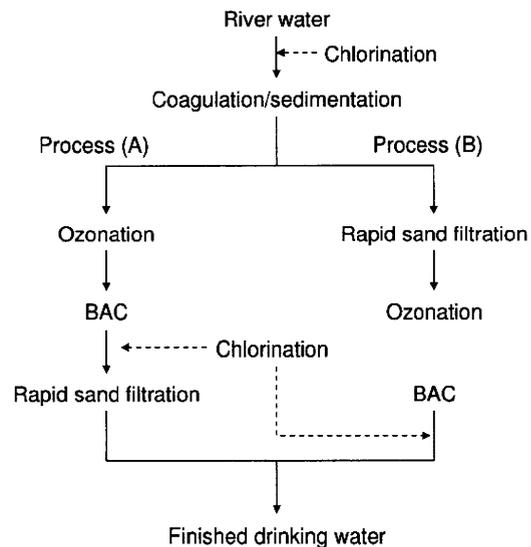


Figure 1 | Treatment processes in a pilot-scale plant.

was treated by coagulation and sedimentation, two treatment processes (A) and (B) were compared. In Process (A), rapid sand filtration was placed after ozonation-BAC treatment. On the other hand, rapid sand filtration was followed by ozonation-BAC treatment in Process (B). Operational conditions of ozonation-BAC treatment were summarized in Table 1.

Sampling

BAC samples were collected from the surface layer of BAC beds in Process (A) (BAC-A) and Process (B) (BAC-B) on 21 September and 26 November of 2007 and 21 February of 2008. In addition to the surface layer samples, middle layer (1 m from the surface) and bottom layer (2 m from the surface) samples from both processes were also obtained on 21 September of 2007.

Nitrification potential test

Nitrification potential of BAC samples was evaluated by batch experiment. In an Erlenmeyer flask, 100 g-wet of BAC sample was mixed with 200 mL of inorganic media containing 5 mg NH₄-N/L as the sole nitrogen source. It was incubated at 20°C with constant agitation. After 0, 8, 16, 24 and 48 hours, ammonium-nitrogen was determined spectrophotometrically using the indophenol-blue method.

Table 1 | Operational conditions of ozonation and BAC treatments

	Operational conditions	Unit	Process (A)	Process (B)
Ozonation	Nominal capacity	m ³ /day	66	45
	Dissolved ozone concentration	mg/L	0.1	0.1
	Contact time	min	5	10
	Retention time	min	5	5
BAC	Nominal capacity	m ³ /day	55	34
	Bed volume	m ³	0.46	0.28
	Bed depth	m	2	2
	Flow regime	–	Down flow	Down flow
	Empty bed contact time	min	12	12
	Linier velocity	m/day	240	240
	Effective diameter of AC	mm	1.2	0.7
	Backwashing interval	hr	72	72
Rapid sand filtration	Grain size: anthracite	mm	1.3	1.3
	Grain size: sand	mm	0.6	0.6
	Line velocity	m/day	120	120
	Backwashing interval	hr	168	72

Nitrite-nitrogen and nitrate-nitrogen were measured with an ion chromatograph (761 Compact IC, Metrohm).

Effects of aluminum on nitrification

The BAC sample collected from the surface layer of the BAC bed in Process (B) on 21 September of 2007 was mixed with the media containing 5 mg NH₄-N/L used for nitrification potential test. Additionally, polyaluminum chloride was added at the final concentrations of 0.02, 0.2 and 2.0 mg Al/L, respectively. The control without addition of aluminum (0 mg Al/L) was also prepared. After incubating them at 20°C with constant agitation for one month, all media were replaced with newly prepared ones without aluminum. Thereafter, nitrification potential was evaluated as described above. The first-order reaction model expressed in the Equation (1) was applied to estimate the rate constants by the least-square regression.

$$C = C_0 e^{-kt} \quad (1)$$

where C = concentration of ammonium-nitrogen (mg N/L), C_0 = Initial concentration of ammonium-nitrogen (mg N/L), k = the first-order rate constant (1/hr), and t = time (hr)

DNA extraction and PCR amplification

DNA was extracted from BAC samples by using a Power-Soil™ DNA kit (Mo Bio Laboratories). Arch-amoAF and Arch-amoAR primers were used for amplification of AOA-*amoA* genes (Francis *et al.* 2005). On the other hand, *amoA*-1F and *amoA*-2R primers specific to *amoA* genes of betaproteobacterial AOB were used for amplification of AOB-*amoA* genes (Rotthauwe *et al.* 1997). The 5' ends of the forward primers (Arch-amoAF and *amoA*-1F) were labeled with 6-carboxyfluorescein (6-FAM). After thermal reactions, PCR products were checked by agarose gel electrophoresis.

Terminal-restriction fragment length polymorphism

Since we could not obtain enough amounts of AOB-*amoA* genes by PCR amplification, terminal-restriction fragment length polymorphism (T-RFLP) analysis was applied to only AOA-*amoA* genes. After the PCR products of AOA-*amoA* genes were digested with *Hha* I (TaKaRa BIO), the 6-FAM-labeled fragments were separated with an ABI Prism® 310 Genetic Analyzer (Applied Biosystems). Fragment analysis was carried out by using GeneMapper™ v3.0 software (Applied Biosystems).

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

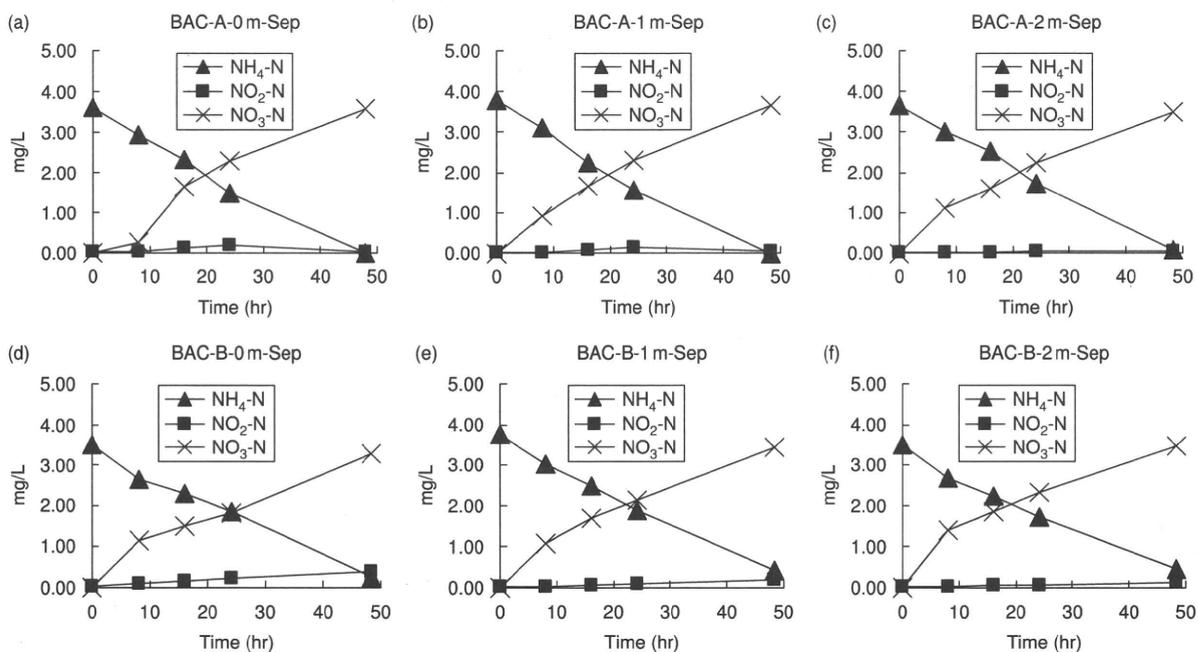


Figure 2 | Nitrification potential of BAC-A and BAC-B on 21 September 2007 collected from surface (0 m), middle (1 m) and bottom (2 m) layers of the BAC beds.

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,

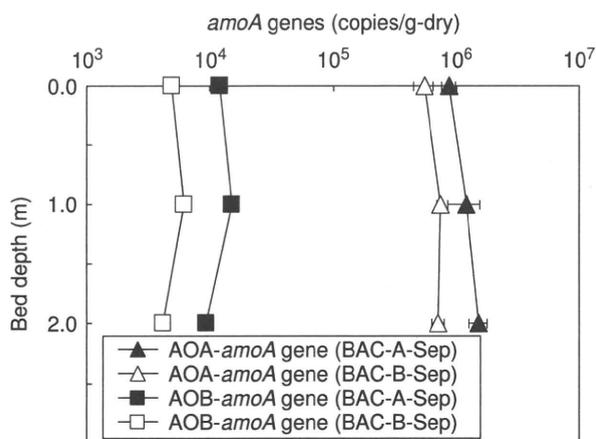


Figure 3 | Vertical distributions of AOA-*amoA* and AOB-*amoA* genes in the BAC beds in Process (A) and Process (B) (21 September, 2007).

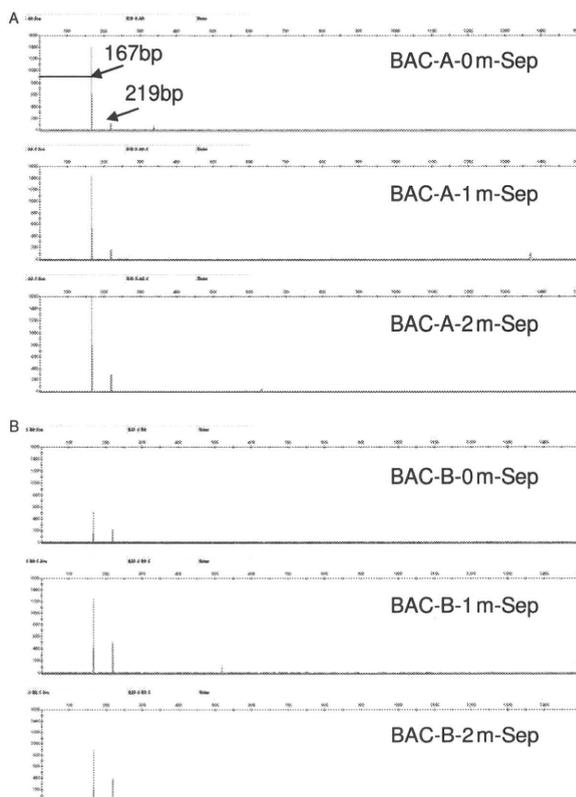


Figure 4 | T-RFLP profiles of AOA-*amoA* genes in the BAC beds in Process (A) and Process (B) (21 September, 2007).

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

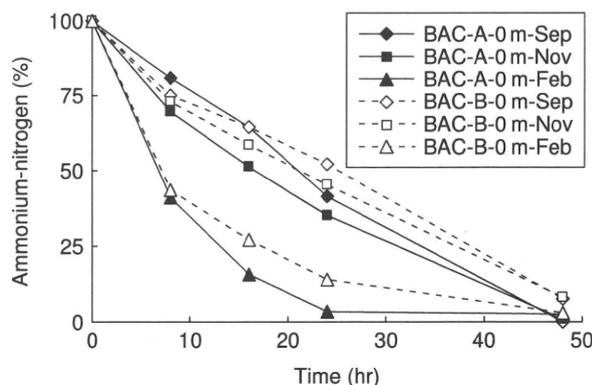


Figure 5 | Seasonal changes of nitrification potential of BAC-A and BAC-B.

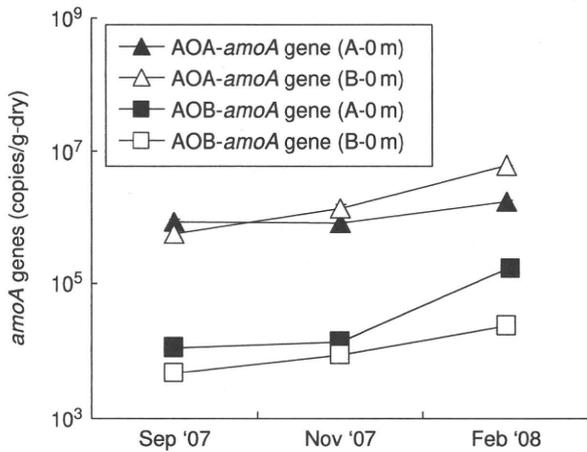


Figure 6 | Seasonal changes in abundances of AOA-*amoA* and AOB-*amoA* genes of BAC-A and BAC-B.

samples were obviously faster in February than those in September and November. BAC-A had slightly higher 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

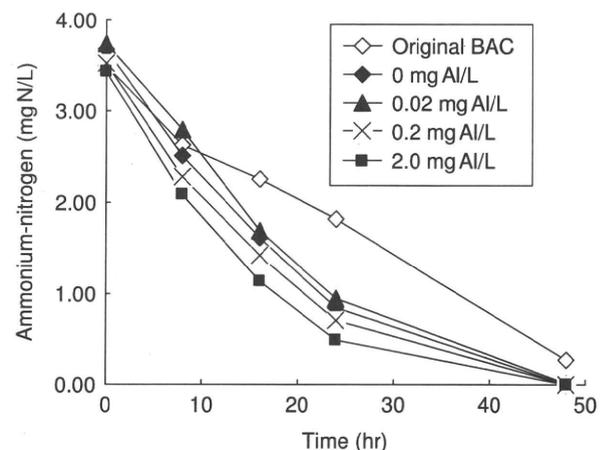


Figure 7 | Effects of aluminum on nitrification potential. Original BAC indicates BAC sample before one-month incubation.

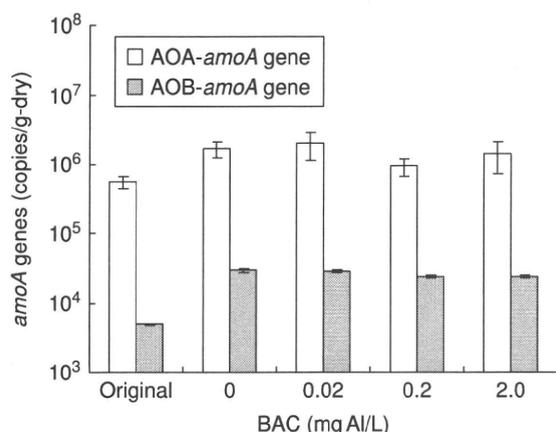


Figure 8 | Effects of aluminum on abundances of AOA-*amoA* and AOB-*amoA* genes. Original BAC indicates BAC sample before one-month incubation.

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.

ACKNOWLEDGEMENTS

This work was partially financed by Grant-in-Aid for Young Scientists (B) (no. 20760358) from Japan Society for the Promotion of Science (JSPS).

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