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研究成果の刊行物・別刷

[Original Paper]

Advances in Asian Environ. Eng.
Vol. 8, No. 1, p. 13-20 (2009)

Detection of Bacterial Regrowth in Water Distribution System Using Endotoxin as an Alternative Indicator

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ABSTRACT

Endotoxin concentrations and their fractions, which can be measured in a short time, were focused as new indicators for regrown bacteria in distribution systems instead of traditional heterotrophic plate count (HPC) method. It was found that almost all part of endotoxin existed as free endotoxin in tap water. Once chlorine residual was neutralized, HPC in several samples were increased after 7 days incubation. The concentrations of cell-bound and total endotoxin were increased drastically, and the ratios of free endotoxin were decreased relatively. The biofilm accumulation was monitored under continuous flow condition using annular reactors at different concentrations of chlorine residual. There were trends toward increasing HPC numbers in the effluent of AR with biofilm accumulation. The concentrations of cell-bound and total endotoxin were also increased with HPC numbers in effluent, and could be indicators for regrown bacteria only in the situation where significant bacterial regrowth was occurred (HPC > 5000 CFU/mL).

Keywords: Bacterial regrowth, Heterotrophic plate count (HPC), Endotoxin, Assimilable organic carbon (AOC), Water distribution system

INTRODUCTION

Disinfection of finished water is considered as an important treatment to supply microbiologically safe drinking water. In Japan, free chlorine of 0.1 mg/L is required at each end of distribution systems, and it works very effectively to control infectious risks by bacterial agents. However, many people have complaints about odor in drinking water, particularly chlorinous odor (Itoh *et al.*, 2007). The chlorine disinfection also causes formation of disinfection by-products (DBPs), such as trihalomethane and haloacetic acid. It has been widely recognized that the chlorine level should be reduced as low as possible to mitigate odor and DBPs problems. But, in a situation decreased chlorine residual, an advanced monitoring for bacterial regrowth and organic control are highly required at the same time in order to minimize microbiological risks, because bacteria in finished water could grow easily using small amount of biodegradable organics such as assimilable organic carbon (AOC) (van der Kooij, 1981).

At present, in water quality standard for drinking water in Japan, the standard values of standard plate count (SPC; 100 CFU/mL) and *Escherichia coli* (not detected), and the provisional targeted value of heterotrophic plate count (HPC; 2,000 CFU/mL) are established as indicators for bacteria. It is well-known that HPC is an excellent indicator for integrity of water treatment process and hygienic status of water distribution system, HPC data in finished water or during water treatment process therefore have been accumulating currently. However, it usually takes 1 week for HPC measurement, so it seems unrealistic to establish water quality monitoring system based on HPC data in distribution systems, which have relatively short detention time in Japan. Thus, new indicators for regrown bacteria, which can be tested rapidly and in practical use, are highly concerned to manage microbiological drinking water quality.

Endotoxin is an outer membrane component of gram-negative bacteria and cyanobacteria, and one of bacterial toxins acting on human. Endotoxin can be measured using simple procedure within 1 hour. There are several reports about endotoxin concentrations in water resources and drinking water and their removal during drinking water treatment (Anderson *et al.*, 2002; Sykora *et al.*, 1980). Endotoxin could be released from bacterial cells with cell multiplication or cell damage caused by chlorination, and it was cited as free endotoxin. Total endotoxin represents sum of two types of endotoxin, cell-bound endotoxin and free endotoxin. The information on free endotoxin was limited, while the concentrations of total endotoxin were described in many reports. In our previous research, it was found that large part of endotoxin in finished water existed as free endotoxin in dissolved organic fraction (Ohkouchi *et al.*, 2007), because almost all parts of bacteria were inactivated by chlorine disinfection. Conversely, it is expected that increase of cell-bound endotoxin could be a good indicator for regrown bacteria.

In this paper, endotoxin was examined as an alternative indicator for bacterial regrowth. First, tap water samples were collected in two different water distribution areas, and the concentrations of endotoxin and the ratios of free endotoxin to total endotoxin, defined here as the ratio of free endotoxin, were determined. Then those changes along with bacterial regrowth were examined in batch mode experiment, by incubation of tap

water samples after neutralizing chlorine residual. Finally, the biofilm accumulation under continuous flow condition was examined using two annular reactors at different concentrations of chlorine residual, to confirm applicability of endotoxin as an indicator for bacterial regrowth in water distribution system.

MATERIALS AND METHODS

Sampling Methods

Two typical water treatment plants (A and B) and their distribution systems were selected. The water treatment plant A had treatment processes including flocculation, sedimentation, rapid sand filtration, and chlorination. Forty and six water samples were taken in the distribution system from plant A (DS-A) at two different seasons, May - June 2007 and January 2008, respectively. In plant B, the surface water was treated by flocculation, sedimentation, rapid sand filtration, ozonation, biological activated carbon adsorption, and chlorination. Six water samples were taken in distribution system from plant B (DS-B) in January 2008. The water samples after 5 min flashing were collected in the glass bottles treated by heat sterilization at 250 ℃ for 2 hours for chemical or microbiological analyses except for AOC measurement. For AOC measurement, the carbon-free glass bottles were prepared by thermal treatment at 550 ℃ for 4 hours. The samples for chemical or bacterial analyses were processed within 4 h after sampling. For endotoxin assay, water samples were preserved at −80 ℃ after fractionation by centrifugation.

Bacterial Regrowth in Batch Mode Experiments

To neutralize chlorine residual, the autoclaved solution of sodium thiosulfate was added to 25 tap water samples taken in DS-A as a final concentration of 0.03 %. Then, each sample were incubated at 20 ℃ for a week under light-protected condition. The samples were taken at day 0, 1, and 7, respectively, and provided for HPC and endotoxin measurement.

Biofilm Accumulation in Continuous Flow Reactor Systems

Two annular reactors (ARs) Model 1320 LS (BioSurface Technologies Corporation, Bozeman) were used in this investigation. The AR is consisted of an outer glass cylinder and a rotating inner drum. Twenty removable polyvinyl chloride (PVC) coupons are mounted on the drum surface. Each PVC coupon has a wetted surface area of 17.9 cm^2. By rotating inner drums at 84 rpm, it could simulate a shear stress, which was equivalent to a velocity of 0.4 m/s in a PVC pipe with 125 mm diameter. The outside of outer each glass cylinder was covered with aluminum foil. Tap water at Katsura campus of Kyoto University was adjusted their chlorine residuals at 0 and 0.1 mg Cl_2/L with addition of sodium thiosulfate solution, respectively, and was pumped to each AR at flow rate of 8.3 mL/min. These AR systems were operated at 20 ℃. The detention time in each reactor was 2 hours. The flow diagram of these systems was illustrated in **Fig. 1**. The biofilm were accumulated on the surface of 20 PVC coupons, so one or two removed coupons were provided for measurements of HPC and total bacterial cells regularly. The biofilm samples were detached from each coupon by scratching using rubber policeman after loosen by sonication for 2.5 minutes with appropriate volume of autoclaved phosphate buffer.

Analytical Methods

Culture-based and direct enumeration of bacteria

HPC bacteria were enumerated by pour plate procedure with R2A agar incubated at 20 ℃ for 7 days. Total bacterial cells were enumerated by staining with 4',6-diamidino-2-phenylindole (DAPI). The bacterial cells in 1 mL water samples or culture broth were collected on 0.2 μm black polycarbonate membrane filter (Nihon Millipore K.K., Tokyo) and then the DAPI solution (1 μg/mL) were added onto the filter. After 10 min staining, the DAPI solution was removed by vacuum filtration. The filters were air-dried and mounted on slide glasses with cover slips. The fluorescence images were observed using epifluorescence microscope,

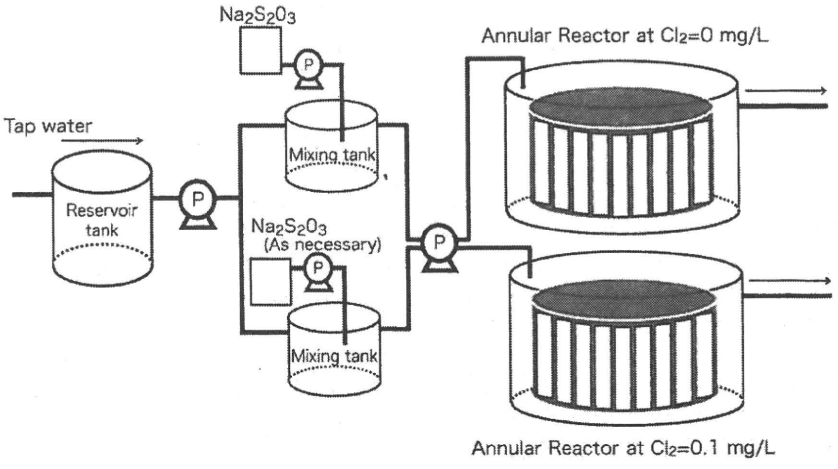

Fig. 1 Flow diagram of annular reactors for biofilm accumulation test.

MICROPHOTO-FX (Nikon, Tokyo) under UV excitation. Each image was captured by digital camera PDMC IIi (Nikon, Tokyo) and the image processing was carried out with software, ImagePro version 4.1 for Windows.

Endotoxin Endotoxin in water samples was fractionated by centrifugation at 14,000 rpm for 10 min, and the supernatant fraction was used for free endotoxin determination. Each endotoxin was determined by endpoint-colorimetric microplate method using Endospecy ES-50M Set and Toxicolor DIA-MP Set (Seikagaku Kogyo, Tokyo). The formation of diazo-compounds was monitored using microplate reader (Model 550, Bio-Rad, Tokyo) at 545 nm with reference wavelength at 650 nm. Endotoxin from *E. coli* strain O113:H10 was used for calibration. The samples were diluted serially using endotoxin-free water. The pipet chips and microplates guaranteed of endotoxin-free were used for this analysis.

Organic carbon Total organic carbon was analyzed using TOC 5000 analyzer (Shimadzu, Kyoto). Assimilable organic carbon (AOC) was determined by slightly modified procedure reported by van der Kooij *et al.* (1982). One mL mineral solution was added to 100 mL sample pasteurized at 70 ℃ for 30 min. Then, *Pseudomonas fluorescens* strain P17 (ATCC 49642) and *Aquaspirillum* sp. strain NOX (ATCC 49643) were inoculated. The inoculated samples were incubated at 20 ℃. With interval of a few days, the bacterial cells were enumerated by counting colonies formed on R2A agar plate separately. The AOC-P17 and AOC-NOX concentrations were calculated from the maximum colony counts divided by each yield factor, which were determined preliminary using acetate as a sole carbon source, respectively. The yield factors in this study were determined as 4.53×10^6 CFU/μg-C for P17, 1.54×10^7 CFU/μg-C for NOX, respectively.

Chlorines Free and combined chlorines were determined by DPD-Ferrous titration method according to the Standard Methods (1998).

Statistical Analysis

All statistical analyses were performed with GraphPad Prism ver. 4.0 for Macintosh (GraphPad software Inc., San Diego). To compare the differences between two groups of samples, a nonparametric *t*-test was performed. Significant differences were determined with a level of $p < 0.01$ in all analyses.

RESULTS AND DISCUSSION

Water Quality Parameters Associated with Bacterial Regrowth

The averages of water quality parameters in two different distribution systems were compared in **Table 1**. In both distribution systems, total chlorine residuals not less than 0.3 mg/L in average were detected, and HPCs were inactivated sufficiently. The average TOC content in DS-A was higher than that in DS-B. It seemed that TOC was removed effectively by ozonation and biological activated carbon adsorption processes in DS-B. The average AOC content in DS-B, however, was slightly lower than that in DS-A, but the difference was not significant. This result suggested that advanced water treatment system consisted of ozonation and GAC or BAC had only a definite improvement in AOC removal. It has been well-known that ozonation increased AOC concentration by degrading high molecular weight organics to low molecular weight and polar compounds (Hammes *et al.*, 2006). Then, some parts of AOC was removed during BAC process (Hu *et al.*, 1999), but the overall efficiency of AOC removal was not improved significantly.

Total and free endotoxins in DS-B were higher than those in DS-A as opposing to organic carbons. The reason has not been identified yet, but in our previous research, the same level of endotoxin, 10 EU/mL approximately, was found in finished water at other water treatment plant located in the same river basin of Plant B, which has a similar treatment processes including ozonation and BAC (Ohkouchi *et al.*, 2007). Two possible explanations are following; 1) Endotoxin concentration in raw water of Plant B is higher than that of Plant A, 2) BAC process causes to increase endotoxin in finished water. The average ratio of free endotoxin to total endotoxin was slightly lower in DS-B, but the difference was not significant.

On the other hand, in comparison of AOC concentrations in summer and winter in DS-A, the average AOC in winter was three-fold greater than that in summer. In winter, biological activities in water and biofilm were decreased because of low water temperature, so larger amount of AOC was remained through the water distribution. van der Kooij (1992) has proposed that AOC level for prevention of bacterial regrowth was less than 10 μg-C/L, but all our AOC data exceeded that biologically stable level substantially. Besides, there were no differences in the average endotoxin levels and the average ratios of free endotoxin in both seasons.

These results suggested that the ratios of free endotoxin to total endotoxin were usually very high in the water distribution systems which were sufficiently

Table 1 Average of parameters associated with bacterial regwowth in tap water.

Parameter	DS-A		DS-B
	May-June 2007	January 2008	January 2008
Sample numbers	n=40	n=6	n=6
TOC (mg-C/L)	1.8±0.25	–	1.3±0.13*
AOC (μg acetate-C/L)	59.8±15.6	173.9±43.5*	136.0±36.2*
AOC-P17	39.5±13.8	135.6±41.7*	104.7±37.1*
AOC-NOX	20.3±9.6	38.3±11.0*	31.2±3.9
Chlorine residual (mg Cl_2/L)	0.52±0.13	0.30±0.06*	0.31±0.11*
Free chlorine	0.40±0.12	0.25±0.06	0.21±0.10*
Combined chlorine	0.12±0.02	0.088±0.03	0.096±0.02
HPC (CFU/mL)	0.3±0.5	0.0±0.0	0.8±1.9
Total endotoxin (EU/mL)	1.48±0.69	1.16±0.13	6.72±1.85*#
Free endotoxin (EU/mL)	1.46±0.56	1.08±0.23	5.52±0.95*#
Ratio of free endotoxin	1.01±0.15	0.93±0.18	0.85±0.19

Values are the means with standard deviation.

* $p < 0.01$, compared with the samples taken in early summer in distribution system supplied by DS-A.

# $p < 0.01$, compared with the samples taken in winter in distribution system supplied by DS-A.

chlorinated, while concentrations of total endotoxin were relatively constant in each distribution system. But total endotoxin could vary depending on source water or treatment processes.

Bacterial Regrowth in Batch Mode Experiments

Among examined 25 samples, the bacterial regrowth phenomena after 7 days incubation were observed in only 6 samples. In these samples, HPC numbers ranged from 2.3×10^3 to 4.7×10^5 CFU/mL. Endotoxin levels and the ratios of free endotoxin between regrown samples and not-regrown samples were compared in **Fig. 2**. On day 1 after neutralizing chlorine residual, there were no significant differences in all parameters (data not shown). On day 7, it was found that total endotoxin concentrations were increased up to 10 - 40 EU/mL in water samples observed regrowth, while free endotoxin showed little increase. Next, the cell-bound endotoxin concentrations were determined by subtracting free endotoxin from total endotoxin. They were significantly higher in regrown samples ($p < 0.01$), and they represented large proportion of total endotoxin increases. Then, the ratios of free endotoxin were decreased significantly ($p < 0.01$) in regrown samples. It has been believed that free endotoxin was released from bacterial cells with multiplication or environmental stresses. Although almost all part of endotoxin existed as free endotoxin in coexistence of some stressors, such as disinfectant residual in tap water, the cell-bound endotoxin seemed to be increased along with bacterial regrowth once the stressor was removed. These results indicated that cell-bound endotoxin or the ratios of free endotoxin could be considered as useful indicators for regrown bacteria.

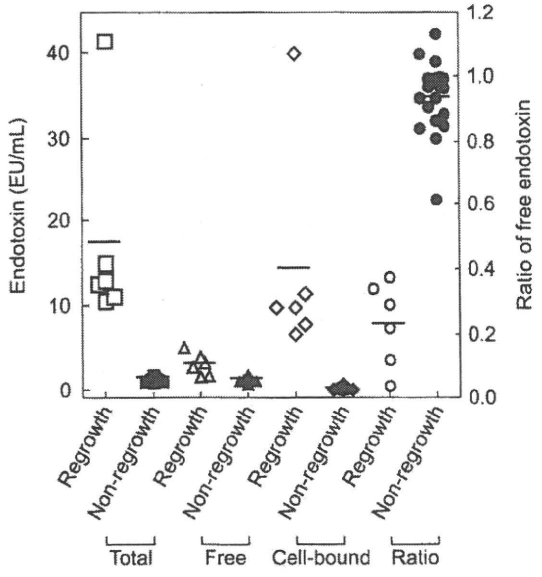

Fig. 2 Comparison of endotoxin between regrown and non-regrown samples.

Biofilm Accumulation in Continuous Flow Reactor Systems

Effects of chlorine residual on biofilm accumulation on PVC coupons The biofilm accumulation on PVC coupons were examined under continuous flow conditions. The time-dependent changes of HPC and total bacterial cells in biofilm were shown in **Fig. 3**. The HPC in biofilm on PVC coupons in AR without chlorine residual reached a stationary phase after 100 days operation, and the maximum biofilm density was approximately 5×10^5 CFU/cm^2. In AR with chlorine residual of 0.1 mg/L, the actual concentrations of chlorine residual were 0.07 $\pm$ 0.05 mg/L. The HPC in biofilm fluctuated after 85 days operation, but the maximum density of biofilm was approximately 2-log lower than that in AR without chlorine residual. At initial phase of biofilm accumulation, the total bacterial cells in AR without chlorine residual remained at higher level than corresponding HPC, but the percentages of HPC were increased drastically. In AR with chlorine residual, the total bacterial cells remained 10 times higher than HPC in average for the entire period.

The biofilm accumulation rates were calculated based on each HPC data until around day 110. The biofilm accumulation rate of 0.078 day^{-1} in AR without chlorine residual was twofold greater than that in AR at chlorine residual of 0.1 mg/L (0.040 day^{-1}). Pederson (1990) has reported that the doubling time of total number of microorganisms in biofilm was 11 days using biofilm reactors fed tap water with chlorine residual of

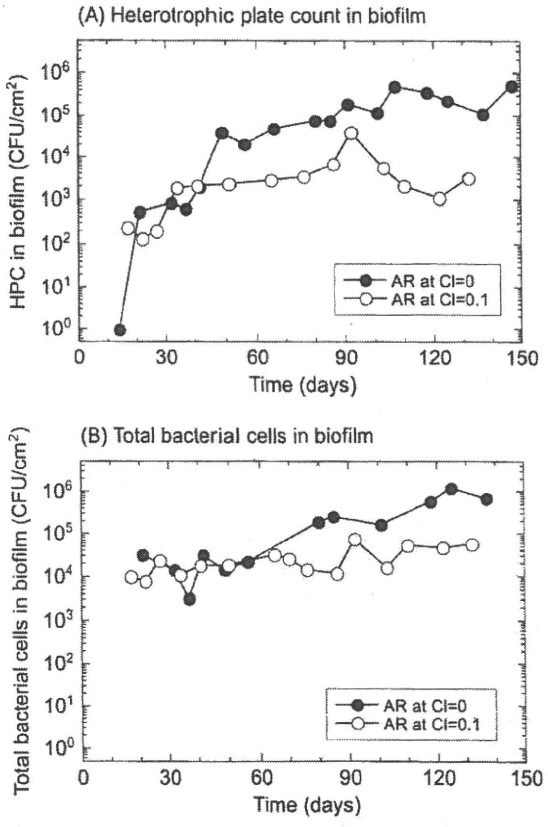

Fig. 3 Changes of HPC and total bacterial ells in biofilm.

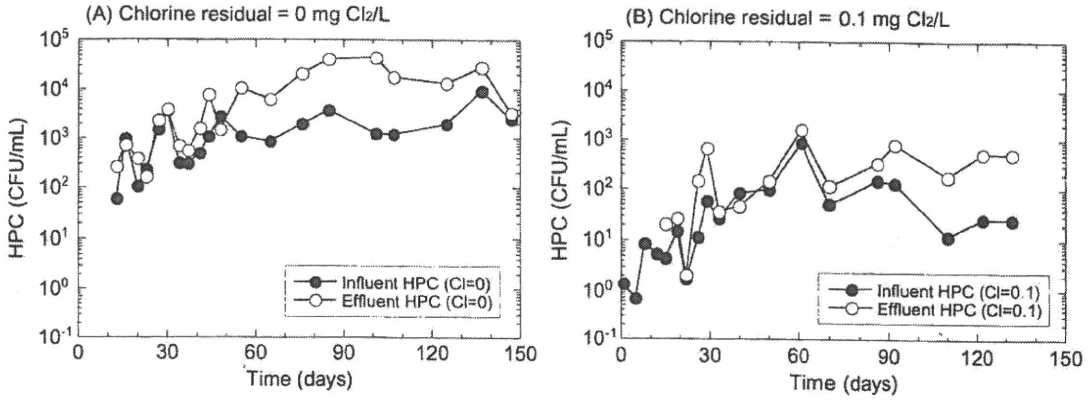

Fig. 4 Changes of HPC in influent and effluent water of each AR.

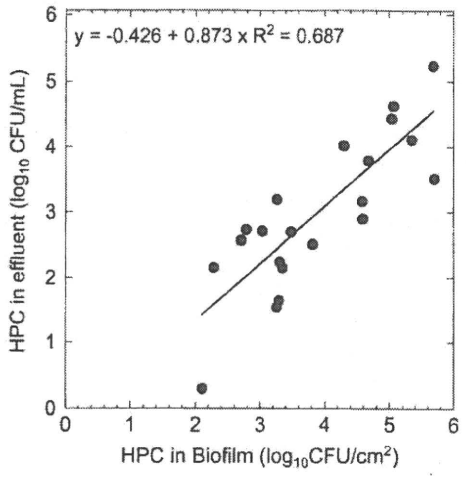

Fig. 5 Relationship between HPC in biofilm and HPC in effluent of AR.

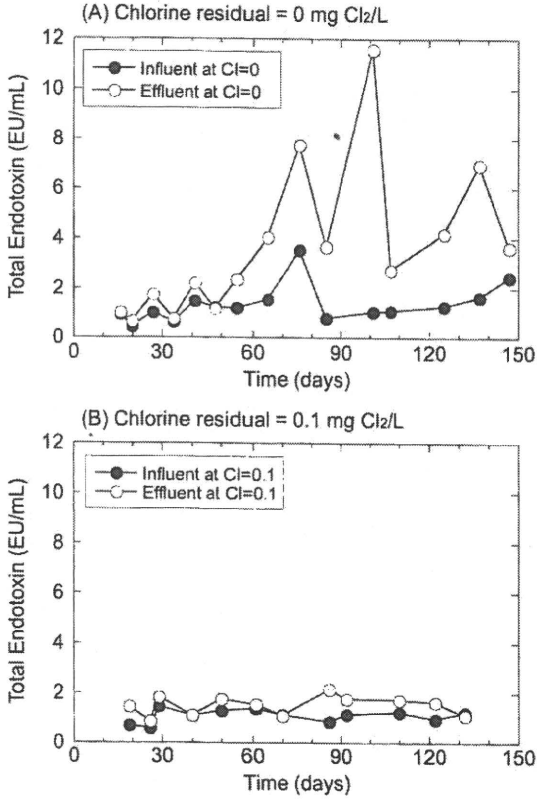

Fig. 6 Changes of endotoxin in influent and effluent of AR.

0.1 mg/L. The biofilm accumulation rate in his research was calculated as 0.063 day^{-1}, and our results were nearly equal to his result. These results indicated that low concentration of chlorine residual such as 0.1 mg/L could not prevent biofilm formation inside distribution pipes, but it could slow progression of biofilm in terms of accumulation rate, total amount of bacterial cells, and culturable bacterial cells in biofilm.

Changes of HPC in effluent from AR The time-dependent changes of HPC in effluent from each reactor were shown in **Fig. 4**. During all phases of biofilm accumulation, there were trends toward increasing HPC numbers in the effluent from both ARs. The correlation between HPC in biofilm and HPC in effluent of ARs was shown in **Fig. 5**. The measurement dates of HPC in biofilm were not always corresponding to those of HPC in effluent, therefore only the data, whose time intervals were within 1day, were used for this analysis. After logarithmic transformation of all HPC data, the HPC in effluent from ARs was increased proportionally with HPC on biofilm, and the slope of linear regression line and the correlation coefficient (R^2) were 0.873 and 0.687, respectively. It is not practical to take biofilm samples from surface of pipes and provide HPC test frequently. This correlation suggested that HPC in bulk water phase, which could be taken easily and frequently, could be a surrogate indicator for progression of biofilm inside pipes.

Endotoxin The changes of total endotoxin in effluent were shown in **Fig. 6**. In effluent from AR without chlorine, total endotoxin was increased and fluctuated significantly after 50 days, while no significant increase of total endotoxin was observed in case of AR without chlorine. The relationship between HPC in water phase and cell-bound endotoxin was shown in **Fig. 7 (A)**. In fact, bacterial regrowth was occurred immediately after adjusting chlorine residual, therefore, all HPC data in both influent

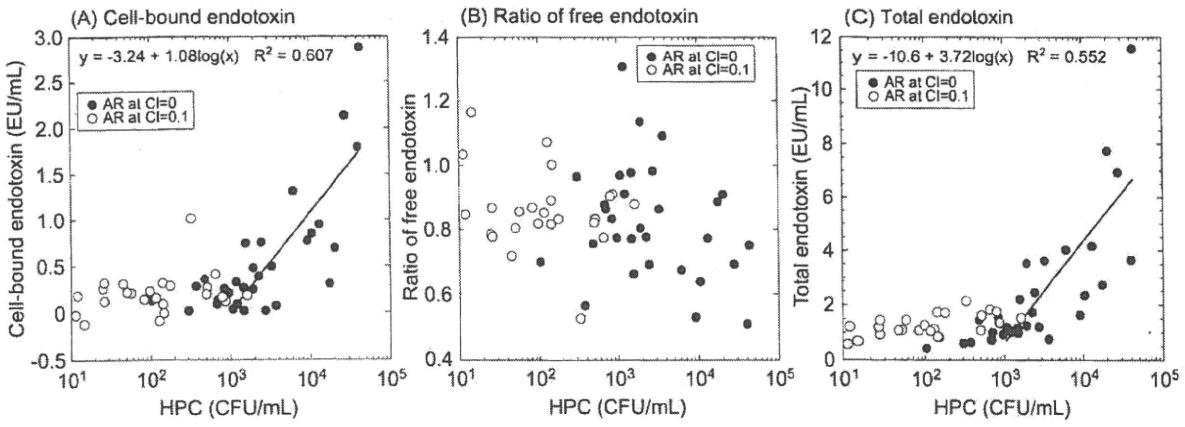

Fig. 7 Relationship between endotoxin and HPC in effluent.

and effluent were used for the following analysis. When HPC numbers in water phase were 1000 CFU/mL or higher, the cell-bound endotoxin tended to increase with HPC numbers. Especially in the range of over 5000 CFU/mL, the concentrations of cell-bound endotoxin were greater than 0.5 with one exception. However, these increased amounts were smaller than those observed in regrown samples in batch mode experiment described above. These results suggested that growth of bacteria in water phase contributed little to the increases of HPC in effluent because of short detention time, although Manuel *et al.* (2007) have reported that the specific growth rate in bulk water phase was much greater than that in biofilm. It was estimated paradoxically that HPC numbers in effluent were increased by HPC came from biofilm. As a result, it seemed that the amount of cell-bound endotoxin in effluent was affected by the difference in membrane integrities of bacterial cells in biofilm under continuous flow condition and in stagnant water as in batch mode experiment.

On the other hand, a poor correlation between HPC in water phase and ratios of free endotoxin (**Fig. 7 (B)**) was obtained. One possible explanation is that not only cell-bound endotoxin, but also free endotoxin were increased simultaneously under continuous flow condition, because there were not only chlorine residual but also more other stressors for bacteria. Total endotoxin followed a similar pattern with cell-bound endotoxin as shown in **Fig. 7 (C)**. The concentrations of total endotoxin were greater than 2.0 with one exception at HPC levels of over 5000 CFU/mL. And the regression slope of total endotoxin was greater than that of cell-bound endotoxin. Based on above results, it is recognized that total endotoxin could be a better and more sensitive indicator for regrown bacteria rather than indicators related to cell-bound endotoxin including ratios of free endotoxin. However, it should be also noted that these indicators of endotoxin could work effectively only in the situation that significant bacterial regrowth, such as over 5000 CFU/mL, was occurred.

CONCLUSIONS

As an indicator for regrown bacteria in distribution system, the applicability of endotoxin, which can be measured quickly, was examined in this study. In tap water samples taken in two different distribution systems, there was a difference in total endotoxin concentrations. It was suggested that endotoxin levels in water source or BAC treatment process could affect the concentrations of total endotoxin in finished water. It was also confirmed that large part of endotoxin in tap water existed as free endotoxin. Once chlorine residual was neutralized, bacteria started to grow in several water samples. In these samples, the concentrations of cell-bound endotoxin were increased drastically. At the same time, the concentrations of total endotoxin were also increased, and the ratios of free endotoxin were decreased relatively. The biofilm accumulation was also monitored using two annular reactors under continuous flow conditions. It was found that HPC in effluent of ARs changed reflecting the progression of biofilm accumulation. There was a positive correlation between the concentrations of cell-bound endotoxin or total endotoxin and HPC numbers in water phase, therefore cell-bound or total endotoxin could be indicators for regrown bacteria only when significant bacterial regrowth, such as over 5000 CFU/mL, was occurred. However, a poor correlation between the ratios of free endotoxin and HPC numbers in water phase was observed.

ACKNOWLEDGEMENT

We thank Mr. Tsukasa Kusano for sampling of tap water and their analysis. We also thank Mr. Suguru Ishikawa and Mr. Kentaro Kobayashi for helping another 12 sampling. This work was supported in part by a Grant-in-aid for Scientific Research from the Ministry of Health, Labour and Welfare (H17-Health-025).

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[Received: June 23, 2008]
[Accepted: December 1, 2008]

African Journal of Microbiology Research Vol. 4 (11) pp. 1128-1134, 4 June, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 © 2010 Academic Journals

Full Length Research Paper

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

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Accepted 19 May, 2010

The infectivity rates of Qβ 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, Qβ 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 (Liltved 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 their 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 polymerrase 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 (Qβ 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, Qβ phage and its cell host, *E. coli*, were obtained from the American type culture collection (23631-B1 and 13965, respectively).

Qβ 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 Qβ 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/KIO_3) according to Rahn et al. (2003). For this study, UV dose determined by chemical actinometry was equal to 5.72 mJ/cm^2 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 10^5 to 1 x 10^6 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 µl portion of each irradiated samples was removed in order to prepare serial dilutions in PBS buffer. A volume equal to 100 µl 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).

Qβ phage replication experiments

To investigate the presence of active bacteria in the UV-irradiated sample, Qβ 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 Qβ phage. After infection and at the indicated time intervals (each 5 min after infection), 100 µl was removed from different suspensions and after a cumulative dilution, a volume of 100 µl 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. Qβ 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 Qβ phage's replication and to determine different kinetic parameters related to the phage adsorption to the host cells under different UV irradiation conditions.

Simulating Qβ phage's growth

To simulate Qβ phage adsorption to the host cells, using the model employed by Levin et al. (1977) and reported by Abedon (2001). The modelling of Qβ 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).

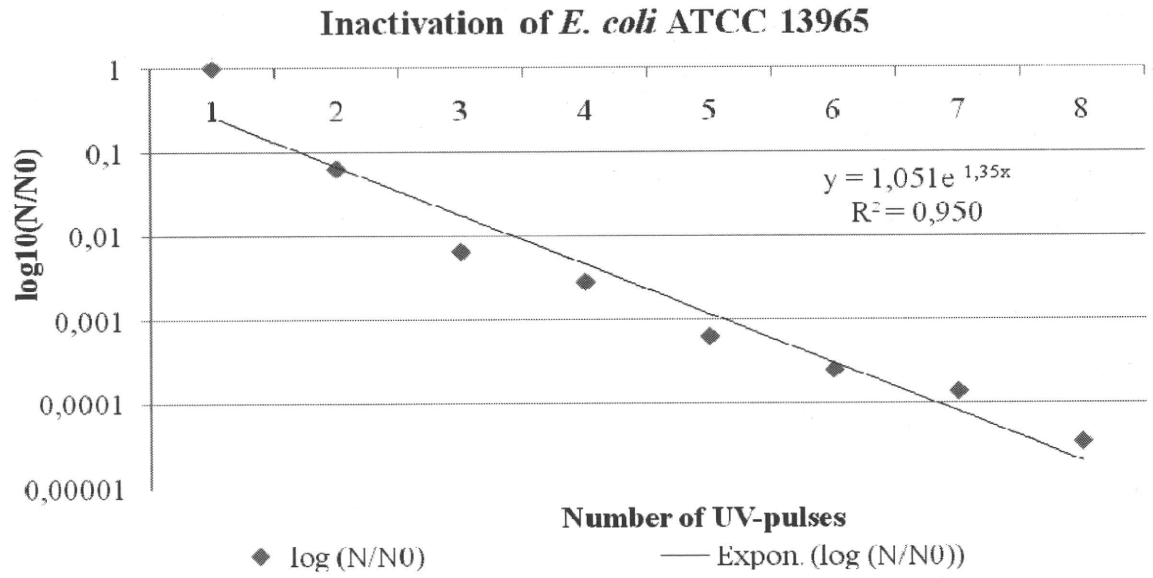

Figure 1. The Kinetic of *E. coli* ATCC13965 inactivation following exposure to UV-C irradiation according to the model of Chick-Watson, *y*: Reduction = N/N_0 with N_0: Number of viable and cultivable cell before exposure to UV light; *N*: Number of viable and cultivable cell after exposure to UV-C irradiation, x = $I^n t$ 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 Qβ phage

The irradiated host cell respectively by 0, 8, 12, 18, 25 and 30 UV pulses infected Qβ 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 (τ) (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).

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

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, τ is number of UV pulse and *n* is threshold level of series-event modem, *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 Qβ 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 Qβ phage during a single cycle of infection.

The lytic Qβ 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 Qβ 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):

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

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 Qβ 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 Qβ 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 Qβ 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

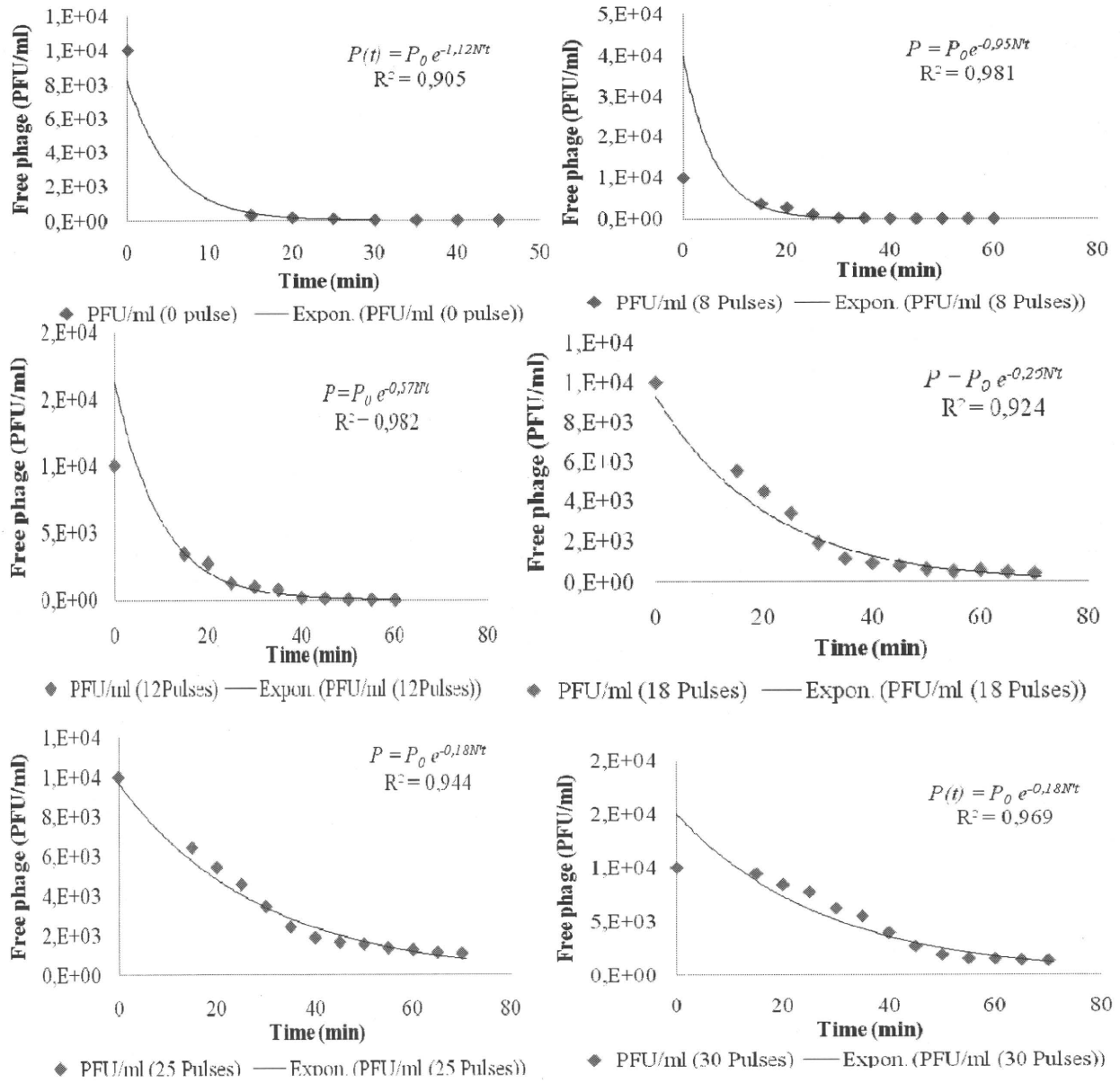

Figure 2. Qβ phage adsorption kinetics dependence on host UV irradiation state (UV irradiation by 0, 8, 12, 18, 25 and 30 UV pulses), P_0: the initiated free-phage concentration at time zero (t_0), $P(t)$: the free-phage concentration at time (t), K': the phage adsorption constant and N': the density 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).

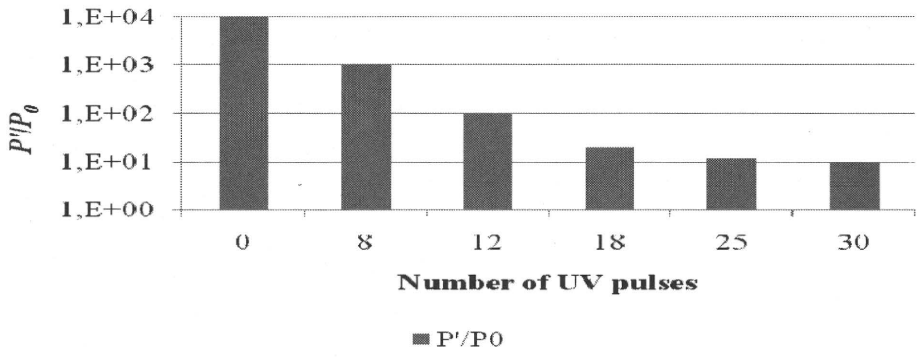

Figure 3. Qβ phage titers dependence on host UV irradiation state after 18 h of incubation at 37°C, P_0: the Qβ phage titter after incubation in the presence of unirradiated bacteria, P': the Qβ phage titres after 18 h of incubation at 37°C in the presence of irradiated *E. coli* by 8, 12, 18, 25 and 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 Qβ 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 Qβ 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 Qβ 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 Qβ 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 ofVBNC 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 Qβ RNA; as an essential factor for *rpoS* translation (Muffler et al., 1996).

HF-I constitute a part of the Qβ 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 Qβ 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 Qβ 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

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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 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.

doi: 10.2166/wst.2010.204

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 m^3/day with two different processes is shown in Figure 1. After river water 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.

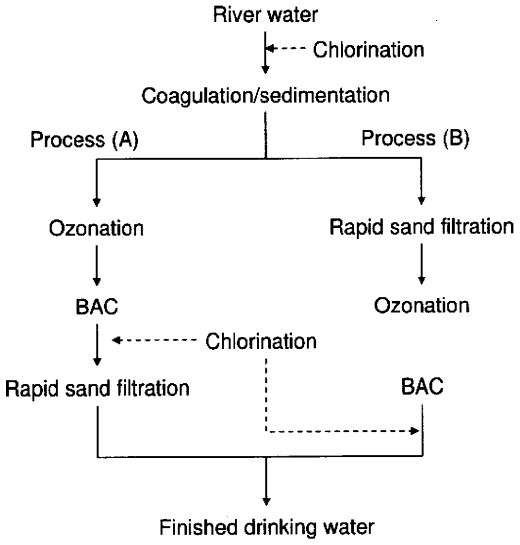

Figure 1 | Treatment processes in a pilot-scale plant.

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 NH_4-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: anthractice	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 NH_4-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 PowerSoil™ 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).