where L_E is the RLU of E_2 at 1.0×10^{-9} M; L_c is the RLU of the control cell lysate (in 10% DCC FBS medium); and L_t is the RLU of the test chemical and sample. The relative luciferase activity is expressed as a mean value of test results of triplicate samples.

Analytical procedures

TOX and TOC were measured by a TOX-10 Σ analyzer (Mitsubishi Chemical Corporation) and a TOC-5000A analyzer (Shimadzu), respectively. E_2 and 4-nonyl phenol (4-NP) were measured by an enzyme-linked immunosorbent assay (NEOGEN Corporation) and a gas chromatograph with a mass spectrometer, respectively (Ministry of the Environment of Japan, 1998). The chlorine concentration in mg-Cl_2/L was measured by the DPD ferrous titrimetric method (Clesceri *et al.*, 1998).

RESULTS AND DISCUSSION

Behavior of the estrogenic effect in water treatment

Figure 1 shows the results of the MVLN assay of Lake Biwa raw water and treated waters. Sample waters were concentrated using the XAD7HP resin. It clearly shows that coagulation and treatment with granular activated carbon reduced the estrogenic effect of the Lake Biwa water. The estrogenic effect nearly disappeared after treating with activated carbon. Figure 2 shows the water quality of raw and treated waters from Lake Biwa. Figures 1 and 2 show that the reduced estrogenic effect by granular activated carbon treatment is consistent with the removal of TOC.

On the other hand, chlorination increased the estrogenic effect of waters. It is noteworthy that chlorination newly produced the estrogenic effect in the water although the estrogenic effect nearly disappeared after treating with activated carbon. Figure 3 shows TOX produced by the chlorination.

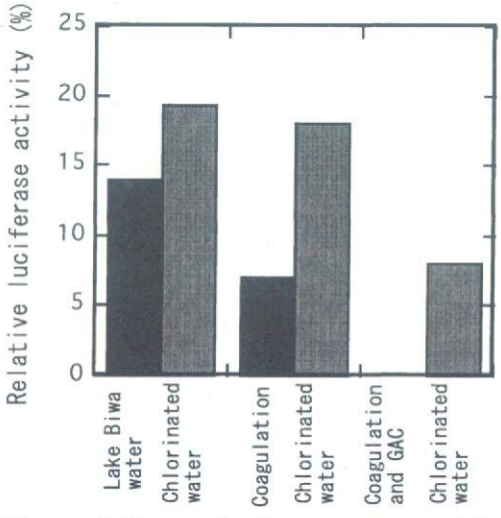

Figure 1 Change in the estrogenic effect of Lake Biwa water.

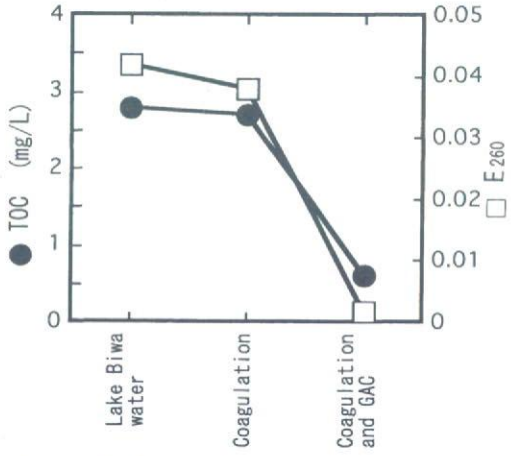

Figure 2 Water quality in coagulation and GAC adsorption.
E_{260} is ultraviolet absorbance at 260 nm.

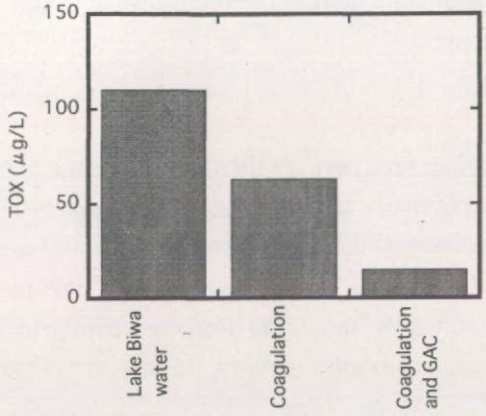

Figure 3 TOX procuced by chlorination in Lake Biwa water and its treated waters.

Itoh *et al.* (2000a) found that commercial humic acid exhibits the estrogenic effect, which increases upon chlorination. In addition, Itoh *et al.* (2000a) demonstrated that the estrogenic effect of concentrated Lake Biwa water using the XAD7HP resin increases up to 2.3 times upon chlorination. The reasons that chlorination increases the estrogenic effect could be 1) chlorine produces by-products such as organochlorine substances, which are estrogenic; 2) a low-molecular-weight fraction, which may bind to the estrogen receptor in a cell, increases due to the oxidation and hydrolysis caused by chlorination; and 3) chlorine releases estrogenic substances, which interact with humic substances in the aqueous environment. Itoh *et al.* (2000b) revealed that the main factor affecting the increase in the estrogenic effect is the effect of chlorination by-products.

The results shown in Fig. 1 suggest that the estrogenic effect is formed due to the reaction of chlorine with organic matter that remains after water treatment. It should be emphasized that this phenomenon is very similar to the formation of THMs in the drinking water treatment process, that is, natural organic matters (NOMs) are major precursors for both the estrogenic effect and THMs.

Characteristics of the estrogenic effect of drinking water and sample preparation procedure

In fact, the results shown in Fig. 1 were obtained by focusing on humic substances in the water, since the water samples were concentrated using the XAD7HP resin. In addition to humic substances, individual compounds such as E_2 and alkyl phenols are present in natural water. Itoh *et al.* (2003b) examined the recovery efficiencies of solid phase extraction procedures to concentrate estrogenic individual compounds. Consequently, this study used the OASIS HLB resin and dichloromethane as an adsorbent and a desorbing solvent, respectively.

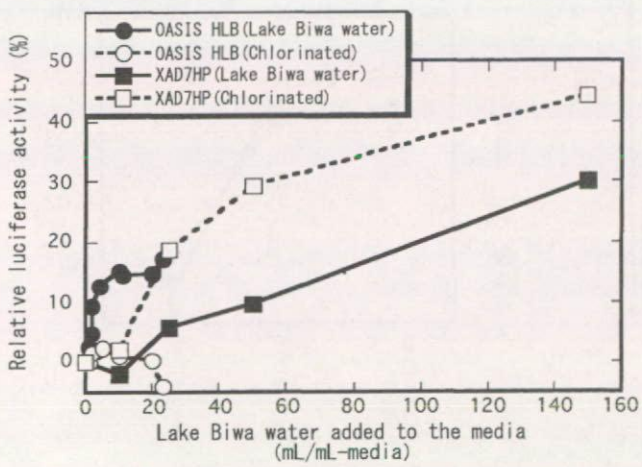

Figure 4 Comparison of sample preparation procedures on the detection of the estrogenic effect.

Figure 4 shows the results of the MVLN assay of samples concentrated by the OASIS HLB resin. It indicates that chlorination caused the estrogenic effect of Lake

Biwa water to nearly disappear. This direction of change is reverse to that shown in Fig. 1.

In contrast, chlorination increased the estrogenic effect of Lake Biwa water concentrated by the XAD7HP resin. This direction of change is consistent with that shown in Fig. 1. Thus, Fig. 4 clearly reveals that the effect of chlorination was completely reversed between the samples concentrated by the OASIS HLB resin and the XAD7HP resin. It is supposed that humic substances and their chlorination by-products are recovered by the extraction using the XAD7HP resin and NaOH. Individual compounds and their by-products are supposed to be recovered by the extraction using the OASIS HLB resin and dichloromethane (Itoh *et al.*, 2003a). It seems that different types of compounds recovered by the two concentration methods used resulted in different outcomes with respect to their estrogenic effects.

Itoh *et al.* (2000a) showed that E_2 and 4-NP contribute to the estrogenic effect of Lake Biwa water. Figure 5 and Table 1 show the changes in the concentrations of these compounds during chlorination and the change in the estrogenic effect of E_2, respectively. In all cases, 1 mg/L of chlorine was added. Chlorine altered both compounds and the estrogenic effect of E_2 drastically decreased. Thus, E_2 and 4-NP are examples of compounds that show a decreased estrogenic effect upon chlorination.

The effects of chlorination of BPA, 4-NP, estrone (E_1), E_2, estriol (E_3), and 17 α-ethynylestradiol (EE_2) on the estrogenic effect have been reported (Aizawa, 2002; Hu *et al.*, 2002; Kuroto-Niwa *et al.*, 2002; Hu *et al.*, 2003; Kitanaka *et al.*, 2003; Lenz *et al.*, 2003; Tabata *et al.*, 2003; Deborde *et al.*, 2004; García-Reyero *et al.*, 2004; Lee *et al.*, 2004; Nakamura *et al.*, 2006). In fact, some chlorinated derivatives or intermediates during chlorination of BPA and 4-NP show stronger estrogenic effect than parent compounds, however, it seems that the estrogenic effect of these compounds eventually decreases after the chlorination with chlorine dosage typically used in practice.

Thus, chlorination can either increase or decrease the estrogenic effect. In other words,

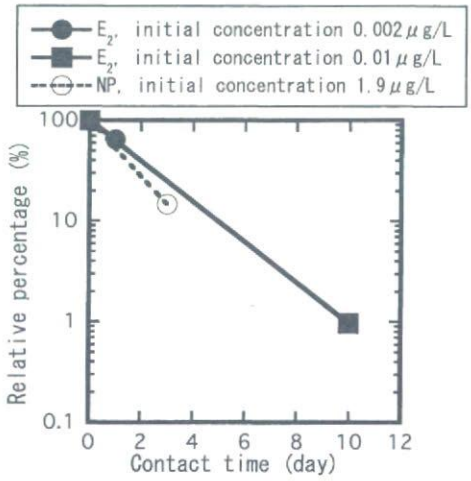

Figure 5 Decomposition of 17β-estradiol (E_2) and 4-nonyl phenol (NP) by chlorine.

Table 1 Change in the estrogenic effect of 17-β estradiol by chlorination.

	Relative luciferase activity (%)
Before chlorination	91.0
1 day after chlorination	3.3

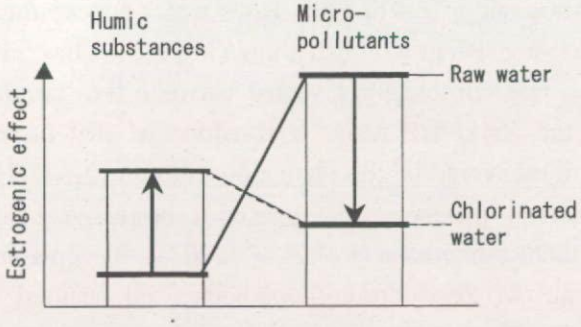

Figure 6 Change in the estrogenic effect of organic matter in natural water by chlorination.
↑↓ : Change by chlorination

organic matters of which estrogenic effect increases or decreases after chlorination are present in natural water. Figure 6 illustrates these changes. It shows that a) humic substances have some estrogenic effect (Itoh *et al.*, 2000a), b) chlorination increases the estrogenic effect of NOMs as shown in Figs. 1 and 4, and c) chlorination decreases the estrogenic effect of micro-pollutants. The important point is that the overall estrogenic effect in chlorinated drinking water is the sum of the increased activity and the decreased activity after chlorination.

The detection of the increase or decrease upon chlorination is dependent on the sample preparation procedure. Based on Fig. 4, the preparation procedure using the OASIS HLB resin is recommended to detect the estrogenic effect in Lake Biwa raw water. The preparation procedure using the XAD7HP resin, however, has to be used to detect the estrogenic effect in drinking water treated with chlorine, because the increased estrogenic effect after chlorination cannot be detected with the procedure using the OASIS HLB resin.

Effect of hydrolysis on the estrogenic effect formed by chlorination

The change in the estrogenic effect after chlorination of humic acid was examined. After finishing the chlorination of humic acid as described in **Chlorination of humic acid**, the pH of the chlorinated solution was adjusted to 7.0 or 10.0, and the change in the estrogenic effect was measured by the MVLN assay. The final concentration of the chlorinated solution in the culture medium was 25 mg/L as TOC. Trace of residual chlorine (less than 0.05 mg-Cl_2/L) was detected

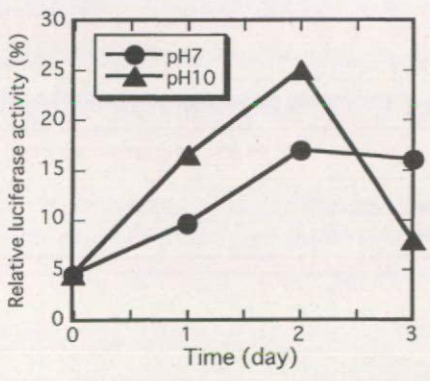

Figure 7 Change in the estrogenic effect of chlorinated humic acid.

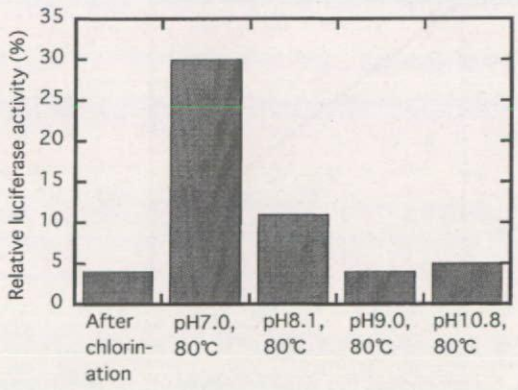

Figure 8 Effect of pH and water temperature on the estrogenic effect of chlorinated water.

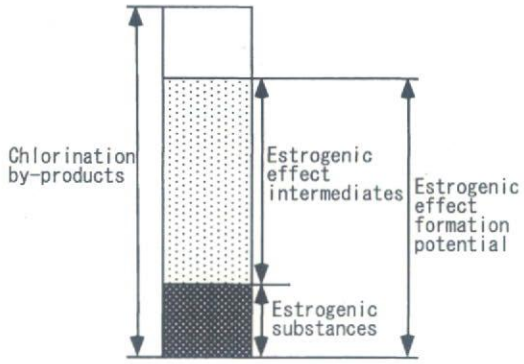

Figure 9 Components of the estrogenic effect in drinking water.

after the chlorination, but one day after adjusting the pH, residual chlorine was not detected.

Figure 7 shows the result of the assay. The estrogenic effect of the chlorinated solution increased even without residual chlorine at pH 7. It is known that the concentration of THMs increases while in the distribution system. The obtained result suggests that some part of the estrogenic effect in drinking water also increases over time after chlorination. The increase in estrogenic effect was faster at pH 10.0 than at pH 7.0, which is reasonable because the hydrolysis rate increases as the pH increases.

Figure 8 shows the effect of pH and water temperature. The estrogenic effect increased under the condition of pH 7 and 80℃ for 1 hour, however, the increased estrogenic effect decreased under the alkaline condition and 80℃. In addition to this, Fig. 7 shows that the increased estrogenic effect began to decrease after two days at pH 10. These results suggest that hydrolysis increases the estrogenic effect in chlorinated water, but further hydrolysis decreases the effect. Conditions of pH and water temperature in these experiments are for examining the effect of hydrolysis, and it does not intend to predict the estrogenic effect of drinking water in the actual water supply system. However, since drinking water is maintained at the neutral pH and the normal temperature in the actual water supply system, it seems that the estrogenic effect would increase in the distribution system.

Figure 9 illustrates this phenomenon. Based on Figs. 7 and 8, the estrogenic substances formed just after chlorination are part of the chlorination by-products. The components, which are called the "estrogenic effect formation potential" and the "estrogenic effect intermediates", can be defined in a chlorinated humic acid solution. The "estrogenic effect intermediates" change into estrogenic substances over time. On the other hand, the "THM formation potential" and the "THM intermediates" in the formation process of THMs have definitions that are similar to those illustrated in Fig. 9. This also suggests that to decrease the estrogenic effect of drinking water, NOMs in addition to suspected EDCs should be removed before chlorination.

CONCLUSIONS

Coagulation and treating with activated carbon decreased the estrogenic effect of Lake Biwa water, but chlorination increased the estrogenic effect. This phenomenon is similar to the formation of THMs during the drinking water treatment process since NOMs are major precursors for both the estrogenic effect and THMs.

It was found that organic matters of which estrogenic effect increases or decreases after chlorination are present in natural water. Thus, it is important to remember that the estrogenic effect of chlorinated drinking water is the sum of the increased activity and the decreased activity

after chlorination. The detection of the increase or decrease upon chlorination is dependent on the sample preparation procedure.

The estrogenic effect of chlorinated water increased even without residual chlorine. The components, which are called the "estrogenic effect formation potential" and the "estrogenic effect intermediates", are defined in a chlorinated humic acid solution. The process for the estrogenic effect formation is similar to the process for THMs formation. The obtained results suggest that to decrease the estrogenic effect of drinking water, NOMs in addition to suspected EDCs should be removed before chlorination.

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Detection of bacterial regrowth in water distribution system using endotoxin as an alternative indicator

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Key words: *bacterial regrowth, heterotrophic plate count (HPC), endotoxin, assimilable organic carbon (AOC), water distribution system*

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 was 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 that significant bacterial regrowth (HPC>5000 CFU/mL).

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.

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[2] Graduate Student
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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 recognized widely that the chlorine level should be reduced as low as possible to mitigate odor and DBPs problems. But, in a situation that decreased chlorine residual, an advanced monitoring for bacterial regrowth and organic control are highly required in order to minimize microbiological risks at the same time. It is 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 the 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 properly.

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. But the information about endotoxin exist in water phase 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 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 indicator for bacterial regrowth in water distribution system.

MATERIALS AND METHODS

1. 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-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, biologically 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 °C 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 °C for 4 hours. The samples for chemical or bacterial analysis were processed within 4 h after sampling. For endotoxin assay, water samples were preserved at -80 °C after fractionation by centrifugation.

2. 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 distribution system of Plant A as a final concentration of 0.03 %. Then, the each sample were incubated at 20°C for a week under light-protected condition. The samples were taken at 0, 1, and 7 days, respectively, and provided for HPC and endotoxin measurement.

3. 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 grass 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 125 mm diameter PVC pipe. 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°C. 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 coupons were removed regularly, and were provided for measurements of HPC or total bacterial cells. 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.

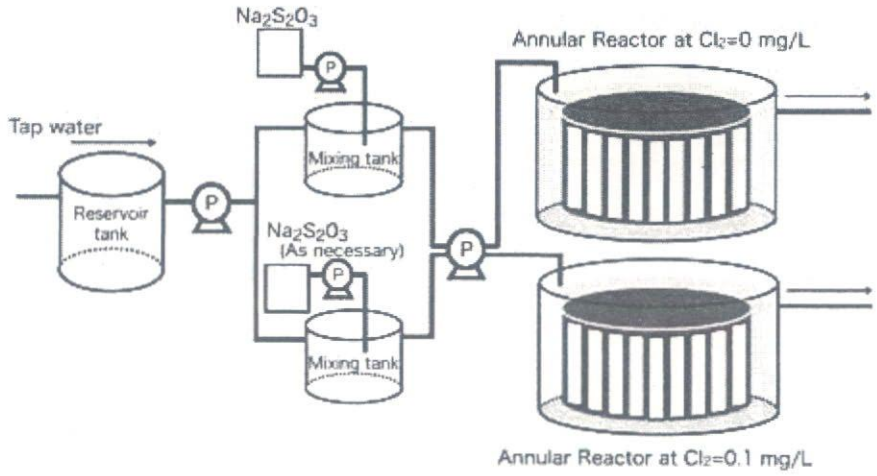

Fig. 1 Flow diagram of annular reactors for biofilm accumulation test

4. Analytical methods

Culture-based and direct enumeration of bacteria HPC bacteria were enumerated by pour plate procedure with R2A agar incubated at 20 °C for 7 days. Total bacterial cells were enumerated by staining with 4',6-diamidino-2-phenylindole (DAPI). The bacterial cells in 1mL water samples or vulture 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 filter. After 10 min staining, the DAPI solution was removed by vacuum filtration. The filters were air-dried and mounted on slide-grasses with cover slip. The fluorescence images were observed under MICROPHOTO-FX epifluorescence microscope (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 The 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) 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 microplate 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) were 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 75°C 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°C. 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 the yield factors, 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 established method (APHA-AWWA-WEF, 1998).

5. Statistical analysis

All statistical analyses were performed with GraphPad Prism ver. 4.0 for Macintosh (GraphPad software Inc., San Diego). A Kruskal-Wallis test was carried out to compare the differences between water qualitiy parameters in different distribution systems and seasons. 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 analysis.

RESULTS AND DISCUSSION

1. 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 were detected in average, 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 a definite improvement in AOC removal. It has been well-known that ozonation increased AOC content 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 totally 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,

which is located at near place of Plant B, and 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.

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.

On the other hand, in comparison of AOC contents in summer and winter in DS-A, the average AOC in winter was three-fold greater than that in summer. In winter, biological activity 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 biostable level substantially. Besides, there were no differences in the average endotoxin level and the average ratios of free endotoxin in both seasons.

2. 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. At 1 day after neutralizing chlorine residual, there

were no significant differences in all parameters (data not shown). At 7 days, it was found that total endotoxin concentrations were increased in the range of 10 to 40 EU/mL of water samples observed regrowth, while free endotoxin showed little increase. The cell-bound endotoxin concentrations were determined by subtracting free endotoxin from total endotoxin, and they represented large proportion of total endotoxin increases. Then, the ratios of free endotoxin were determined to compare decreased dramatically. It has been believed that free endotoxin was released from bacterial cells with multiplication or environmental stresses. Although almost all part of endotoxin was existed as free endotoxin in a situation of co-existence of some stressor, such as disinfectant residual in tap water, the cell-bound endotoxin seemed to be increased along with regrowth of bacteria once the stressor was removed. These results indicated that cell-bound endotoxin or ratios of free endotoxin in addition to total endotoxin could be considered as useful indicators for regrown bacteria.

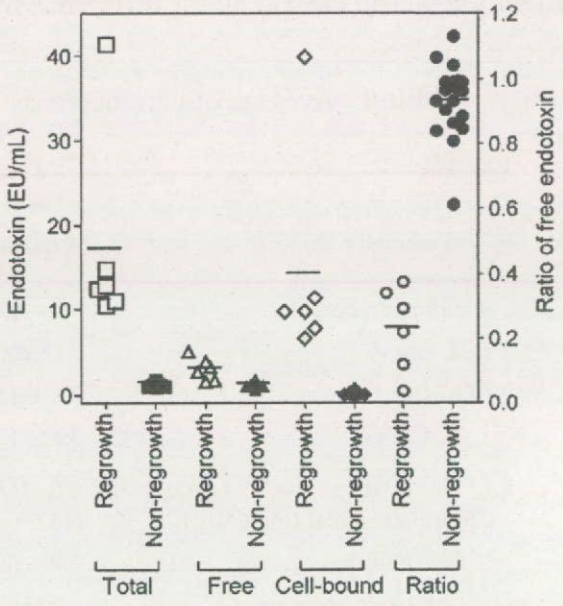

Fig. 2 Camparison of endotoxin between regrown and non-regrown samples.

3. Biofilm accumulation in an annular reactor

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 change 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±0.05 mg/L. The HPC in biofilm

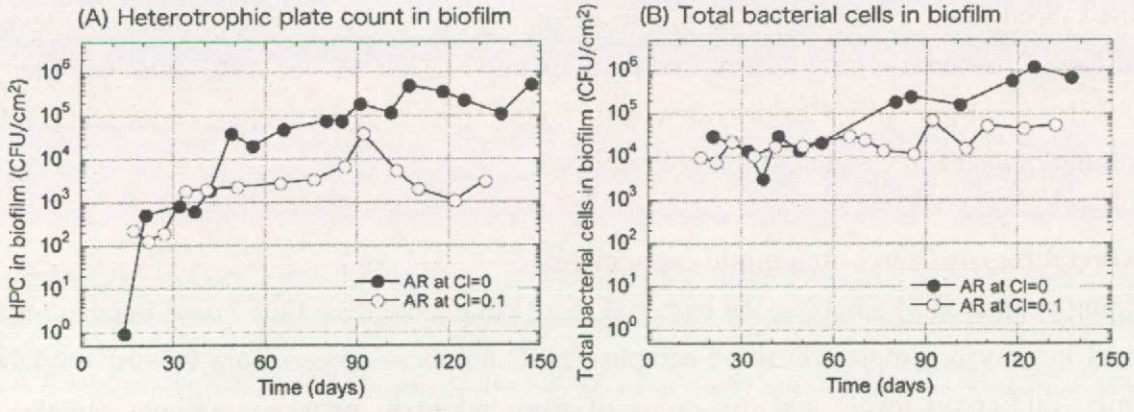

Fig. 3 Changes of HPC and total bacterial cells in biofilm.

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 110 days. 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 0.1 mg/L. The biofilm accumulation rate 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 and amount.

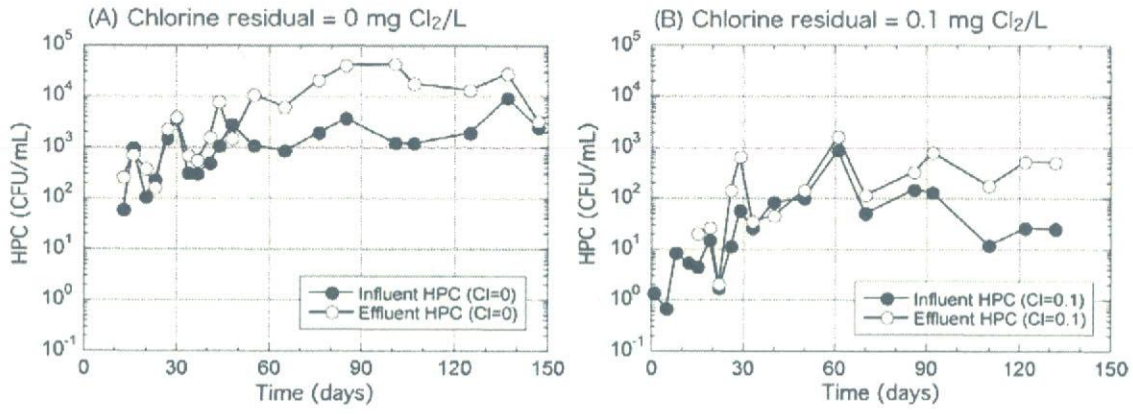

Fig. 4 Changes of HPC in influent and effluent of each AR.

Changes of HPC in effluent from ARs The time-dependent change of HPC in effluent each reactor was shown in Fig. 4. During all phase 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, which was increased in ARs, was increased proportionally with HPC in 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 test HPC frequently. This correlation proved that HPC in bulk water phase 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, regrowth of bacteria was occurred immediately after adjusting chlorine residual, therefore, all HPC data in both influent 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 be increased 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, nevertheless 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 integrity of bacterial cells in biofilm and in stagnant water as in batch mode experiment.

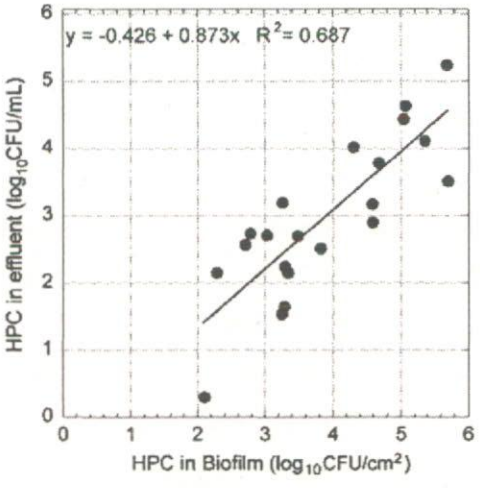

Fig.5 Relationship between HPC in biofilm and HPC in effluent of AR.

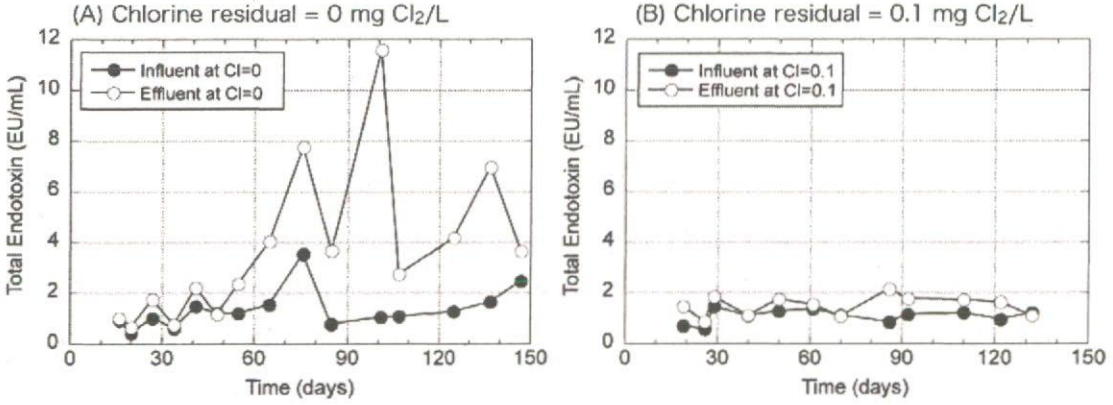

Fig. 6 Changes of endotoxin in influent and effluent of AR.

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 more other stressors for bacteria than chlorine residual. Total endotoxin followed a similar

pattern with cell-bound endotoxin as shown in Fig. 7 (C). The concentration of total endotoxin were greater than 2.0 with one exception at HPC levels of over 5000 CFU/mL, and the regression slope 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. 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.

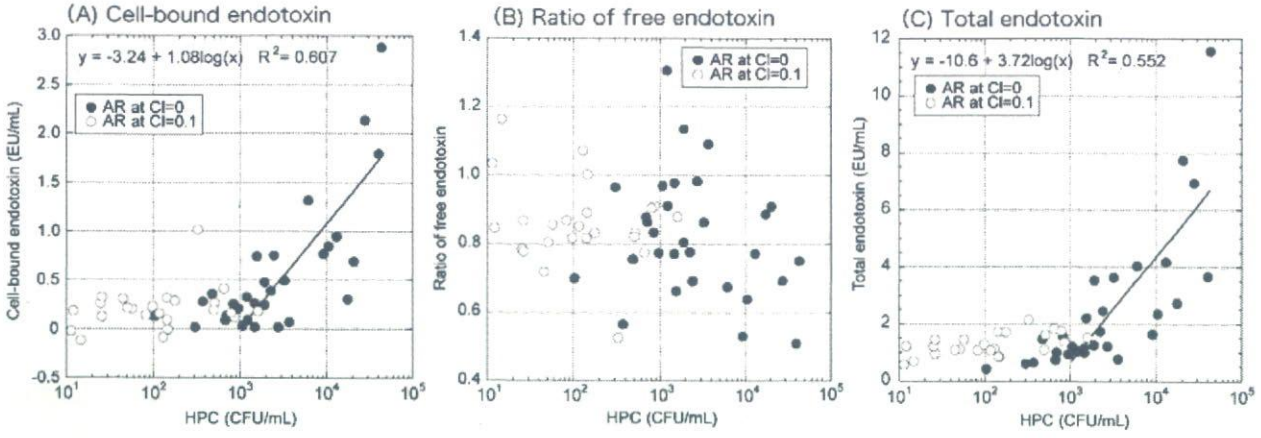

Fig. 7 Relationship between endotoxin and HPC in effluent.

CONCLUSIONS

As indicator for regrown bacteria in distribution system, which can be measured in a short time, endotoxin 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 raw water or BAC treatment process could affect the concentrations of total endotoxin. 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 was changed reflecting the progression of biofilm accumulation. There was 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.

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26

従属栄養細菌の迅速定量を目的としたブロモデオキシウリジンラベル化DNAの定量方法に関する基礎的検討

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Primary investigation of measurement of DNA labeled with bromodeoxyuridine for rapid quantification of heterotrophic bacteria.

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1．はじめに

現在の水道システムでは塩素消毒を行い、給配水過程において塩素を残留させることで感染症の原因となる微生物を不活化させ、リスクを低減させている。しかし、塩素剤の添加によって消毒副生成物やカルキ臭が生成し、健康への影響や水道水の快適性低下が問題となっている。そのため、消毒副生成物やカルキ臭の生成抑制を目的として、残留塩素濃度の低減の検討が進められている。その一方で、給配水過程での微生物の再増殖や生物膜の形成も問題となっており、残留塩素濃度と微生物の衛生面での安全性との兼ね合いが今後の課題となっている。従来、微生物に関する水質基準項目として一般細菌が用いられてきたが、厚生科学審議会生活環境水道部会水質管理専門委員会によって見直しが行われ、一般細菌に代わる指標として従属栄養細菌が取り上げられた。従属栄養細菌は、一般細菌よりも本来的な水中細菌数を表すことから、浄水処理過程や消毒過程での細菌の挙動評価、あるいは塩素消失や滞留による水質悪化の結果としての微生物再増殖といった水道システムでの微生物汚染の状況評価に適している[1]。そのため2007年の水質基準の改正を経て、2008年4月より新たに水質管理目標設定項目として従属栄養細菌が追加され、現在各地で従属栄養細菌の存在量に関するデータの蓄積が進められている。しかし、従属栄養細菌数の測定には7日間を要することから、給配水時間が短い我が国の水道システムにおいては従属栄養細菌数測定結果のフィードバックによる水質管理は実現が難しいと考えられ、培養法に代わる迅速定量法の確立が必要である。

そこで本研究では、従属栄養細菌数の迅速測定法を確立するため、有機物存在下におけるDNA合成に着目した検出方法を検討する。具体的には、モデル微生物として、*Pseudomonas fluorescens* P17（ATCC 49642; P17株）および *Aquaspirillum* sp. NOX（ATCC 49643; NOX株）を使用し、微生物増殖に伴うDNA合成時にチミジンの類似体である5-ブロモ-2'-デオキシウリジン（以下、BrdUと記載）を用いて標識を行い、その定量的測定を行う。これらの検討を通してBrdUラベル化法が従属栄養細菌数の迅速測定法として適用可能であるかを検討する。

2．実験方法

2.1．従属栄養細菌数の測定

試料中の微生物濃度の測定については、R2A寒天培地を用いた平板培養法を用いた。

2.2．BrdUラベル化法による微生物の定量

本研究で行うBrdUラベル化法は、培養細胞の増殖アッセイにおいてはよく使用されているが、微生物検出を目的とした検討については海洋微生物の生産速度測定[2), 3), 4)]への適用が報告されているのみである。本研究ではHamasakiらによる方法[2)]を参考にし、多検体同時処理が可能となるよう96穴マイクロプレートを用いた測定方法へと改良し、また、取り込まれたBrdUを定量するために、酵素標識された抗体を用いる酵素抗体法を採用した。主な実験の流れとしては、まず1日目に段階的に希釈した微生物懸濁液を用いたBrdUラベル化反応、96穴マイクロプレートへの微生物細胞の固定を行った。そして、2日目に前処理として内因性ペルオキシダーゼと細胞壁の処理、最後に酵素抗体法を利用してBrdU標識DNAの検出を行い、平板培養法で求めた微生物濃度との関係を調べた。簡易的な測定操作の流れを図1に示す。ここで、細胞固定には4％パラホルムアルデヒドリン酸緩衝液、内因性ペルオキシダーゼの不活化には3％ H_2O_2 溶液、細胞壁の消化にはペプシン（2 mg/mL : 0.01 N HCl溶液）とリゾチーム（3 mg/mL : TE緩衝

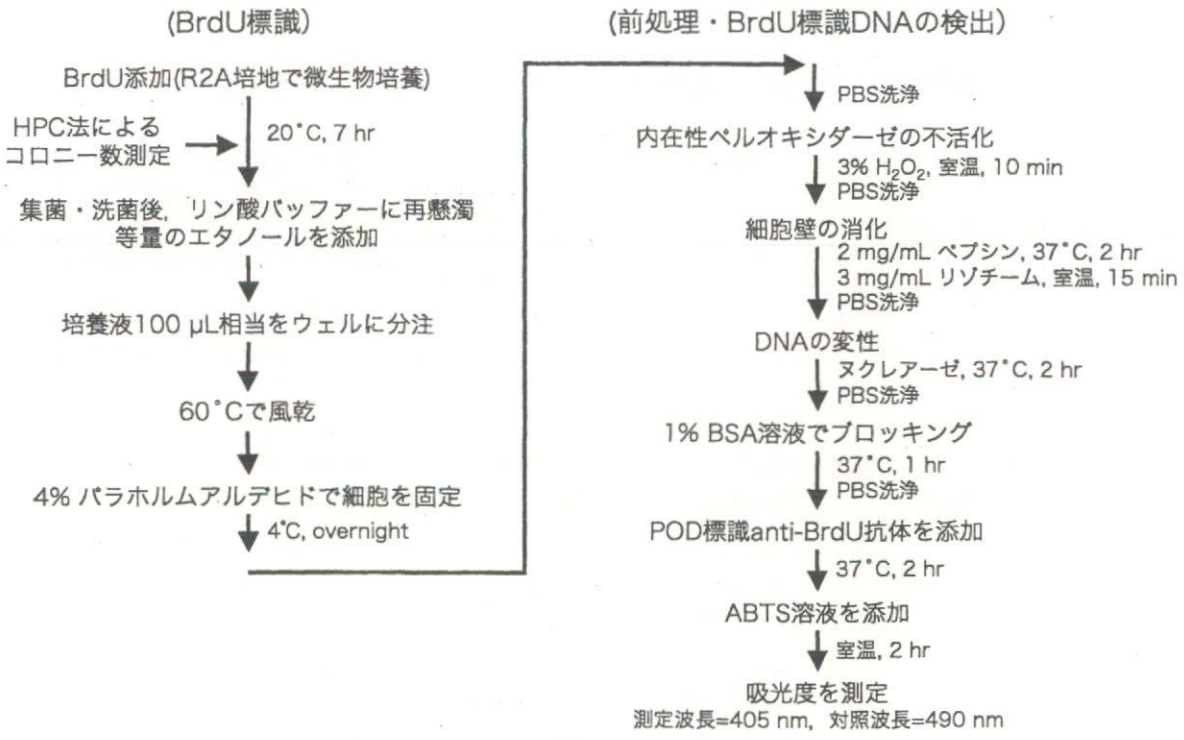

図1 P17株細胞数と吸光度の関係

液）を用いた。そして、ペルオキシダーゼ基質としては、ABTS基質 (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) を使用した。本研究では主に以下の条件について検討を行った。

1) BrdU濃度 10 μM, 1 μM, 100 nM, 10 nM
2) BrdUラベル化反応時間 3, 5, 7時間（P17株） 5, 6, 7, 8時間（NOX株）
3) マイクロプレートの種類 表面処理の有無
4) anti-BrdU抗体濃度 40 mU/mL, 200 mU/mL
5) ウシ血清アルブミン (BSA) 溶液を用いたブロッキングの有無

最後に同一実験条件下で3回実験を行い再現性の確認を行った。

3. 実験結果と考察

BrdU濃度がBrdU標識DNA量（＝吸光度）に与える影響を図2および図3に示す。BrdU標識DNA量は、R2A寒天培地で計測したP17株およびNOX株の細胞数の対数値に比例して増加したが、微生物濃度が高い領域では、およそ1000-10000 CFU/mLを境に吸光度の値が減少した。そこで1000 CFU/mL以下の範囲で回帰式の傾きを比較した結果、P17株、NOX株ともにBrdU濃度が1 μMの時に最も大きな吸光度の変化が得られた。この結果より、BrdU濃度を1 μMと設定した。その他の測定条件についても同様の検討を行った。以下に決定した測定条件を示す。

1) BrdU濃度 1 μM
2) BrdUラベル化反応時間 7時間
3) マイクロプレートの種類 ポリスチレン製 表面処理なし
4) anti-BrdU抗体濃度 200 mU/mL
5) BSA溶液を用いたブロッキング有り

以上で決定した測定条件を用いて、3回繰り返し測定を行った。P17株とNOX株の細胞数とBrdU標識DNA量との関係を図4および図5に示す。また、細胞数1 log当たりの吸光度変化量（回帰式の傾き）とバックグラウンド値を表1に示す。バックグラウンド値は細胞数0.01 CFU/mLとして算出される吸光度値と定