Behaviors of physiologically active bacteria in water environment and chlorine disinfection

K. Sawaya, N. Kaneko, K. Fukushi and J. Yaguchi

ABSTRACT

Direct microscopic methods using several fluorescent staining were applied to estimate the proportion of physiologically active bacteria in the water environment and evaluate the efficacy of disinfection with chlorine. 4′,6-diamidino-2-phenylindole (DAPI) was used to determine total bacterial numbers, and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was chosen for direct detection of respiring bacteria. BacLight™ kit was used to assess bacterial membrane integrity. Bacteria with growth potential were enumerated using the DVC method and microcolony technique. The total bacterial number in river was $8 \times 10^6 \sim 3 \times 10^{10}$ cells/mL, and colony forming units on R2A medium were $1 \times 10^4 \sim 4 \times 10^5$ cfu/mL. In the case of wastewater treatment plant, 1 ~ 10% of total bacterial cells could form colonies. Physiologically active bacteria in river and wastewater treatment plant determined by fluorescent staining were much higher than those obtained by plate counting. The effect of chlorine on the physiological viability of *Escherichia coli* was also investigated. Microscopic viable bacteria were even more chlorine resistant than culturable bacteria. The inactivation rate coefficients of direct viable bacteria were one-second to third those of culturable bacteria.

Key words | chlorine disinfection, culturability, fluorescent staining, river, viable counts

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INTRODUCTION

Enumeration of viable or active bacteria in aquatic environment has relied on viability assays using conventional plate counting techniques for decades. However, these methods underestimate the total number of viable or active bacteria because of the viable but non-culturable physiological state (Roszak & Colwell 1987; Byrd *et al.* 1991). Viable but non-culturable bacteria fail to form colonies on established media but remain viable. Therefore, the traditional enumeration may cause overestimates of disinfection efficiencies on the basis of colony-forming ability in water supply and wastewater treatment system.

The objectives of this research were to determine the proportion of metabolically active bacteria in the water environment and estimate the actual efficacy of disinfection with chlorine using several direct microscopic techniques other than plate counting.

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MATERIALS AND METHODS

Chemicals and organisms

We purchased the DNA-binding 4′,6-diamidino-2- phenylindole (DAPI) and sodium hypochlorite from Wako Pure Chemical Industries, Ltd. (Japan). The redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was obtained from

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Polysciences Inc. The LIVE/DEAD Bacterial Viability Kit (BacLight™) was purchased from Molecular Probes Inc. *Escherichia coli* JCM1649^T obtained from the RIKEN BioSource Center (Saitama, Japan) was used in the experiment of chlorine disinfection.

Water samples

River water was collected from five rivers located in the southeast of Aomori Prefecture, northern Japan. Water samples were taken 3 times from December 2004 to November 2005. Municipal wastewater, secondary and chlorinated effluent were obtained from two sewage treatment plants and the college wastewater treatment facility at Hachinohe, Aomori.

Bacterial enumeration

The presence of culturable bacteria in water samples was determined by plate counts on R2A (Reasoner & Geldreich 1985) and PGY (Peptone Glucose Yeast extract) agar medium. Five fluorescent staining techniques were applied to obtain direct visualization of bacteria as shown in Table 1. Water samples were vacuum-filtered onto black polycarbonate membranes (Advantec; pore size 0.20 μm) prior to staining. 4′6-diamidino-2- phenylindole (DAPI) was used to determine total bacterial numbers, and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was chosen for direct fluorescent detection of respiring bacteria (Rodriguez *et al.* 1992; Pyle *et al.* 1995). BacLight kit was used to assess bacterial membrane integrity (Boulos *et al.* 1999; Molecular Probes 2003). Bacteria with growth potential were enumerated using the DVC method suggested by Kogure *et al.* (1979) and microcolony technique (Kawai *et al.* 1999).

A BX41 epifluorescence microscope (Olympus Co.) equipped with a mercury burner was used for all direct counts. DAPI stained bacteria were observed with a U-MWU2 excitation filter, and respiring bacteria were viewed with a U-MNIB2 excitation filter as well as BacLight stained bacteria.The number of bacteria was estimated from counts of 10 microscopic fields (at 1000 ×) for each sample.

Table 1 | Characteristics of 5 fluorescent staining used in this study

Assay	Total direct counts	Viable counts: DVC	Viable counts: Microcolony	CTC	BacLight
Bacteria trap	Black polycarbonate membrane (pore size 0.2 μm)				
Reagent	–	Nalidixic acid	–	CTC	–
Culture medium	–	R2A	R2A	1/2R2A (without phosphate)	–
Staining	DAPI	DAPI	DAPI	CTC-DAPI double staining	SYTO9/propidium iodide
Microscopic observation	Fluorescent blue	Elongated or enlarged growing cells	Microcolony	CTC-formazan (red)	viable-fluorescent green nonviable-fluorescent red
Physiological parameter	DNA binding	Division inhibition	Growth potential	Respiratory activity	Membrane integrity

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River water incubation

River water sample was taken from the Mabuchi River on Nov. 21, 2005. The Erlenmeyer flask containing 50 mL of river water was incubated at 20°C with shaking at 140 rpm for more than 60 days. Bacterial counts were performed by the direct microscopic methods shown in Table 1 and plate counting technique.

Chlorine disinfection

Sodium hypochlorite was diluted in ultra-pure water (Wako Pure Chemical Industries, Ltd.) to give a 200 mg/L chlorine stock solution. The overnight culture of *E. coli* JCM 1649^T on R2A medium was prepared. Batch experiments were performed in 300 ml mixed reactors at 20°C. The cell suspension and chlorine solution were added to the batch reactors containing sterile phosphate-buffered water at pH 7.2. After the desired contact time, 100 mg/L of sodium thiosulfate neutralized the residual chlorine. Samples were taken before and after chlorine disinfection. Measurements of culturable bacteria were conducted using m-Endo (Difco) and R2A medium. The residual chlorine concentrations assayed by the DPD method ranged from 0.05 to 1.2 mg/L.

RESULTS AND DISCUSSION

Physiologically active bacteria in river

Figure 1 shows the number of total and culturable bacteria in the Niida River between Jan. and Oct. 2005. Total bacteria were counted by DAPI and BacLight (live + dead) staining. Enumeration of culturable bacteria was performed using the two culture media, R2A and PGY medium. The total bacterial numbers were $2 \times 10^7 \sim 3 \times 10^{10}$ cells/mL, whereas colony forming units were $3 \times 10^4 \sim 4 \times 10^5$ cfu/mL. DAPI or BacLight staining detected $10^3 \sim 10^6$ -fold more bacterial counts than the conventional plate counting method. Figure 2 shows the percentage of active bacteria determined by four direct fluorescent counts to total bacteria in the Niida River. Physiologically active bacteria in the river were much higher than those obtained by plate counting. BacLight (live) staining technique resulted in the highest viable count, approximately 90% of the total bacteria were detected. Actively respiring bacteria, as determined by CTC

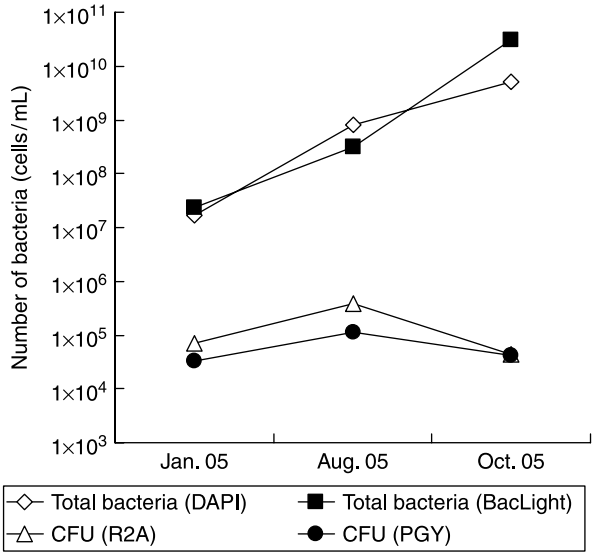

Figure 1 | Changes in number of bacteria in the Niida River.

reduction, accounted for less than 20% of the total bacteria. The direct enumeration of viable bacteria obtained by the DVC and microcolony methods was intermediate between BacLight and CTC viable counts. No significant differences were observed in bacterial enumeration for the five rivers investigated. The total bacterial number in river was $8 \times 10^6 \sim 3 \times 10^{10}$ cells/mL, and colony forming units on R2A medium were $1 \times 10^4 \sim 4 \times 10^5$ cfu/mL.

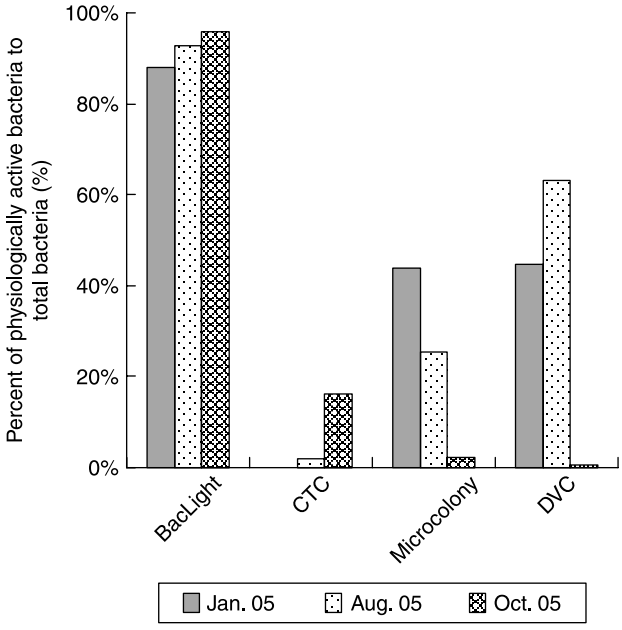

Figure 2 | Percent of physiologically active bacteria to total bacteria in the Niida River.

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Physiologically active bacteria in wastewater treatment plant

The behaviors of physiologically active bacteria were investigated in the two sewage treatment plants and the college wastewater treatment plant. Figure 3 shows the number of total and viable bacteria in M sewage treatment plant. 1 ~ 10% of total bacterial cells in municipal wastewater, secondary and chlorinated effluent could form colonies. These results were similar to those obtained from other treatment plants. It has been shown that the proportion of culturable bacteria in wastewater was significantly higher than that in river. Figure 4 shows the percent of viable bacteria determined by four direct fluorescent counts to total bacteria in M sewage treatment plant. Bacteria with no damaged membrane detected by BacLight (live) staining represented less than 40% of the total direct counts, other viable counts were always lower than BacLight counts. In three treatment plants, the numbers of viable bacteria were almost intermediate between total and culturable counts. The college wastewater treatment plant with low F/M ratio reduced all the viable counts only to less than 20% of the total bacteria.

River water incubation

River water was incubated to monitor the growth and survival of bacteria inherent in river with shaking at 20°C for more than 60 days. Figure 5 shows the number of total and culturable bacteria during incubation periods. The level of DAPI direct counts remained at approximately that of the initial concentration of bacteria throughout the course of the experiment. At the beginning of the experiment, culturable counts on R2A medium were ca.10^4 cfu/mL, almost 5 log units lower than the DAPI counts. A rapid increase (1 log unit) in culturable counts was observed after incubation for 3 days. Since the temporary rise, the number of culturable bacteria had gradually declined approximately 1.5 log units for 64 days. As shown in Figure 6, four fluorescent viable counts also increased above the initial levels within 15 days. These rises in culturable and viable counts indicate that a part of non-culturable bacteria recovered culturability, because the DAPI counts were almost unchanged over the course of the experiment. The recovery may be caused by a change in temperature, resulting from the transfer from the river to the incubation chamber. After incubation for 28 days, all the direct viable counts were less than 10% of the total bacteria, and those determined by the DVC and microcolony techniques showed a subsequent decrease to below 10^4 cells/mL.

Chlorine disinfection

The effect of chlorine on the physiological viability of *E. coli* was investigated. Before chlorine treatment, the total DAPI counts were approximately 1.0 log units higher than the

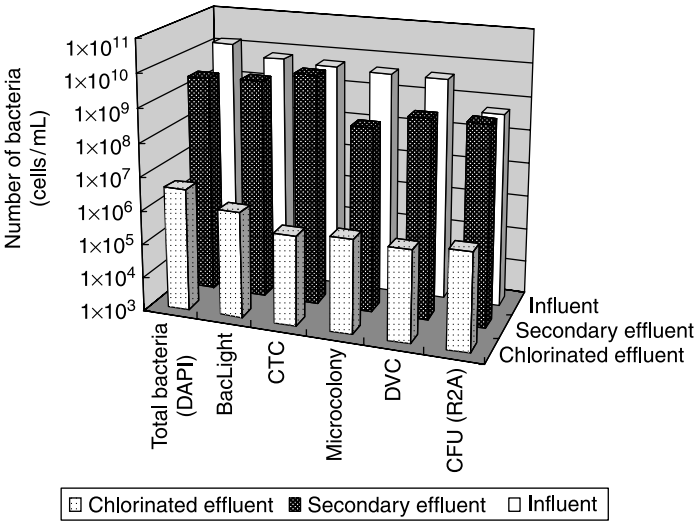

Figure 3 | Bacterial number in M sewage treatment plant.

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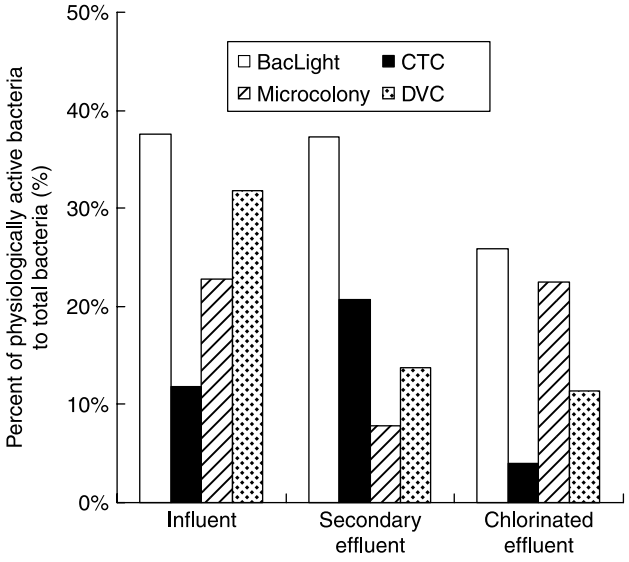

Figure 4 | Percent of physiologically active bacteria to total bacteria in M sewage treatment plant.

colony counts on R2A medium, and four direct viable counts lay between these two bacterial numbers. The total DAPI counts were not influenced by an increase in chlorine dosages, while direct viable counts decreased up to 3 log units with increasing chlorine concentrations. Culturable cells were the most sensitive to chlorine, and showed a decline of 6 log units at the residual chlorine concentration of 1.2 mg/L. It was found that direct viable cells were even more chlorine resistant than culturable cells. These results agree with the biofilm disinfection by Yu & McFeters (1994).

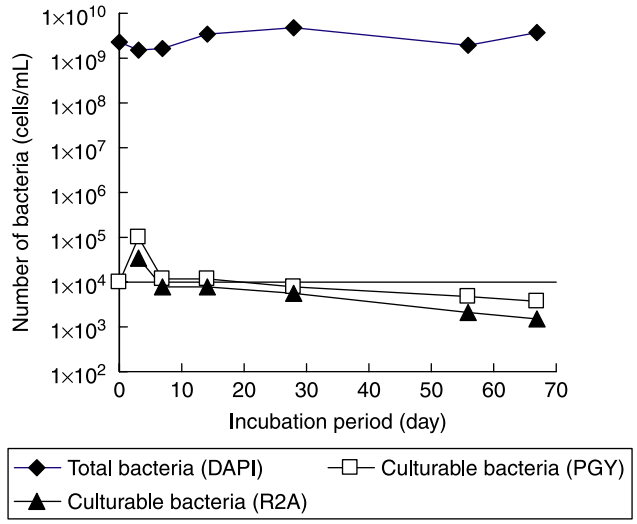

Figure 5 | Survival of total and culturable bacteria in the river water.

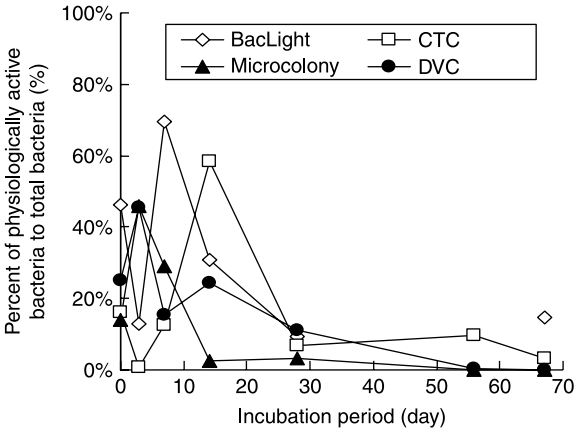

Figure 6 | Survival of physiologically active bacteria in the river water.

Applying a simple first-order reaction to the microbial inactivation led to the relationship between the inactivation rates and *CT* (chlorine concentration × contact time) values described in Equation (1).

$$\mathrm{Ln}(N/N_0) = -kCT \quad (1)$$

N; bacterial number after chlorine treatment
N_0; bacterial number before chlorine treatment
k; inactivation rate coefficient
C; residual chlorine concentration
T; contact time

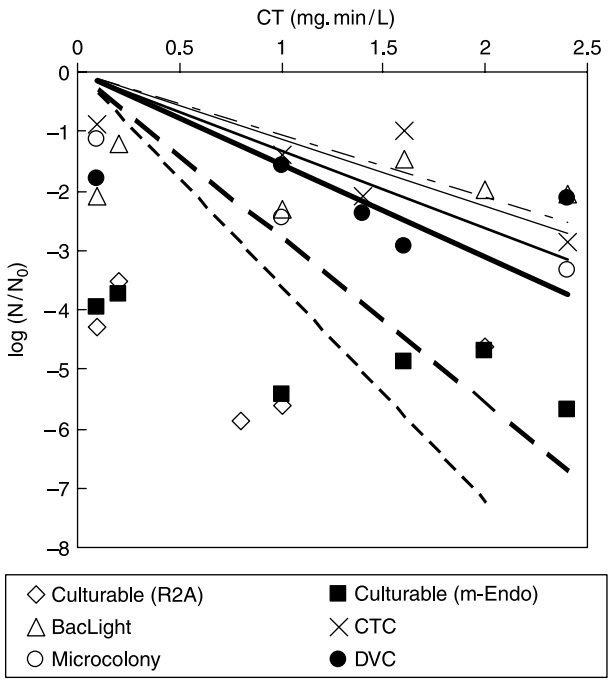

Figure 7 | Effects of chlorine on the physiological activity of *E. coli*.

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Table 2 | Inactivation rate coefficients of *E. coli* treated by chlorine

Inactivation rate coefficient	*k* (L/mg/min)
Culturable (R2A)	8.3
Culturable (m-Endo)	6.4
BacLight	2.3
CTC	3.0
Microcolony	3.7
DVC	3.0

Figure 7 shows the relationships between the inactivation rates and *CT* values. The inactivation rate coefficients calculated are given in Table 2. The inactivation rate coefficients of direct viable cells were one-second to third those of culturable cells. Results obtained in this study suggested that conventional plate counting overestimated the efficacy of disinfection treatment.

CONCLUSIONS

Several fluorescent staining techniques were applied to determine the proportion of physiologically viable bacteria in the water environment and estimate the actual efficacy of disinfection with chlorine. A great number of bacteria in the water environment remained physiologically active, while most could not form colonies on conventional media. Microscopic viable *E.coli* cells were even more chlorine resistant than culturable cells. The inactivation rate coefficients of direct viable cells were one-second to one-third those of culturable cells. These findings will require us to reconsider whether conventional culture methods are sufficient to assess water quality in aquatic environment and disinfection efficacy in order to ensure public health safety.

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