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Application of Epifluorescence Microscopy for Measurement of Bacterial Population in Water Supplies

Young-Hwan Rhee* and Seung-Yee Shin**

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用水中 細菌計數를 위한 螢光檢鏡法의 應用

李 英 煥*·愼 升 包**

Abstract

Methods for the measurement of aquatic bacteria can be divided into two groups. The first group of these methods is based on the 'replicon' concept that live bacterial cells, when diluted and transferred to a suitable medium, produce colonies. These methods distinguish living from dead bacteria, but they massively underestimate bacterial numbers. The second group of enumeration methods uses visual counting technique using specific apparatus such as a microscope. These methods are generally direct and simple, but it is very hard to distinguish between live and dead bacteria and between small particle and bacteria. Recently developed technique in staining methods has provided a reliable method of visual determination of aquatic bacteria. This uses epifluorescence microscopy to measure the total bacterial population. In order to present the fluorescence microscopy as a new methodology for the determination of bacterial numbers in water supplies, data were obtained from chlorine and monochloramine doses added to samples. Total counts by fluorescence microscopy were compared with standard plate count method. The total number of bacteria in water supplies can be determined with fluorescence microscopy. This technique allows better resolution of small bacteria and differentiation of particle from bacteria. Chloramine was found to persist longer in natural waters and prevent bacterial regrowth.

Introduction

The high quality of drinking water is being emphasized more so today than ever because of its disease producing potential. It has been recognized for quite some time that the microbial water quality is often related to spread of disease. However, the microbial ecology of water supplies that influences the water quality changes have not been examined systematically.

Methods for the enumeration of bacteria in aquatic environment can be divided into two groups. The first group of enumeration methods is based on the 'replicon' concept that live bacteria cells, when

**Department of Civil Engineering, College of Engineering, University of Missouri-Columbia, USA*
(Presently: Department of Agricultural Chemistry, Chonnam National University, Kwangju, Korea;
전남대학교 농대 농화학과)
***Department of Food Science & Nutrition, Collge of Agriculture, University of Missouri-Columbia, USA*

diluted sufficiently and transferred to a suitable medium, produce colonies.[1] These methods distinguish living from dead bacteria, but they massively underestimate aquatic bacterial population because the media most commonly used do not allow the growth of all bacteria present, and also multitude of bacteria in a clump produce only one colony. The second group of bacteria enumeration methods uses visual counting technique using specific apparatus, such as a microscope using different staining methods.[2] These methods are comparatively simple and direct, but it is very hard to distinguish between live and dead bacteria, and their accuracy depends on the extent of dead bacterial cells present in the sample. It can be difficult to define death in bacteria as well as in other biological forms, but enumeration is facilitated by the fact that dead cells tend to lyse. Recent advances in staining methods has provided a reliable method of visual determination of bacteria in an aquatic system. This method uses fluorescence microscopy[3,4,5] to determine the total bacterial numbers in water supplies.

To produce a high quality water, the entire bacterial population of water supply should be known with more precision. That is, more attention should be given to determine the total numbers of bacteria present in a sample instead of a certain indicator groups having a certain biochemical trait.

The principal objective of the present study was: 1) to present the fluorescence microscopy as a new methodology for the measurement of bacterial population in water supplies, and comparing it with standard bacterial plate count method commenly used; and 2) to evaluate this method for determining the disinfection efficiency of disinfectants, chlorine and chloramine.

Materials and Methods

A. Sampling and culturing procedure with disinfectant

The batch studies were conducted to observe the changes of bacterial numbers following the application of disinfectants, chlorine and chloramine, under aerobic conditions at room temperature. For this study, water samples were taken from the surface of Flat Branch Creek, Columbia, Missouri in USA. These samples were stirred with a magnetic stirrer in Erlenmeyer flask respectively. Requisite amounts of chlorine and chloramine were added to these flasks, and the initial pH, alkalinity, hardness, bacterial numbers of each sample were measured immediately. Commercial bleach (sodium hypochlorite) was used as the source of hypochlorous acid while monochloramine was prepared using ammonium chloride and commercial bleach solution. Temperature, pH, and disinfectant residuals were determined as a function of time. Bacterial counts were determined by two different methods, fluorescence microscopy and the standard plate count method.[1] Several drops of 1 molar sodium thiosulphate solution were used for neutralizing the disinfectant residuals in samples taken for standard plate counts.

A 1 ml seed of *Escherichia* (*E. coli*) was inoculated into 10 ml of lauryl sulphate lactose broth and incubated for 24 hours at 32°C. Then, pour technique using standard plate count agar was used for counting the *E. coli* after 48 hours of incubation at 32°C.

B. Direct filuorescence microscopy

Membrane filters (0.2 μm pore size, 25 mm diameter, Nucleopore Co.) were stained before use by soaking 2 to 24 hours in a solution of 0.2% irgalan black dye(Chemical Index; acid black 107) dissolved in 2% acetic acid. After being dyed, each filter was individually rinsed by dipping several times in distilled water. Then, it was dried on absorbent filter paper and stored for subsequent use. These filters were autoclaved before use.

It was essential that the bacteria were examined while still moist, so the filters needed to be counted within an hour. Otherwise, it was possible to use formaldehyde to preserve the samples for several days or weeks,[3,5] and to prevent bacterial numbers to change during the time of sample collection and measurement.

The water sample containing 2% final concentration of formaldehyde were stained with acridine orange (AO) to give 0.01% solution, then incubated for 2 or 3 minutes, and filtered through membrane filters.

After the last drop of water had passed through

the filter, the damp filter was placed on a microscope slide and fresh type B Cargille immersion oil was placed on it. A cover slip was placed over it and immersion oil was added again. These preparations were kept in refrigerator until bacterial numbers were counted.

The number of bacteria per milliliter were estimated from a count of at least 10 randomly chosen microscope fields and a total of at least 200 bacteria were counted. It was attempted to keep 20 to 50 bacteria per each microfield. An eyepiece micrometer disk ($60\times90\ \mu m^2$) was employed for these counting. All bacterial shapes different from debris, yeasts, fungi, etc, were counted.

The microscope used in this investigation was a Leitz Ortholux equipped with an MPV microscope photometer and a fluorescence vertical illuminator. The light source was a 150W mercury lamp. A heat absorbing filter and both a TK 495 nm and a K 495 nm filter were employed. Bacteria were counted through a 95X fluorite oil immersion objective coupled with 10X eyepiece to provide a total magnification of 950X. A Leicaflex SL 35 mm camera was used for taking microphotographs.

C. Analytical methos

Standard methods[1] were employed for the measurements of alkalinity, pH, hardness, concentration of disinfectant residues. Amperometric titration method was used to determine disinfectant concentration.[1]

Results and Discussion

Routine microscopic counts were made using the epifluorescent technique as described earlier with a total magnification of 950X after pretreatment with formaldehyde. Fig. 1 shows the appearance of a typical field of an acridine orange-stained sample under the epifluorescence illuminator. Both active and dead cells are seen as red in color whereas inactive cells fluoresce green.[3] Some of the cells are blurred because they are not in the same plane of focus. The greatest advantage of this method is the ease of differentiation of bacteria from debris and other organisms as reported by previous investigators.[6]

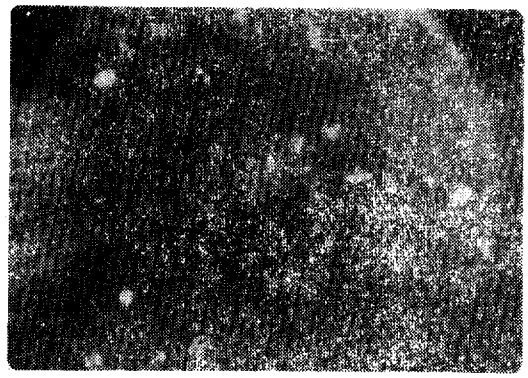

Fig. 1. Appearance of a typical field of an acridine orange-stained bacteria under epifluorescence(magnification 950X)

Table 1. Enumeration of pure cultures of *E. coli* by standard plate count(SPC) and acridine orange direct count (AODC) (Incubation temperature for the plate counts was 32±1°C)

Replication	Enumeration of *E. coil* (cell counts/ml)	
	AODC	SPC
1	6.88×10^7	6.60×10^7
2	8.37×10^7	8.00×10^7
3	1.01×10^8	9.90×10^7

In order to assess this method, *E. coli* was cultured in laboratory. Assuming that almost all of the *E. coli* cells were alive and that there were few unlysed cells under the culturing conditions, the total counts, including the red and green fluorescing cells of *E. coli* determined by this method, were compared to results obtained using standard plate count method as shown in Table 1. Even though total counts (AODC) by fluorescence microscopy were not perfectly consistent with viable counts (SPC), the two methods gave essntially the same results. These results are in agreement with those reported by Ecker.[7] Therefore, the assumption that all the bacteria were stained rapidly and specifically and transferred without loss to the membrane filter surface where they were counted was justifiable.

In order to examine the inhibition effects of different disinfectants on the bacterial growth in the batch tests using fluorescence microscopy and standard plate count method, data were obtained from

Table 2. Analysis of Flat Branch Creek water (May 19, 1981)

Temperature:	21°C
pH:	7.7
Alkalinity:	3.8 meq/l
Hardness:	4.8 meq/l
SPC:	2.0×10^4/ml
AODC:	2.3×10^5/ml

different chlorine doses (2.0 and 4.0 mg/l) and monochloramine (2.2 mg/l) added to samples. Table 2 shows the sample water quality before disinfection while Table 3 shows the data obtained from the each batch test after disinfection. The data of Table 3 were also plotted in curves as shown in Fig. 2. Significant inhibition was observed in total and viable counts during the first 30 minutes using chlorine dosages of 2.0 mg/l and 4.0 mg/l. With the disappearance of the chlorine residuals, the bacteria began to regrow. As compared to free chlorine, chloramine is a much less reactive compound. Therefore, it persists over a much longer period of time in aquatic environments. The effect of the persistence of chloramine is reflected in the continued reduction in viable counts. In the present study, chloramine was found to be as effective as chlorine initially and superior to chlorine in preventing bacterial regrowth.

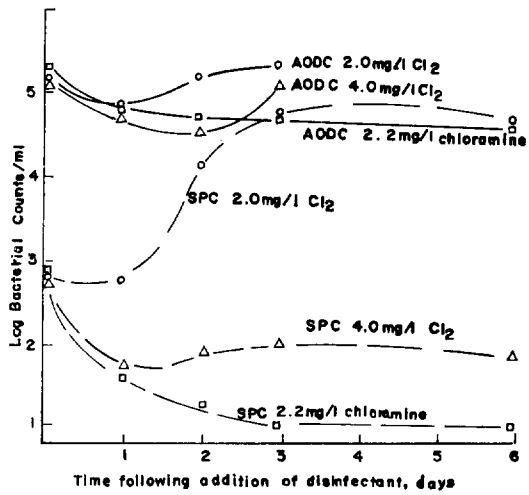

Fig. 2. Disinfection of Flat Branch Creek water with chlorine and chloramine Enumeration of bacteria by acridine orange direct count (AODC) and standard plate count (SPC)

Table 3. Disinfection of Flat Branch Creek water with chlorine and chloramine. Enumeration of bacteria by acridine orange direct count(AODC) and standard plate count(SPC)

Disinfectant applied (mg/l)	Elapsed time (days)	Disinfectant residual (mg/l)	SPC (number/ml)	AODC (number/ml)
Chloramine, 2.2	0.02	2.2	640	170000
	1	1.8	32	57000
	2	1.6	21	48000
	3	1.3	<10	42500
	6	1.2	<10	32000
Chlorine, 2.0	0.02	0.3	620	153000
	1	Trace	520	71000
	2	ND	12600	141780
	3	ND	51000	198000
	6	ND	37000	NM
Chlorine, 4.0	0.02	1.8	540	113000
	1	0.2	45	42500
	2	ND	70	28000
	3	ND	80	113000
	6	ND	60	NM

NM: Not measured
ND: Not detected

Temperature: 24~25°C
pH: 7.8~8.0

要 約

水中微生物의 計數方法은 크게 둘로 나누인다. 첫째 方法은 replicon槪念에 基礎를 둔 것으로 살아있는菌과 죽은 菌을 구별할 수 있으나 使用되는 培地가 生理的으로 다른 多樣한 細菌들로 구성된 水中細菌의 生育에 적합하지 않아서 全 細菌數를 計數할 수 없다.

둘째 方法은 직접 검경하여 計數하는 方法으로 살아있는 菌과 죽은菌, 細菌과 particle의 區別이 곤란하다 그러나 최근 細菌染色術의 發達로 水中細菌을 肉眼으로 쉽게 區別하여 計數할 수 있는 方法이 可能하게 되었다. 이 方法은 螢光顯微鏡을 使用하여 acridine orange로 染色된 水中의 全細菌數를 測定하는 方法이다.

따라서 本 硏究는 上水道源의 細菌汚染度를 測定하는데 새로운 方法으로서 epifluorcscence microscopy를 提示하는데 있으며 이의 使用性可能與否를 診斷하기 위해 chlorine과 chloramine을 試料水에 處理하여 경시적으로 細菌數를 調査하여 平板法과 比較하였다.

試料水(sample water)의 全細菌數는 螢光檢鏡法에 依해 正確히 測定되었으며 分離能力(resolution)에 있어서 卓越하였고 또한 經濟的이고도 간편하였다. 그리고 消毒殺菌劑로는 chloramine이 鹽素보다 水中細菌에 미치는 影響은 훨씬 컸다.

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