The Journal of Microbiology, September 2003, p.262-265 Vol. 41, No. 3

NOTE

Freezing Seawater for the Long-term Storage of Bacterial Cells for Microscopic Enumeration

Jung-Ho Hyun[1,*] and Eun-Jin Yang[2]

[1]*Marine Microbiology Laboratory, Korea Ocean Research and Development Institute, Ansan, P.O. Box 29, Seoul 425-600, Korea*
[2]*Department of Oceanography, Inha University, Incheon 402-751, Korea*

(Received July 10, 2003 / Accepted August 18, 2003)

Although enumerating bacterial cells is a fundamental step in understanding microbial ecosystems in marine environments, substantial decrease in bacterial counts with increasing sample storage time hampers the accurate estimation of bacterial biomass. We compared the variations in bacterial cell numbers caused by freezing and thawing of sample bottles or slides. Bacterial counts of seawater samples frozen only once in a sampling bottle yielded approximately 95% of the original numbers after 90 days, whereas 80% of the original count was obtained for samples prepared on slides. Only 67% and 58% of the original counts were recovered in samples repeatedly frozen and thawed in bottles or on slides, respectively. The results indicated that freezing a seawater sample in a bottle increased the consistency of the epifluorescence microscopic enumeration of bacterial cells.

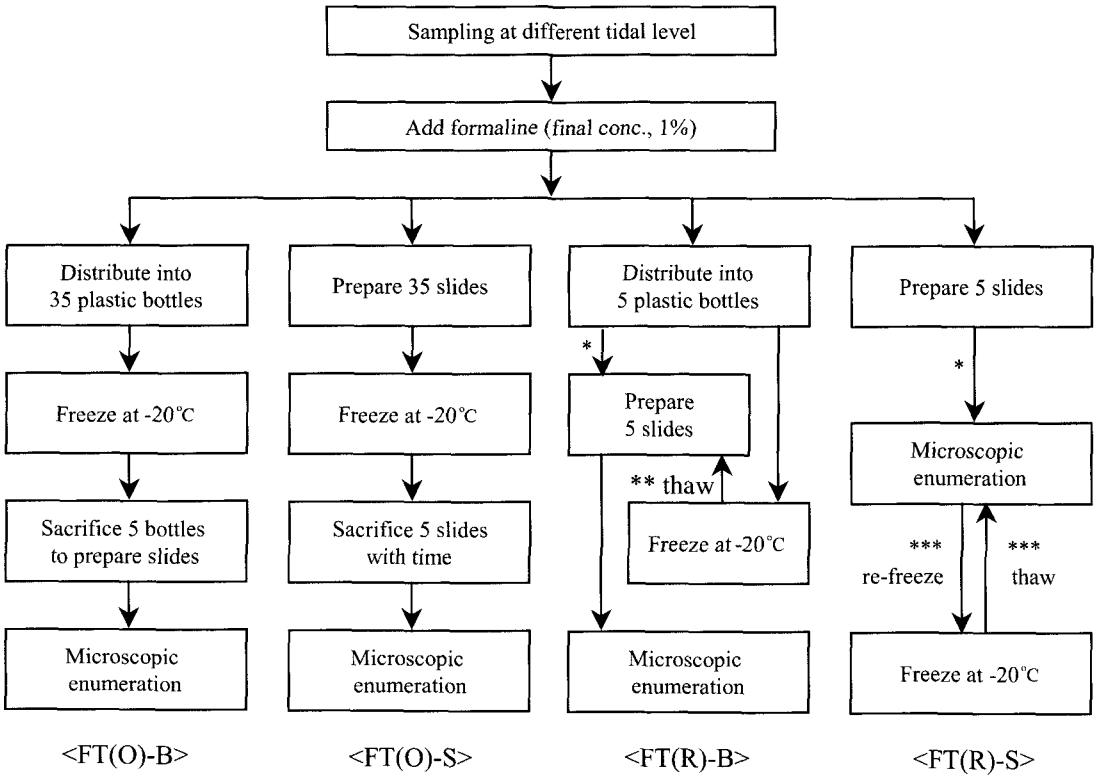
Key words: freezing seawater, storage of bacterial cells, microscopic enumeration

Bacteria in the marine environments play a significant role as both a trophic link and as a carbon sink for photosynthetically fixed organic carbon in the microbial food web (Pomeroy, 1974; Azam *et al.*, 1983). Generally, bacterial numbers in the surface mixed layer of the ocean vary from 0.2 to 3.0×10^6 cells/ml (Turley, 1993), and bacterial biomass constitutes the majority of the microbial biomass (Fuhrman *et al.*, 1989; Caron *et al.*, 1995). Therefore, enumerating bacterioplankton is an essential step in the study of aquatic planktonic ecosystems.

The general procedure used to enumerate bacterial cells consists of: (1) sampling water, preserving it with fixatives, and storing it in the field; and (2) filtering it onto black polycarbonate filters (0.2 μm pore size) after staining with a fluorochrome dye, such as acridine orange or 4,6-diaminidino-2-phenylindole (DAPI), and then counting the cells using epifluorescence microscopy in the lab (Hobbie *et al.*, 1977; Porter and Feig, 1980). Although the optimal conditions for each procedure and the use of fluorochrome dyes have been thoroughly reviewed (Kepner and Pratt, 1994), the storage of water samples after adding the fixatives remains problematic. Porter and Feig (1980) proposed the storage of water samples at 4°C, but we have frequently experienced a substantial decrease in bacterial cell numbers with prolonged storage at 4°C, as it has been reported in several papers (Turley and Hughes 1992; Kepner and Pratt 1994). On the other hand, Turley (1993) reported that prepared slides of bacterial samples could be stored frozen at -20°C for up to 70 days without a significant loss in cell numbers, but it is generally difficult to prepare the slides during an oceanic cruise over a short sampling distance. Furthermore, it may take several weeks before the samples arrive at a lab. Consequently, we speculated that freezing seawater samples would more effectively preserve bacterial cells. Interestingly, none of the papers dealing with sample storage (see Turley, 1993; Kepner and Pratt, 1994) tested freezing seawater samples in sampling bottles. Here, we report that freezing offers a promising means to keep cell numbers in seawater samples constant during long-term storage.

Experiments were performed on March 14, 15, and 26, 2002, near Palmi Island, at the mouth of Gyeonggi Bay (37°21'N; 126°29'E) and the inner harbor of the port of Incheon. The surface water temperature ranged from 6.2 to 7.9°C and the salinity from 30.1 to 30.5 psu. Since the area is characterized by a huge tidal range (*i.e.*, 3 m during a neap tide and 8 m during a spring tide), which markedly affects bacterial growth and viability (Hyun *et al.*, 1999), we collected water samples at high and low tide at Palmi

* To whom correspondence should be addressed.
(Tel) 82-31-400-6244; (Fax) 82-31-406-2495
(E-mail) jhhyun@kordi.re.kr



* Samples at time zero.
** Samples were repeatedly frozen and thawed for the preparation of slides at each time point.
*** Slides were repeatedly frozen and thawed at each time point.

Fig. 1. Schematic outline of the experimental design. FT(O)-B and FT(O)-S denote samples frozen and thawed only once in a bottle or on a slide, respectively, while FT(R)-B and FT(R)-S denote samples frozen and thawed repeatedly in bottles and on slides, respectively.

station. The concentration of suspended particulate matter (SPM) ranged from 35.2 mg/l at high tide to 55.3 mg/l at low tide.

Surface water samples for enumerating bacterial cells were preserved with formalin (final conc. 1%) and stored in an ice chest. A total of 4 manipulations were performed in the laboratory within 3 h of water sampling (Fig. 1). First, seawater samples were dispensed into thirty-five 20-ml plastic bottles, and frozen at -20°C. Five bottles were used for cell counts at the designated times. These samples were, therefore, frozen and thawed only once in a bottle (FT(O)-B, where O=once and B= bottle). Second, 35 slides were prepared at once, and frozen at -20°C. Five slides were used at each time point for bacterial counts. These samples were frozen and thawed only once on slides (FT(O)-S, where S=slide). For the third treatment, samples were dispensed into five 20-ml plastic bottles. Five slides were prepared from each bottle for the bacterial counts, and the reminder of the water in the bottles was re-frozen until the next time point (FT(R)-B). For the fourth treatment, five slides were prepared and used for bacterial cell counts repeatedly at each time point (FT(R)-S). For samples FT(R)-B and FT(R)-S, the re-frozen bottles or slides were thawed at the time of the next cell count. The bacterial cell numbers corresponding to each treatment were measured in five replicates. To prepare the slides for microscopic enumeration, the samples were stained with DAPI, filtered through Nucleopore filters (0.2-μm pore size, black), and mounted on slides with immersion oil (Cargille type A). Bacteria were enumerated using an epifluorescence microscope (Nikon type 104) equipped with a mercury lamp (HB-10101 AF), a UV excitation filter, and a BA 420 barrier filter.

Of all the manipulations, bacterial counts of the samples frozen only once in bottles (FT(O)-B) yielded the most consistent numbers during long-term storage (Table 1). Bacterial cell counts in FT(O)-B remained consistent for at least 3 months for samples collected at high tide (low SPM), and for 3 weeks for samples collected at low tide (high SPM). The lower cell counts after 3 weeks in water samples collected at low tide may be associated with the attachment of bacterial cells to particles. Weinbauer *et al.* (1998) reported that counts of cells stained with DAPI were low because the background fluorescence was high for samples containing a high detritus or sediment content. By contrast, bacterial cell counts in FT(O)-S were significantly lower than the original counts after 1 or 2 weeks and the bacterial cell counts in both FT(R)-B and

Table 1. Counts of bacterial cell (cells/ml) in bottles and on slides that were subject to be frozen/thawed only once at each time or frozen/thawed repeatedly at each time point. Numbers in the parentheses indicate standard deviation

Manipulation : Frozen and thawed repeatedly				
Location	Elapsed days	n	in bottles (FT(R)-B)	on slides (FT(R)-S)
Palmi Island (High tide)	0	5	9.53 (± 0.79)	9.49 (± 0.78)
	7	5	9.63 (± 0.66)	8.57 (± 0.27)
	14	5	9.50 (± 0.71)	8.26 (± 0.54)*
	21	5	9.59 (± 0.73)	8.10 (± 0.62)*
	30	5	9.58 (± 0.74)	8.08 (± 0.56)*
	60	5	9.58 (± 0.76)	7.92 (± 0.40)*
	90	5	9.55 (± 0.56)	7.98 (± 0.32)*
Palmi Island (Low tide)	0	5	12.70 (± 0.41)	12.70 (± 0.41)
	7	5	12.56 (± 0.95)	12.67 (± 0.87)
	14	5	12.25 (± 0.52)	12.03 (± 0.90)*
	21	5	12.07 (± 1.08)	10.98 (± 0.67)*
	30	5	11.39 (± 0.72)*	10.66 (± 0.93)*
	60	5	11.51 (± 0.75)*	10.05 (± 0.64)*
	90	5	11.35 (± 0.66)*	10.00 (± 0.35)*
Palmi Island (Low tide)	0	5	12.06 (± 0.74)	12.06 (± 0.74)
	7	5	11.73 (± 0.72)	12.04 (± 0.97)
	14	5	11.70 (± 1.15)*	11.61 (± 0.79)*
	21	5	10.92 (± 0.58)*	10.95 (± 0.54)*
	30	5	9.92 (± 0.64)*	10.66 (± 0.79)*
	60	5	8.71 (± 0.48)*	8.91 (± 0.45)*
	90	5	8.24 (± 0.56)*	8.07 (± 0.39)*
Incheon port (High tide)	0	5	14.17 (± 0.68)	14.17 (± 0.68)
	7	5	14.14 (± 1.13)	11.89 (± 1.08)*
	14	5	13.48 (± 0.90)*	10.87 (± 0.89)*
	21	5	11.89 (± 0.85)*	10.37 (± 0.74)*
	30	5	10.23 (± 0.70)*	8.73 (± 0.80)*
	60	5	9.18 (± 0.86)*	7.18 (± 0.43)*
	90	5	9.20 (± 0.53)*	7.09 (± 0.62)*

*significantly different from the original counts as determined by t-test ($P<0.05$).

FT(R)-S decreased significantly after 1 week.

When we plotted the average cell count for each manipulation, no significant change with time was observed for FT(O)-B (Fig. 2). Approximately 95% of the original numbers were counted after 90 days, while 80% were counted for FT(O)-S. Both FT(R)-B and FT(R)-S showed a consistent decrease in cell count, to 67 and 58% of the original counts after 90 days, respectively. In particular, there was a steep decrease in repeatedly frozen and thawed samples in bottles (FT(R)-B) after 2 weeks.

Since Turley (1993) reported that prepared slides of bacterial samples may be stored frozen at -20°C for up to 70 days with no significant loss in cell numbers, frozen slides have remained the method of choice for sample storage for bacterial counts (Kepner and Pratt, 1994). However, our results indicate that cell counts from frozen slides remain constant for only up to 2 weeks, which implies that frozen slides are not the best choice for sample storage during a long cruise. We also demonstrated that bacterial cell numbers were significantly underestimated after the repeated freezing and thawing of seawater samples both in bottles and on slides. In conclusion, our results indicate that freezing seawater is an easy and effective way for long-term storage of water samples for bac-

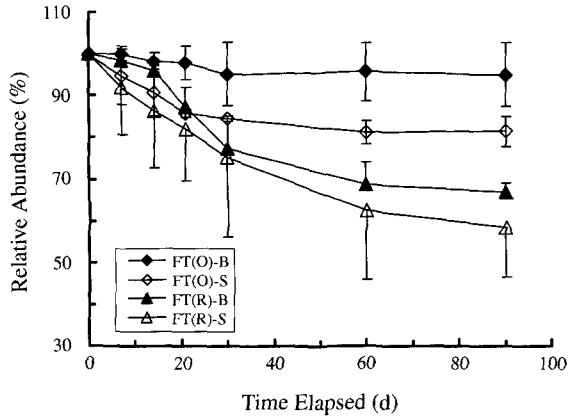


Fig. 2. Relative abundance of bacterial cells with time as compared with the first counts in the lab. Each data point is an average of five replicates for each treatment. Bars indicate the standard deviation of the average. The abbreviations are the same as used in Fig. 1.

terial counts on most oceanic cruises with short sampling distance.

This research was partially supported by the regional research center for coastal environments of the Yellow Sea at Inha University, funded by MOST and KOSEF (PN-49700), and by the basic research program of the Korea Ocean Research and Development Institute (PE83500). We thank two reviewers for their helpful comments on the early version of this manuscript.

References

Azam F., T. Fenchel, J.G. Field, J.S. Gray, L.A. Meyer-Reil, and F. Thingstad. 1983 The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* 10, 257-263.

Caron, D.A., H.G. Dam, P. Kremer, E.J. Lessard, L.P. Madin, T.C. Malone, J.M. Napp, E.R. Peele, M.R. Roman, and M.J. Youngbluth. 1995. The contribution of microorganisms in particulate carbon and nitrogen in surface waters of the Sargasso Sea near Bermuda. *Deep-Sea Res.* 42, 943-972.

Fuhrman, J.A., T.D. Sleeter, C.A. Carlson, and L.M. Proctor. 1989. Dominance of bacterial biomass in the Sargasso Sea and its ecological implications. *Mar. Ecol. Prog. Ser.* 57, 207-217.

Hobbie, J.E., R.J. Daley, and S. Jasper. 1977. Uss of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 33, 1225-1228.

Hyun, J.-H., J.K. Choi, K.H. Chung, E.J. Yang, and M.K. Kim. 1999. Tidally induced changes in bacterial growth and viability in the macrotidal Han river estuary, Yellow Sea. *Estuar. Coast. Shelf Sci.* 48, 143-153.

Kepner, Jr., R.L. and J.R. Pratt. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiol. Rev.* 58, 603-615.

Pomeroy, L.R. 1974. The ocean's food web: A changing paradigm. *BioScience* 24, 499-504.

Porter, K.G. and Y.S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* 25, 943-948.

Turley, C.M. 1993. Direct estimates of bacterial numbers in seawater samples without incurring cell loss due to sample storage, p. 143-147. *In* P.F. Kemp, B.F. Sherr, E.B. Sherr, and J.J. Cole (eds.), Handbook of methods in aquatic microbial ecology. Lewis Publishers, Boca Raton, FL.

Turley, C.M. and D.J. Hughes. 1992. Effects of storage on direct estimetes of bacterial numbers of preserved seawater samples. *Deep-Sea Res.* 39, 375-380.

Weinbauer, M.G., C. Beckmann, and M.G. Hofle. 1998. Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. *Appl. Environ. Microbiol.* 64, 5000-5003.