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# Effects of Fixation and Storage on Flow Cytometric Analysis of Marine Bacteria

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**Flow cytometry (FCM) is now becoming a routine tool for the enumeration and optical characterization of bacteria in marine environments. We investigated the effects of sample fixation and storage upon flow cytometric determination of marine bacteria. Fixed and unfixed seawater samples were analyzed by FCM immediately aboard ship and/or later in the laboratory, and the appearances of the fluorescence signals and bacterial counts of these samples were compared. Fixation and storage led to the formation of multiple peaks in fluorescence histograms; this was also seen in 22 out of 36 samples frozen in liquid nitrogen. Fixation did not, but storage did induce a decrease of bacterial counts: a rapid decrease during the first 3 days followed by a slower decline. The decline of cell numbers in stored samples was expressed by a regression model. Our studies indicate that precaution is necessary when interpreting the data from fixed and/or stored marine bacterial samples analyzed by FCM. The possibility that the procedure of fixation and storage leads to the appearance of high DNA and low DNA bacterial groups should be considered.**

Keywords:
· Flow cytometry,
· marine bacteria,
· fixation,
· storage,
· HDNA bacteria,
· LDNA bacteria.

## 1. Introduction

Bacterial abundance is an essential parameter when we quantify the contribution of bacteria to carbon cycling and nutrient regeneration in ecosystems. Since the late '70s, total direct count methods using fluorochrome stains such as acridine orange or 4′,6-diamino-2-phenylindole (DAPI) and epifluorescence microscopy (EFM) have become standard (Kepner and Pratt, 1994), because most naturally occurring communities cannot be cultured on agar media (Kogure *et al*., 1979). There are problems with these methods, however; they are generally time consuming and differences in estimates among observers can be large (Nagata *et al*., 1989). Compared with microscopic techniques, flow cytometry (FCM) offers the advantages of higher speed, better objectivity, and greater accuracy. In addition, it is possible to separate physiologically different cells (Davey and Kell, 1996). Owing to the recent development of fluorescent dyes and a high-performance system, FCM is now becoming a routine tool in marine microbiology (Gasol and del Giorgio, 2000).

Unless water samples are analyzed immediately, they must be fixed and preserved until analysis. Samples for FCM are generally flash-frozen in liquid nitrogen (Troussellier *et al*., 1995; Marie *et al*., 1996; Gasol and del Giorgio, 2000) and stored at –80°C until analysis after fixation. If liquid nitrogen is not available, the samples may be kept in a freezer or refrigerator until analysis (del Giorgio *et al*., 1996; Lebaron *et al*., 1998; Troussellier *et al*., 1999; Button and Robertson, 2001; Jochem, 2001). Effects of preservation on bacteria in refrigerated seawater samples have been pointed out using EFM and FCM. The counts tend to decrease during storage (Turley and Hughes, 1992; Troussellier *et al*., 1995; Decamp and Rajendran, 1998; Shibata *et al*., 2006). It was suggested that bacterial attachment to the inner wall of the container (Turley and Hughes, 1992), remaining protease activity (Gundersen *et al*., 1996) or autolysis of non-nucleoid-visible cells (Vosjan and van Noort, 1998) may be the causes of bacterial loss in preserved samples. On the other hand,

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Table 1. Sampling locations, depth, date and sum of sample sizes of the seawater samples used in each experiment.

| Experiment | Sample name | | Sampling location | | Sampling depth (m) (date, day/mo/yr) | | $N$[a] |
|---|---|---|---|---|---|---|---|
| Comparison between FCM counts and EFM counts<br>Change in counts over storage time | KT-00-12[b] | Stn A | Sagami Bay | 35°00′ N, 139°20′ E | 0, 20, 75, 150, 400, 500, 600, 800, 1000 | (09/09/00) | 9 |
| | KT-00-12[b] | Stn B | Ocean side of the Kuroshio | 33°01′ N, 141°18′ E | 0, 10, 20, 30, 50, 75, 100, 150, 200, 500, 750, 1000, 1500, 2000, 2500, 3000 | (10/09/00) | 16 |
| | KT-00-12[b] | Stn C | Shore side of the Kuroshio | 33°30′ N, 138°30′ E | 0, 10, 20, 30, 50, 75, 100, 150, 200, 300, 500, 750, 1000, 1500, 2000, 2500, 3000 | (11/09/00) | 17 |
| Fixation and storage effects on FCM appearance (open ocean)<br>Change in counts over storage time | KT-01-10[b] | Stn B | Ocean side of the Kuroshio | 33°00′ N, 142°00′ E | 0 | (10/07/01) | 1 |
| | KT-01-10[b] | Stn C | Axis of the Kuroshio | 34°19′ N, 140°49′ E | 0 | (11/07/01) | 1 |
| | KH-01-3[c] | Stn 1 | South Pacific Ocean | 0°00′ S, 160°00′ E | 0 | (08/12/01) | 1 |
| | KH-01-3[c] | Stn 5 | South Pacific Ocean | 20°00′ S, 160°00′ E | 0 | (13/12/01) | 1 |
| | KH-01-3[c] | Stn 7 | South Pacific Ocean | 30°00′ S, 160°00′ E | 0 | (16/12/01) | 1 |
| | KH-01-3[c] | Stn 9[d] | South Pacific Ocean | 40°00′ S, 160°00′ E | 0 | (19/12/01) | 1 |
| Fixation and storage effects on FCM appearance (coast)<br>Change in counts over storage time | Otsuchi Bay | Stn A | Otsuchi Bay | 39°20′ N, 141°55′ E | 1, 5[e], 20 (22/05/01), 1 (30/05/02) | | 5 |
| | Otsuchi Bay | Stn B | Otsuchi Bay | 39°20′ N, 141°57′ E | 1, 7.5, 20[e], 35 | (22/05/01) | 4 |
| | Otsuchi Bay | Stn C | Otsuchi Bay | 39°21′ N, 141°59′ E | 1, 7.5, 25, 50 | (22/05/01) | 4 |
| | Otsuchi | | Pier in Otsuchi Bay | | 0 | (29/05/02) | 1 |
| | Harumi Pier | H3 | Pier in Tokyo Bay | | 0 | (26/06/01) | 1 |
| | Harumi Pier | H3 (frozen with LiqN) | Pier in Tokyo Bay | | 0 | (18/10/02) | 1 |
| Fixation and storage effects on FCM appearance (coast)<br>(frozen with LiqN) | Aburatsubo Inlet | A1, A2, A3, A4, A5 | Inlet of Sagami Bay | | 0 | (24/01/05) (21/02/05) (25/07/05) (30/11/05) (21/12/05) | 12 |
| | Harumi Pier | H1, H2, H3, H4, H5 | Pier in Tokyo Bay (Port of Tokyo) | | 0 | (27/01/05) (19/03/05) (28/11/05) (07/12/05) | 8 |
| | Daiba | D1, D2, D3, D4, D5 | Pier in Tokyo Bay (Port of Tokyo) | | 0 | (11/03/05) (15/03/05) (04/12/05) (08/12/05) (10/12/05) | 11 |
| | Yokohama | Y1, Y2, Y3, Y4 | Pier in Tokyo Bay (Port of Yokohama) | | 0 | (11/03/05) (09/12/05) | 4 |
| Comparison on counts in stored samples<br>Change in counts over storage time | KT-01-5[b] | Stn 2 | Sagami Bay | 35°06′ N, 139°22′ E | 0, 10, 20, 30, 50, 75, 100, 200, 400, 600, 800, 900 | (08/05/01) | 12 |
| | KT-01-5[b] | Stn 3 | Sagami Bay | 35°08′ N, 139°23′ E | 0, 10, 20, 30, 50, 75, 100, 200, 300, 400 | (08/05/01) | 10 |
| | KT-01-5[b] | Stn 4[f] | Suruga Bay | 34°42′ N, 138°36′ E | 0, 10, 20, 30, 50, 75, 100, 200, 500, 1000, 1500, 1900 | (10/05/01) | 12 |
| | KT-01-5[b] | Stn 6 | Suruga Bay | 34°59′ N, 138°39′ E | 0, 10, 20, 30, 50, 75, 100, 200, 400, 700, 1000 | (10/05/01) | 11 |

FCM, flow cytometry; EFM, epifluorescence microscopy; LiqN, liquid nitrogen.
[a]Sum of sample sizes.
[b]Cruise of R/V *Tansei-maru*, Ocean Research Institute, The University of Tokyo.
[c]Cruise of R/V *Hakuho-maru*, Ocean Research Institute, The University of Tokyo.
[d]Not used in the experiment on change in counts over storage time.
[e]Not used in the experiment on fixation and storage effects on FCM appearance.
[f]Not used for the comparison on counts in stored samples.

as to effects of fixation, there seems to be little investigation and agreement. Some researchers mention that green fluorescence of bacteria stained with SYBR- and SYTO-type dyes decreased after fixation in flow cytometric analysis (del Giorgio *et al*., 1996; Guindulain *et al*., 1997; Lebaron *et al*., 1998; Troussellier *et al*., 1999), and above all, del Giorgio *et al*. (1996) pointed out that the decrease in fluorescence may potentially affect estimates of abundance and also estimates of cell DNA content, which are based on fluorescence of stained cells. Unfortunately, none of these authors report their data on changes in bacterial counts, so fixation effects on bacterial abundance in seawater samples has remained obscure. Although Marie *et al*. (1999) pointed out the need for fixation of bacterial samples when a nucleic acid-specific stain is used, to our knowledge, the actual data supporting the necessity is still lacking.

Two factors, i.e., a change in fluorescence intensity and cell lysis, may cause a decrease in bacterial counts after fixation and storage, and these two factors might also affect the FCM appearance of bacterial assemblage. Many researchers have reported the presence of flow cytometric bacterial subpopulations in seawater discriminated by different green fluorescence intensity (Li *et al*., 1995; Jellett *et al*., 1996; Marie *et al*., 1997; Gasol *et al*., 1999; Yanada *et al*., 2000; Zubkov *et al*., 2001; Servais *et al*., 2003). The possible involvements of fixation and storage in the appearance of such bacterial subpopulations have never been seriously investigated.

We examined the effects of sample fixation and storage upon flow cytometric analysis on both appearance of the fluorescence signal and estimates of bacterial abundance. Fixed and unfixed seawater samples were immediately analyzed by FCM to check the fixation effects, and fixed samples were analyzed again later for the storage effects. The results suggest that the process of fixation and storage causes apparent changes in the appearance of bacterial subpopulations and a decrease in bacterial counts.

## 2. Materials and Methods

### *2.1 Experiments and seawater samples*

KT-00-12, KT-01-10 and KT-01-5 samples used in the experiments (Table 1) were collected during cruises of R/V *Tansei-maru*, and KH-01-3 samples were collected during cruise of R/V *Hakuho-maru*, of Ocean Research Institute, The University of Tokyo. Twelve-liter Niskin bottles (General Oceanics) mounted on a CTD/rosette were used for sampling at various depths in these cruises. For Otsuchi Bay samples, a 10-liter Van Dorn water sampler was used to collect seawater from several depths. Surface water samples at various locations were taken with a plastic bucket or a sterile polypropylene bottle.

#### *2.1.1 Comparison between FCM counts and EFM counts*

Seawater collected from various depths at three sampling stations during the KT-00-12 cruise were fixed and used to examine the relationships between FCM counts and EFM counts of bacteria (Table 1; KT-00-12 Stn A, B and C, n = 42). Samples for FCM were stained with SYBR Green I (SYBR I) and those for EFM were treated with SYBR I and also with DAPI. Both FCM and EFM counts were obtained after return to the laboratory.

#### *2.1.2 Fixation and storage effects on FCM appearance*

To investigate the fixation and storage effects on FCM appearance of stained bacteria, fixed and unfixed seawater samples from various locations were analyzed immediately aboard ship and/or later in the laboratory. At first, these effects were checked using the samples derived from the open ocean (Table 1; KT-01-10, KH-01-3) and the coastal region (Table 1; Otsuchi Bay, Harumi Pier). They were kept refrigerated at 4°C (n = 20). The effects were also checked afterward using the samples from the coastal region, including the fixed and cryopreserved samples with liquid-nitrogen freezing followed by storage at –80°C (Table 1; Harumi Pier, Aburatusbo Inlet, Daiba, Yokohama; n = 36). When we examined the effect of fixation, the samples were analyzed by FCM immediately after fixation (within 4 h).

#### *2.1.3 Comparison of counts in stored samples*

This experiment was done with seawater obtained from various depths at three sampling stations during the KT-01-5 cruise (Table 1; KT-01-5 Stn 2, 3 and 6; n = 33). Fixed samples were counted aboard ship at 14 to 42 h after fixation and again at 2.5 to 3 months in the laboratory by FCM in order to check any change in bacterial counts during storage.

#### *2.1.4 Change in counts over storage time*

An experiment to reveal the effect of storage time on estimates of bacterial numbers was performed with seawater collected from various locations (Table 1; KT-00-12, KT-01-10, KH-01-3, Otsuchi Bay, Harumi Pier, KT-01-5; n = 108). Bacterial cells in fixed samples were counted by FCM aboard ship and/or later in the laboratory after storage for different periods of time. Prior to the preservation, the ratio of the bacterial number immediately after fixation (within 4 h) to the number in unfixed samples was calculated to check the fixation effect on bacterial counts. Most of the seawater samples used in the experiment of fixation and storage effects on FCM appearance were also used in this check (n = 45).

### *2.2 Fixation and storage*

All fixed samples in the present study were composed of seawater treated with a combination of 1% paraformaldehyde and 0.01% glutaraldehyde. Each fixative solution was filtered through 0.2-$\mu$m-pore-size filters before use. Unless otherwise stated, unfixed and fixed

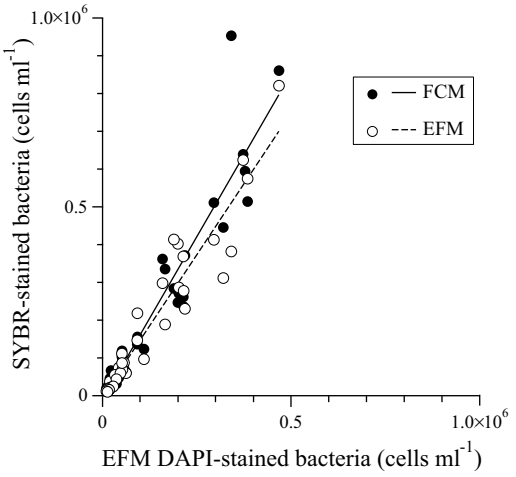


Fig. 1. Relationships between numbers of DAPI-stained bacteria counted by EFM (EFM DAPI-stained bacteria) and counts of SYBR-stained bacteria enumerated by FCM (●, FCM SYBR-stained bacteria) or EFM (○, EFM SYBR-stained bacteria). The regression equations, with EFM DAPI-stained bacteria as the independent variable, were $Y = -1.2 \times 10^4 + 1.7X$, $r^2 = 0.90$ for the dependent variable FCM SYBR-stained bacteria and $Y = -3.7 \times 10^3 + 1.5X$, $r^2 = 0.92$ for EFM SYBR-stained bacteria. Seawater samples were collected during KT-00-12 cruise and fixed.

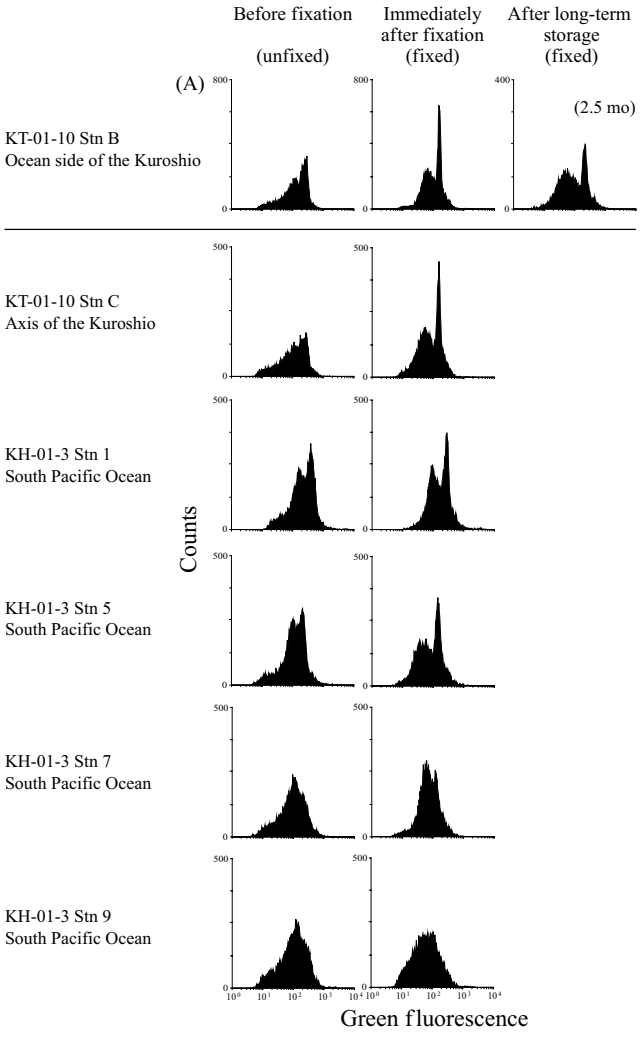


Fig. 2. Green fluorescence histograms from flow cytometric analyses targeting the bacterial assemblage of unfixed samples and fixed samples. Seawater samples were collected from surface water at six different sites in the open ocean. Fixed samples were analyzed both immediately after fixation (within 4 h) and after long-term storage (2.5 months).

samples were stored in a refrigerator at 4°C until use. Fixed and frozen samples were also prepared in the experiment to check the fixation and the storage effects on FCM appearance. After fixation, the samples for cryopreservation were frozen immediately in liquid nitrogen and stored at –80°C until use (Marie *et al.*, 1996), instead of being held at 4°C. In this study, "paraformaldehyde" was prepared from paraformaldehyde powder in our laboratory, in distinction from commercial formalin.

### *2.3 Flow cytometry*

To analyze natural bacterial assemblages by FCM, samples (1.5 ml) were stained with SYBR I (Molecular Probes) at a final concentration of $10^{-4}$ of the stock solution supplied by the manufacturer (Lebaron *et al.*, 1998) and incubated for 30 min in a dark room at ambient temperature. Just before analysis, a given concentration of 2.0-$\mu$m-diameter fluorescent beads (yellow-green carboxylate-modified, Molecular Probes) was added to samples as an internal standard.

FCM was conducted with a PAS-III flow cytometer (Partec GmbH), which has an air-cooled argon laser emitting blue light (wavelength 488 nm) at 30 mW. The threshold to trigger an event was set in the green fluorescence range, and particles were counted in logarithmic mode using FloMax software (Partec GmbH). Bacteria were distinguished from signal noise by plotting side scatter (SSC) against green fluorescence. The flow rate was set at less than 1000 events per second (Gasol and del Giorgio, 2000), and signal data were accumulated until about 20,000 events had been counted. The concentrations of bacterial cells were estimated relative to that of the beads added. For some flow cytometric histograms and analyses of signal intensity, the data were analyzed using WinMDI 2.8 software (Joseph Trotter). The geometric mean was calculated with WinMDI for comparison of the green fluorescence intensity.

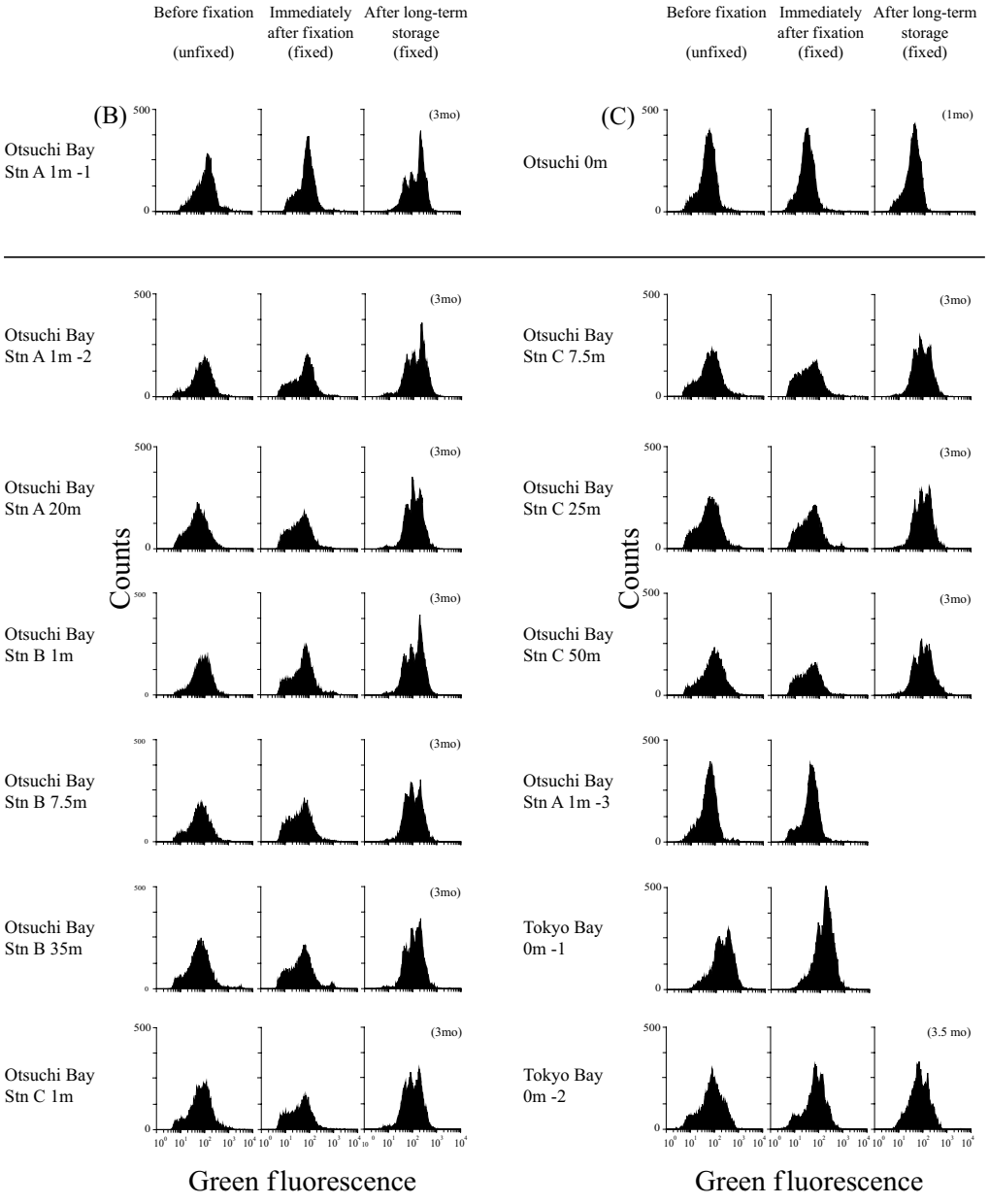


Fig. 3. Green fluorescence histograms from flow cytometric analyses targeting the bacterial assemblage of unfixed samples and fixed samples. Fourteen different seawater samples were collected from the coastal region. Fixed samples were analyzed both immediately after fixation (within 4 h) and after long-term storage (1 to 3.5 months).

*2.4 Epifluorescence microscopy*

For SYBR I microscopic counts, bacteria were trapped on 0.2-$\mu$m-pore-size aluminum oxide membrane filters (Anodisc 25, Whatman). Each filter was laid on a 100-$\mu$l drop of SYBR I solution (final conc., $2.5 \times 10^{-3}$ of the supplied conc.) in a Petri dish for 15 min in a dark room (Noble and Fuhrman, 1998). The filter was then removed, dried, and mounted with SlowFade anti-fade reagent in glycerol (Molecular Probes).

For DAPI microscopic counts, we followed the method proposed by Porter and Feig (1980) with minor modification. Briefly, samples were stained with the dye solution (final conc., 0.5 $\mu$g ml$^{-1}$) for 15 min in a dark room and then filtered through 0.2-$\mu$m-pore-size black polycarbonate membrane filters (Nuclepore). The filters were then mounted on microscope slides with immersion oil (Olympus). Bacterial cells were counted under an Olympus BH-2 or BX51 epifluorescence microscope. No

fewer than 20 fields and 400 cells were counted for each sample.

*2.5 Statistical treatment and model*

The Wilcoxon test was applied to compare bacterial counts or fluorescence intensity of stained bacteria in each experiment.

During the experiment to test change in counts over storage time, bacterial numbers in fixed samples were counted by FCM after storage. Natural logarithms were applied and fitted to the bacterial number and storage time, and the following regression model was obtained:

$$\mathrm{Ln}(Nt_n Nt_0^{-1}) = a\ \mathrm{Ln}(t_n + 1), \qquad (1)$$

where $Nt_0$ is the number of bacteria immediately after fixation, $Nt_n$ is the number of bacteria at time $t_n$, which is the duration of storage (days) after fixation. When samples could not be analyzed immediately after fixation, $Nt_0$ was calculated from the bacterial number in unfixed samples by using the ratio of $Nt_0$ to the number counted in unfixed samples obtained from other sample sets.

## 3. Results

*3.1 Comparison between FCM counts and EFM counts*

Figure 1 shows the relationships between bacterial numbers counted by FCM and EFM among fixed samples. Counts of SYBR-stained bacteria enumerated by FCM (FCM SYBR-stained bacteria) were closely correlated with those of DAPI-stained bacteria counted by EFM (EFM DAPI-stained bacteria) ($r^2 = 0.90$, $P < 0.0001$, n = 42). The intercept (b) of the regression line ($Y = b + cX$; $Y$, FCM SYBR-stained bacteria; $X$, EFM DAPI-stained bacteria) was not significantly different from zero ($P = 0.47$), and the slope (c) of the regression line was 1.7 ($P < 0.0001$). Counts of SYBR-stained bacteria enumerated by EFM (EFM SYBR-stained bacteria) were also closely correlated with those of EFM DAPI-stained bacteria ($r^2 = 0.92$, $P < 0.0001$, n = 41). The intercept (b) of the regression line for these counts ($Y$, EFM SYBR-stained bacteria; $X$, EFM DAPI-stained bacteria) was not significantly different from zero ($P = 0.77$) either, and the slope (c) of the regression line was 1.5 ($P < 0.0001$). The Wilcoxon test showed that FCM SYBR-stained bacteria were significantly different from EFM DAPI-stained bacteria ($P < 0.0001$); on the other hand, they were not significantly different from EFM SYBR-stained bacteria ($P = 0.21$).

*3.2 Fixation and storage effects on FCM appearance*

Flow cytometric histograms of SYBR I-stained bacteria are shown in Figs. 2 and 3. Three typical patterns of changes in the histogram appearance were observed after fixation and storage from 20 sets of data. For surface water collected on the ocean side of the Kuroshio during KT-01-10, the appearance of the histogram showed a drastic change immediately after fixation (within 4 h) (Fig. 2A). Although the peak with higher fluorescence intensity maintained its level of fluorescence, the peak with lower fluorescence intensity shifted lower (leftward) in fixed samples. Similar drastic changes of appearances after fixation were observed in 5 of 6 samples from the surface of the open ocean (Fig. 2). In the coastal water samples, these drastic changes in the histograms were found in 2 of 14 samples after fixation (Fig. 3). In contrast, the histograms of some samples did not change immediately after fixation, but only after long-term storage at 4°C. In the unfixed samples, one peak was observed; this peak was still present immediately after fixation, but three peaks were observed after 3 months of storage (Fig. 3B). This pattern of change in the histograms was observed in 10 of 12 data sets from the coastal samples (Fig. 3). In 1 of 12 samples from the coastal region, no change was observed in the histogram, either immediately after fixation or after long-term storage (Fig. 3C); only one peak was observed throughout the period. Unlike the green fluorescence, the histogram appearances of SSC did not change in any samples (data not shown).

The appearance of the histograms changed even among the samples that were fixed and immediately frozen in liquid nitrogen (Fig. 4). In the green fluorescence histograms of stained bacteria from the surface water of Harumi Pier in Tokyo Bay, one clear peak was observed in unfixed samples (Unfixed, Day0), but two peaks appeared in samples that were analyzed immediately after fixation, frozen in liquid nitrogen, and stored at –80°C for one hour (Fixed·LiqN, Day0). Two peaks were also observed in the samples that were fixed and stored in a refrigerator at 4°C in parallel (Fixed·Ref, Day0), and remained for 2 and 14 days (Day2 and Day4), and also for 33 and 105 days. No difference was observed in the appearance of the histogram between Fixed·Ref samples and Fixed·LiqN samples during the experiment. Including this sample, the emergences of two peaks in green fluorescence histograms after fixation were found in both Fixed·Ref samples and Fixed·LiqN samples in 22 out of 26 seawater collected from the coastal region during the fall and winter months (Oct. to Jan.). On the other hand, among the seawater samples taken during the spring and summer months (Feb. to July), the appearances of the green fluorescence histograms did not change at all after fixation in 10 seawater samples, even if the samples were collected from the same sampling locations during fall and winter.

Means of green fluorescence intensity of stained bacteria were calculated for all unfixed samples and fixed samples used in this experiment investigating fixation and

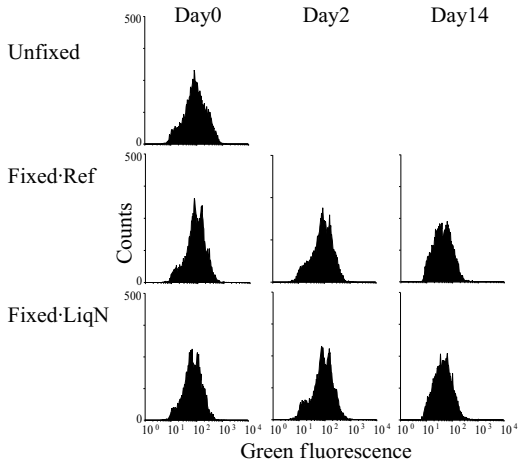


Fig. 4. Green fluorescence histograms of the bacterial assemblage of unfixed samples and fixed samples analyzed immediately after (Day0), 2 days (Day2), and 14 days (Day14) later of treatments. Fixed samples were stored in a refrigerator at 4°C (Fixed·Ref) or frozen in liquid nitrogen and stored at –80°C (Fixed·LiqN). Water samples were collected from the surface of Harumi Pier in Tokyo Bay.

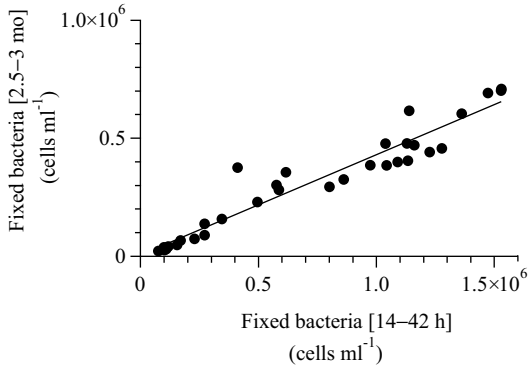


Fig. 5. Relationships between bacterial numbers counted 14 to 42 h after fixation (Fixed bacteria [14–42 h]) and those enumerated 2.5 to 3 months after fixation (Fixed bacteria [2.5–3 mo]). The regression equation, with Fixed bacteria [14–42 h] as the independent variable, was $Y = 5.0 \times 10^3 + 0.43X$, $r^2 = 0.92$ for the dependent variable Fixed bacteria [2.5–3 mo]. Seawater samples were collected during KT-01-5 cruise and analyzed by FCM.

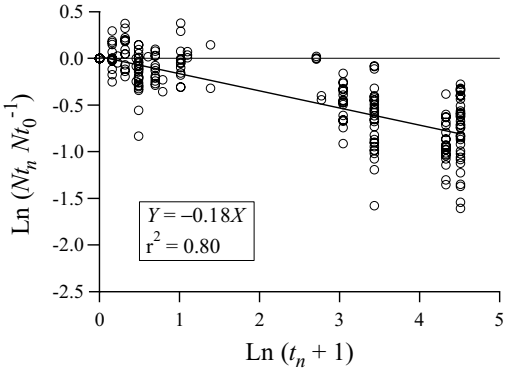


Fig. 6. Changes in the ratio of bacterial counts during storage to the initial counts relative to the duration of storage. The data were plotted using natural logarithmic scales for both axes. Seawater samples were collected from various locations (n = 108), and the plot at day 0 (immediately after fixation) consists of 108 data points. $Nt_0$, number of bacteria immediately after fixation were added; $Nt_n$, number of bacteria at $t_n$ days; $t_n$, storage time (days) after fixation.

storage effects on FCM appearance (n = 55). The fluorescence intensities decreased immediately after fixation in all seawater samples from the open ocean and the coastal region, except in three samples. The ratio of the fluorescence intensity of fixed samples to that of unfixed samples was 0.77 on average both in the open ocean samples (±0.18, 2SD (standard deviation); n = 6) and in the coastal water samples (±0.32, 2SD; n = 49). When the data of green fluorescence intensity between fixed samples and unfixed samples were compared by the Wilcoxon test, a significant difference was found in seawater samples, both of the open ocean ($P < 0.05$) and the coastal region ($P < 0.0001$). As to SSC signals, the ratio of the intensity of fixed samples to that of unfixed samples was 0.97 (±0.28, 2SD) on average.

### *3.3 Comparison of counts in stored samples*

Figure 5 shows the relationships between bacterial numbers counted 14 to 42 h after fixation (Fixed bacteria [14–42 h]) and those counted 2.5 to 3 months after fixation (Fixed bacteria [2.5–3 mo]). Counts of Fixed bacteria [14–42 h] were highly correlated with those of Fixed bacteria [2.5–3 mo] ($r^2 = 0.92$, $P < 0.0001$, n = 31). The intercept (b) of the regression line ($Y = b + cX$) was not significantly different from zero ($P = 0.81$), and the slope (c) of the regression line was 0.43 ($P < 0.0001$). The counts from these samples were compared by the Wilcoxon test, and Fixed bacteria [2.5–3 mo] were significantly different from those of Fixed bacteria [14–42 h] ($P < 0.0001$). On average, the bacterial numbers in fixed samples decreased by 60% during 42 h to 3 months of storage.

### *3.4 Change in counts over storage time*

The natural logarithms of the FCM counts from fixed samples that had been stored for different periods of time were plotted against the natural logarithm of storage time (Fig. 6). Bacterial numbers decreased rapidly during the first 3 days and then declined more slowly. The slope

($a$ = –0.18) of the model (Eq. (1)) was significantly different from zero ($P < 0.001$), and this regression model fits the data well ($r^2 = 0.80$, $P < 0.001$). From 45 data sets, the ratio of the bacterial number immediately after fixation ($Nt_0$) to the number in unfixed samples was 1.02 on average. There was no significant difference between $Nt_0$ and the bacterial number in unfixed samples (Wilcoxon test, $P = 0.31$).

## 4. Discussion

We have shown that fixation and storage affect the flow cytometric determination of marine bacterial populations. Fixation causes an instantaneous decrease in fluorescence intensity and sometimes a change in the appearance of the fluorescence histogram. Although this apparent change did not cause a significant decrease in the number of bacteria counted, it may lead to the appearance of multiple subpopulations. Even with flash freezing with liquid nitrogen immediately after fixation (within 4 h), the change in fluorescence histograms was inevitable. On the other hand, storage caused a logarithmic decline in bacterial numbers, that is, a rapid decrease in the first 3 days followed by a slower decline over a period of up to 90 days.

Among cellular components, the most susceptible ones to the fixatives may be nucleic acids and protein-DNA complexes. For instance, polynucleotides are denatured by formaldehyde (Haselkorn and Doty, 1961), and double-stranded DNA (dsDNA) is reported to be lost to some degree during preservation of *Escherichia coli* with formaldehyde (Cotner *et al*., 2001). As to the association between DNA and stains, it is known that decrease in the fragment size of the dsDNA correlates with reduced SYBR I fluorescence (Vitzthum *et al*., 1999), and that both the quantity of nucleic acid and its topology or degree of supercoiling affect the fluorescence of SYTO 13 staining (Guindulain and Vives-Rego, 2002). Therefore, denaturation or fragmentation of DNA should cause an instantaneous decrease in stain fluorescence. In addition, it is also known that formaldehyde fixation causes redistribution of histone proteins around DNA molecules (Polacow *et al*., 1976), so the protein-DNA complexes in bacteria may also be affected by fixation, resulting in structural changes in the DNA-SYBR I complex and subsequent decrease in the fluorescence intensity. As a preliminary test, we checked the fluorescence intensity of fixed and unfixed cells of *Vibrio parahaemolyticus* cultured in 0.2-$\mu$m-filterd seawater for 18 months. When stained with SYBR I, fixation induced a decrease in the cellular green fluorescence intensity of 55%. When extracted DNA was examined, the fluorescence also instantaneously decreased by 74% after the fixation. Therefore, it is obvious that apparent fluorescence intensity is affected by the complex structure of biomolecules within the cell. Lebaron *et al*. (1998) and Troussellier *et al*. (1999) suggested that fluorescence decreases in fixed cells with depolarized membranes because positively charged dyes, such as SYBR- and SYTO-type dyes, tend to penetrate living cells that are negatively charged because of their membrane potential. This cannot, however, explain all our data, although our results do not contradict the suggestion of these authors.

The changes in fluorescence must be considered when interpreting apparent fluorescence histograms of natural bacterial assemblages. The single peak in the histogram of the unfixed sample became two or three peaks after fixation or storage (Figs. 2, 3 and 4). This change in histogram appearance occurred even when the samples were quickly frozen in liquid nitrogen (Fig. 4). Flow cytometric analyses often indicate the presence of two subpopulations in seawater, called high DNA bacteria (HDNA) and low DNA bacteria (LDNA), discriminated by different green fluorescence intensity after nucleic acid staining (Li *et al*., 1995; Jellett *et al*., 1996; Marie *et al*., 1997; Yanada *et al*., 2000). Lebaron *et al*. (2001) found that HDNA and LDNA groups correspond to the actively growing fraction and inactive fraction of a natural bacterial assemblage, respectively. The ecological significance of these two groups has been recognized (Gasol *et al*., 1999, 2002; Vaque *et al*., 2001; Zubkov *et al*., 2001; Servais *et al*., 2003). In the present investigation, 36% to 51% of the total bacterial population in the fixed open ocean samples showed higher green fluorescence intensity (Fig. 2). These values are consistent with the proportions of HDNA previously reported for water from the open ocean (25%–58%; Gasol and Moran, 1999; Vaque *et al*., 2001). The values from coastal water were 69% to 78% when we calculated the proportion of bacteria in the peaks except the one with lowest fluorescence in the histogram (Fig. 3). They were also consistent with the previously reported values of HDNA from the coastal water (51%–73%; Li *et al*., 1995; Gasol and Moran, 1999; Vaque *et al*., 2001), except for only one value from Tokyo Bay (37%, Fig. 3). Therefore, our results do not contradict the general concept of HDNA and LDNA subpopulations. However, the possibility of a biased appearance of subpopulations after fixation cannot be rejected. The appearance of the two bacterial groups after sample fixation was also evident in the results of Troussellier *et al*. (1999, Fig. 1), although they did not provide any specific comments about it.

The observation of a decline in bacterial counts in fixed seawater samples was consistent with the previous studies (Turley and Hughes, 1992; Troussellier *et al*., 1995; Gundersen *et al*., 1996; Decamp and Rajendran, 1998; Vosjan and van Noort, 1998; Shibata *et al*., 2006). We calculated the rate of decrease in bacterial numbers during storage for 108 samples collected from various

environments, and obtained a slope of –0.18 with a certain degree of reliability ($r^2 = 0.80$) after logarithmic transformation (Fig. 6). Turley and Hughes (1992) found various rates of decline among seawater samples, and, because the pattern of decrease varied among samples, they concluded that one single model was not applicable to all samples. Although variations among samples were noticed, we think it more practical to propose a single model that can provide rough estimates of the rates of decline. Our model predicts that, after 3, 10, and 90 days, bacterial numbers will drop to 78%, 65%, and 45% of their initial levels, respectively. Although these declines may depend on the fixatives used and the storage conditions, our model results are reasonable in comparison with those of some previous studies: the calculated values from our regression model were not different from the values or the range in almost all cases of the previous reports which showed the proportions of the cell numbers remaining in stored samples (Table 2). Based on our regression model, 1 day (ca. 90 %) and 3 days (ca. 80%) after fixation can be regarded as the time allowable for accurate estimation of total bacterial numbers because of the high proportions to the initial bacterial numbers in fixed samples.

The FCM counts of bacteria stained with SYBR I were significantly higher than the EFM counts of those stained by DAPI in this study (slope, 1.7; Fig. 1). The relationships between FCM counts and DAPI-stained EFM counts have been previously investigated in some reports, and linear regression of FCM counts with blue nucleic acid stains (TOTO, TO-PRO, SYTO 13 or Picogreen) vs. EFM counts with DAPI have yielded slopes of the values ranging from 0.76 to 0.96 (Li *et al*., 1995; Troussellier *et al*., 1999; Yanada *et al*., 2000). We infer that a higher value of the slope in our study (1.7) results from the fact that SYBR I has higher fluorescence intensity (Lebaron *et al*., 1998) and a higher fluorescence quantum yield (product information provided by Molecular Probes Inc.) than the other blue nucleic acid stains. In addition, the higher EFM counts with SYBR-I staining than those with DAPI staining (slope, 1.5; Fig. 1) are also attributable to the fact that SYBR I emits much brighter fluorescence than DAPI (Weinbauer *et al*., 1998). The EFM counts of SYBR-I stained bacteria gave virtually the same results as the FCM counts of SYBR I (Fig. 1), so the counts by FCM and EFM will be indifferent if the same stain is used.

Two major causes may account for the apparent decrease in bacterial numbers during storage: cell lysis and a decrease in the fluorescence signal intensity. Because the activities of various enzymes in the cells may not completely cease even after fixation (Gundersen *et al*., 1996), membrane permeability may change (Bullock, 1984) and the membrane may thin (Beveridge *et al*., 1978) or become degraded (Decamp and Rajendran, 1998), resulting

Table 2. Proportion of bacterial cell numbers remaining in stored seawater samples : results from previous studies and calculated value using the regression model in this study.

| Proportion to initial number (%) Avg. (Range) [Calculated value] | Storage time | Fixative | Counting method | Sample size | Reference |
|---|---|---|---|---|---|
| 50 [54] | 29 d | 2.5% glutaraldehyde | EFM with DAPI | 1 | Gundersen *et al*. (1996) |
| 61 (25–93) [51] | 40 d | 2.5% glutaraldehyde | EFM with AO | 18 | Turley and Hughes (1992) |
| 54 (15–100) [47] | 10 wk | 2% formaldehyde | EFM with DAPI | 3 | Vosjan and van Noort (1998) |
| 56 (30–100) [45] | 3 mo | 4% formaldehyde | EFM with *Bac*Light | 4 | Decamp and Rajendran (1998) |
| 46[a] [43] | 16 wk | 2% paraformaldehyde | FCM with DAPI | 1 | Troussellier *et al*. (1995) |
| 45 (41–47)[a] [41–43] | 105–145 d[a] | 0.74% formaldehyde | EFM with DAPI | 3 | Shibata *et al*. (2006) |
| 63 (55–70)[a] [41–43] | 105–145 d[a] | 0.74% formaldehyde | EFM with SYBR I | 3 | Shibata *et al*. (2006) |
| [45] | 3 mo | 1% paraformaldehyde +0.01% glutaraldehyde | FCM with SYBR I | 108[b] | This study |

EFM, epifluorescence microscopy; FCM, flow cytometry; AO, acridine orange; *Bac*Light, LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes).
[a]Calculated from their figures.
[b]Sample size used for the regression model.

in cell lysis and the release of DNA. Even without lysis, a decrease in cell volume during storage (Fry and Davies, 1985; Turley and Hughes, 1992) may result in a decline in fluorescence intensity. An alteration of the cell structure by the fixative or as an effect of storage is expected to be one of the reasons for a change in fluorescence as well as an alteration of the structure of biomolecules within the cell. Bacterial attachment to the inner wall of the container is another possible cause of a decrease in bacterial number during storage (Turley and Hughes, 1992). In our experiments, the test tubes containing fixed seawater were always stirred vigorously before analysis in order to hold the number of attached bacteria to a minimum, so bacterial attachment cannot be a major cause of the bacterial loss shown in this study, if any.

The effects of fixation and storage are expected to vary with both cellular physiological states and taxonomical groups (Suganuma and Morioka, 1979; Vosjan and van Noort, 1998). A majority of inactive cells lost their membrane integrity (Pirker *et al*., 2005), and cells with a compromised membrane should be more susceptible to fixation. Because each sample is composed of physiologically and taxonomically different groups of microbes, the apparent effects of fixation may also vary among the samples. These heterogeneities among cells may lead to the emphasized appearance of HDNA and LDNA bacteria. In the present study, the occurrences of two bacterial subpopulations after fixation varied depending on sampling location (open ocean and coast) and sampling season. Two peaks were more often observed in the open ocean water (5 of 6 samples) than in the coastal water (2 of 14 samples). In addition, even among the coastal water samples taken from the same place, they were more frequently detected during the fall and winter months (22 of 26 samples) but not during the spring and summer months (none of 10 samples). These results seem to indicate that the fixation induces two apparent bacterial subpopulations in seawater with lower bacterial activity, which is probably related to the nutritional condition. To confirm this, we need to measure parameters related to the nutritional condition in the environments and the physiological states of bacterial populations in combination with analyses of the bacterial community structures in further studies.

In conclusion, we have confirmed the effects of both fixation and storage on the flow cytometric analysis of marine bacteria. Fixation has instantaneous effects that may cause a decline in fluorescence intensity, a change in the appearance of fluorescence histograms and an emphasized appearance of HDNA and LDNA of marine bacterial populations. The apparent cell numbers declined continuously during sample storage. Thus, samples should be analyzed as soon as possible after collection, and the duration of storage should be recorded for more accurate estimation of bacterial numbers. To our knowledge, this is the first report of a systematic examination of the effects of fixation and storage on flow cytometric analysis of aquatic bacteria.

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