

Effects of long-term sample storage on the detection of bacterial cells using fluorescence in situ hybridization

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Abstract

Fluorescence in situ hybridization (FISH) is widely used to characterize bacterial community structure. However, a major limitation of the FISH technique is that the effectiveness of target cell detection varies widely over ecosystem types and with differences in methodology. Samples collected at sea often are stored for weeks or months before analysis using FISH, therefore quantifying the effects of storage on the detection of bacterial cells is crucial for comparing studies of bacterial community structure from diverse regions and ecosystems. Presented are the results of a 12-month time-course study during which replicate seawater samples were prepared, stored frozen, and hybridized after 0, 1.5, 3, 6, and 12 months to determine the effects of long-term sample storage on hybridization efficiency and the characterization of community structure. The time-dependent slope of the probe for *Bacteria*, but not the *Cytophaga-Flavobacteria* cluster or the α - and γ -*Proteobacteria*, was significantly different from zero, with a 6.3% change in target cell detection per year. This change in detection was small and within the typical error reported for bacterial counting. We conclude that during this 12-month time-course study there was a minimal effect of long-term storage on the detection of bacterial cells using FISH.

Fluorescence in situ hybridization (FISH) uses fluorescently-labeled, rRNA-targeted oligonucleotide probes that are hybridized to group-specific gene sequences, thus allowing for the identification of unicellular organisms at target taxonomic levels (Glöckner et al. 1996). This technique has been used frequently to study heterotrophic prokaryotes from the domains *Bacteria* and *Archaea*, and less frequently for the *Eucarya*, i.e., nano- and pico-phytoplankton and protists (Lim et al. 1993, Lim et al. 1995, Simon et al. 1995), and photosynthetic picoheterokonts (stramenopiles; Massana et al. 2002). More recently, FISH has been combined with catalyzed reporter deposition (CARD-FISH; Pernthaler et al. 2002) and microautoradiography (MICRO-CARD-FISH; Teira et al. 2004, Herndl et al. 2005), to enhance sensitivity and improve detection of small

or slow-growing cells with low rRNA content (Pernthaler et al. 2002). Despite the limitations of the classical FISH method (DeLong et al. 1989, Amann et al. 1995, Glöckner et al. 1996, Pernthaler et al. 2001), it has been widely used to characterize bacterial community structure in the published literature (e.g., Glöckner et al. 1999, Amann et al. 2001, Bouvier and del Giorgio 2003, Wagner et al. 2003).

One of the major limitations of the FISH technique is the variability in target cell detection (Bouvier and del Giorgio 2003). For example, the percentage of cells detected with the *Bacteria* probe (EUB338) varies from 1% in soil to 100% in enriched culture, with an average of 56% for all ecosystem types (Bouvier and del Giorgio 2003). This variation is also influenced by methodological factors, such as cell fixation, hybridization conditions, mounting solutions, fluorochrome type, and counting methods (Bouvier and del Giorgio 2003, Williams et al. 2004). Another important, yet neglected, factor is the effect of sample storage on the efficiency of target cell detection with rRNA-targeted oligonucleotide probes. Samples collected, preserved, and filtered for FISH often are stored for many weeks or months before hybridization and counting. Studies of the effects of sample storage on the detection and quantitative counting of bacteria (Turley and Hughes 1992, 1994, Turley 1993, Gundersen et al. 1996) and photosynthetic picoplankton (Booth 1987, Hall 1991, Putland and Rivkin 1999) have shown that long-term storage of preserved seawater

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Table 1. Oligonucleotide probe sequences and target bacterial groups

Probe	Sequence	Specificity	Source
EUB338	GCTGCCTCCCGTAGGAGT	Most <i>Bacteria</i> ; not <i>Planctomyces</i>	Amann et al. 1990
CF319a	TGGTCCGTGTCTCAGTAC	<i>Cytophaga-Flavobacteria</i>	Manz et al. 1996
ALF968	GGTAAGGTTCTGCGCGTT	α - <i>Proteobacteria</i>	Glöckner et al. 1999
GAM42a	GCCTTCCCACATCGTTT	γ - <i>Proteobacteria</i>	Manz et al. 1992
NON338	ACTCCTACGGGAGGCAGC	Negative control probe; complementary to EUB338	Wallner et al. 1993

ter samples results in a significant reduction in the abundance of cells detected using epifluorescence microscopy. For example, samples that were filtered, stained with acridine orange (AO), and mounted could be stored frozen (-20°C) for up to 70 days with no significant reduction in cell counts (Turley and Hughes 1992, Turley 1993). Counts of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)-stained bacteria were consistent when stored for up to 24 weeks at 4°C , but decreased significantly after 36 weeks (Porter and Feig 1980). Sample preparation for analyses of bacterial community structure using FISH (Glöckner et al. 1996) differs in several ways from the preparation of samples for DAPI or AO direct counts (Kepner and Pratt 1994); FISH samples are initially preserved, filtered onto $0.2\ \mu\text{m}$ white polycarbonate filters, and stored at -20°C until they are hybridized with oligonucleotide probes, counterstained with DAPI, and counted within 2 to 3 d. Therefore, the effects of a delay between sample collection and sample analysis may differ from those observed with seawater samples that have been preserved, stained, mounted onto glass slides, and stored for weeks to months before counting. Thus, it is not possible to reliably extrapolate the results of previous published studies of the effects of storage on direct counts of bacterial abundance using AO or DAPI to analyses of bacterial community structure using FISH.

Quantitative studies with robust statistical analyses of the time-dependent effects of long-term sample storage on the efficiency of target cell detection using FISH are lacking. Lam and Cowen (2004) assessed the effects of sonication, preservation, and storage on target cell detection in particle-rich seawater samples, however the results for samples that had been prepared using the classical FISH method (fixation with formalin, filtration, and storage at -20°C) were inconclusive and no temporal trends could be determined over the short time course (96 d). Pernthaler et al. (2001) suggested that filters prepared for FISH "can be stored frozen for several months without apparent loss of hybridization signal," but this result has not been quantified.

This study examines the effects of long-term storage on the detection of bacterial cells and on the characterization of bacterial community structure, using four rRNA-targeted oligonucleotide probes. Replicate seawater samples were prepared (preserved and filtered) and stored frozen (-20°C) for up to 1 yr. During this time course, samples were hybridized, counterstained, and counted at time intervals of 0, 1.5, 3, 6, and

12 months to determine the effects of storage on the detection of bacterial cells using FISH.

Materials and procedures

Sampling, cell fixation, and storage—Seawater was collected on 28 May 2004 at Tapper's Cove, Newfoundland and Labrador, Canada ($49^{\circ}39'54''\text{N}$, $52^{\circ}43'38''\text{W}$). Twenty replicate filters (i.e., four filters for each of the five time points) were prepared from a single seawater sample. Each 90 mL replicate seawater sample was fixed with 10 mL of 37% formalin, and filtered (after 1 h) onto a white $0.2\text{-}\mu\text{m}$ polycarbonate membrane filter (Millipore GTTP04700) that was placed over a cellulose prefilter (Millipore AP1504700). The polycarbonate filters were washed with 50 mL of phosphate-buffered saline (sterile and prefiltered), and air-dried over absorbent paper in individual sterile petri dishes. One set of four filters was hybridized and counted immediately, and the other four filter sets were stored at -20°C until analysis at 1.5, 3, 6, and 12 months.

Oligonucleotide probes—In this study, fluorescently-labeled rRNA-targeted oligonucleotide probes (MOBIX Lab) were used for the phylogenetic analysis of heterotrophic prokaryotes from the domain *Bacteria* (EUB338); as well as the intermediate taxonomic levels: α -(ALF968) and γ -(GAM42a) *Proteobacteria*, and the *Cytophaga-Flavobacteria* (CF319a) cluster. The 'nonsense' probe (NON338), which is complementary to the EUB338 probe, was used as a negative control for the detection of non-specific binding and background fluorescence (Glöckner et al. 1996). The target bacterial groups and gene sequences for the oligonucleotide probes employed in this study are shown in Table 1. All oligonucleotide probes were labeled at the 5' end position with the sulfoindocyanine dye, indocarbocyanine (Cy3).

Fluorescence in situ hybridization, DAPI staining, and epifluorescence microscopy—In this research, we used a modification of the protocol of Glöckner et al. (1996). Each filter was cut into five triangular sections, and each section placed onto a microscope slide. To ensure that the side containing the bacteria was facing upwards, a slit was cut in the right edge of each filter section. The filter sections were hybridized with $20\ \mu\text{L}$ of hybridization solution, containing 0.9 M NaCl, 20 mM Tris-HCl (pH 8), 35% formamide (for all probes except ALF968, which required 20% formamide), 0.01% sodium dodecyl sulphate (SDS), and 50 ng of oligonucleotide probe. Each slide was placed in a sealed hybridization chamber containing a

piece of absorbent paper and 1 mL of hybridization buffer (to create a humid atmosphere), and incubated in the dark for two hours at 46°C. Following incubation, filter sections were placed into individual falcon tubes of prewarmed (46°C) washing buffer, containing 70 mM NaCl (for all probes except ALF968, which required 215 mM NaCl), 20 mM Tris-HCl, 5 mM EDTA, and 0.01% SDS, and incubated in the dark for 15 min at 46°C. Each filter section was dried over absorbent paper at room temperature, placed on a glass slide, and counter-stained with 50 µL of DAPI (1 µg/mL) for 1 min (on ice). After staining, each filter section was washed with 1 mL of filtered Milli Q water, dried over absorbent paper and mounted on a glass slide in glycerol medium (Citifluor #1; Citifluor).

The slides either were examined immediately using an Olympus BH-2 epifluorescent microscope, equipped with a ×100 1.30 oil objective, Cy3 filter (41007-HQ) and DAPI filter (UG-1), or were stored at -20°C for a maximum of 2 d before microscopic analysis. As Cy3 fluorescence fades much more rapidly than DAPI fluorescence (Pernthaler et al. 2001), direct counts of hybridized cells were completed first, followed by DAPI counts (under UV light), for total bacteria, in the same field of view. At least 500 hybridized cells were enumerated for each of the filters or, at low abundances of hybridized cells, at least 1,000 DAPI stained cells were counted (Pernthaler et al. 1998). At each time point ($t = 0, 1.5, 3, 6,$ and 12 months), counts were completed for four phylogenetic groups, as well as for the negative control probe (Table 1) on each of four replicate filters. The relative abundances (%) of hybridized cells were determined from the direct counts of hybridized and DAPI stained bacteria, within the same field of view (i.e., [number of hybridized cells for each target group / number of DAPI stained cells] × 100). All probe specific counts were corrected for non-specific binding and background fluorescence by subtracting counts obtained with the NON338 probe.

Statistical treatment and models—A two-way Analysis of Variance (ANOVA) was employed to determine an overall time effect. The response variable was the proportion of bacterial cells detected with each target oligonucleotide probe (reported as % of the total bacterial cells determined by DAPI counts), the independent variable was time (months), and the fixed factor was the probe group. This was followed by an Analysis of Covariance (ANCOVA) to determine if the time-dependent slopes (i.e., percent change per month) differed significantly for each probe group. Further regression analyses were completed for the individual probe groups. For each analysis, the residuals were examined and found to meet the assumptions of linearity, normality, independence, and homogeneity (Seber 1977). All statistical analyses were conducted using Minitab Release 14 and SPSS 13.0.

Assessment

We determined the effects of sample storage on the characterization of bacterial community structure using FISH by conducting a time-course study using four rRNA-targeted oligonucleotide

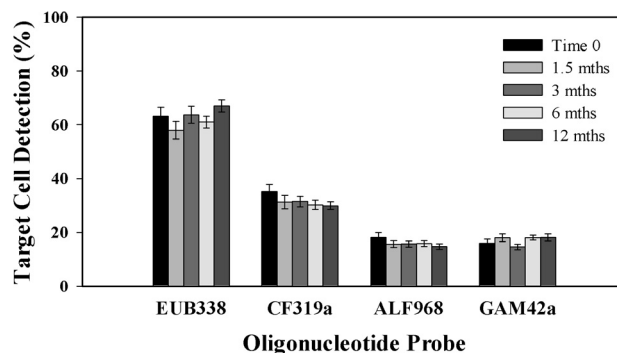


Fig. 1. Mean percentage (%) of bacterial cells detected with target oligonucleotide probes during a one-year time course. Target groups were the *Bacteria* (EUB338), α -(ALF968) and γ -(GAM42a) *Proteobacteria*, and the *Cytophaga-Flavobacteria* (CF319a) cluster. All probe counts were corrected by subtracting counts obtained with the negative control probe NON338. Percentages were computed as cells hybridized with the target oligonucleotide probe divided by the total cells determined from DAPI counts, and mean values were calculated from four replicate filters at each time point. Error bars indicate 95% confidence intervals.

probes to quantify the effects of storage on the detection of bacteria at the highest taxonomic level, the domain *Bacteria* (EUB338); and the intermediate levels, α -(ALF968) and γ -(GAM42a) *Proteobacteria*, and the *Cytophaga-Flavobacteria* (CF319a) cluster.

The proportions of bacterial cells detected with each of the target oligonucleotide probes are shown in Fig. 1. A sequential statistical analysis was employed to determine if target cell detection (%) changed over time. Firstly, a two-way ANOVA was used to examine if all probe groups exhibited the same response; 'time' was the independent variable and 'oligonucleotide probe' was the fixed factor. A significant interaction between 'time' and 'oligonucleotide probe' ($F_{12,71} = 2.83$, $P = 0.004$, $n = 78$) indicated that the probe groups responded differently. Secondly, an ANCOVA was used to determine whether the slopes of the regression of target cell detection (%) versus time was significantly different among the probe groups. The results of the ANCOVA were also statistically significant ($F_{3,71} = 4.92$, $P = 0.004$, $n = 78$), indicating that the probe groups responded differently over time. Thirdly, regression analyses were used to assess the effects of storage for each probe group. No significant differences in target cell detection were observed for the CF319a, ALF968, or GAM42a probes. Only the EUB338 probe had a time-dependent slope that was statistically significant (slope = 0.529, $P = 0.037$, $n = 20$); the percent change in target cell detection was 6.3% per year, which is small relative to the typical counting error of $\pm 10\%$ for FISH (reported by Glöckner et al. 2000, Pernthaler et al. 2001), and $\pm 15\%$ for AO or DAPI direct counts (reported by Knap et al. 1996). We conclude that during this 12-month time-course study, there was a minimal effect of long-term sample storage on target cell detection using FISH and the resultant analyses of bacterial community structure. A statistically significant effect was observed only for the EUB338

Table 2. Results of Model I regression analysis of percent target cell detection for the EUB338 probe as a function of time (months) during a one-year time course. Values reported are the slopes of the regression (i.e., percent change per month) with P and n values in parentheses. Slopes that are significantly different ($P=0.05$) from zero are underlined.

	1.5 months	3 months	6 months	12 months
Initial	-3.480 (0.145; 8)	0.260 (0.883; 12)	-0.086 (0.870; 16)	<u>0.529</u> (0.037; 20)
1.5 months	-	3.990 (0.063; 8)	0.404 (0.557; 12)	<u>0.769</u> (0.007; 16)
3 months	-	-	1.030 (0.302; 8)	0.563 (0.104; 12)
6 months	-	-	-	<u>1.200</u> (0.021; 8)

probe ($P=0.037$, $n=20$) and there was no statistically significant difference in target cell detection with the EUB338 probe over the first 6 months ($P=0.870$, $n=16$; Table 2).

Discussion

The results of this study have important implications for the interpretation of field studies where bacterial community structure has been characterized from seawater samples that have been preserved, filtered and stored for up to 12 months. Although there have been several studies of the effects of sample storage on direct counts of bacteria (Turley and Hughes 1992, 1994, Turley 1993, Gundersen et al. 1996) and photosynthetic picoplankton (Booth 1987, Hall 1991, Putland and Rivkin 1999), we are not aware of any previously published quantitative studies of the effects of long-term sample storage on the efficiency of target cell detection using FISH. Given that sample preparation for analyses of bacterial community structure using FISH (Glöckner et al. 1996) differs from the preparation of samples for DAPI or AO direct counts (Kepner and Pratt 1994), it is not possible to reliably extrapolate the results of previous published studies of the effects of storage on direct counts of bacterial abundance using AO or DAPI (Porter and Feig 1980, Turley and Hughes 1992, Turley 1993) to analyses of bacterial community structure using FISH.

Accurate estimates of absolute abundance of bacteria and the relative abundances of specific phylogenetic groups are crucial in quantifying the relationship between bacterial community structure and ecosystem function. Methodological factors, such as sample storage, may contribute to variation in the detection of bacterial cells using FISH, and thus must be addressed to accurately compare studies of bacterial community structure from different ecosystems as well as samples from the same ecosystem that have been analyzed at different times.

Comments and recommendations

As there was no statistically significant difference in target cell detection over the first 6 months of the time course, we

conclude that samples for FISH can be prepared (preserved and filtered) and stored at -20°C for up to 6 months with no significant change in target cell detection, and stored for up to 12 months with a minimal ($<7\%$) effect of storage. More recently, FISH has been combined with catalyzed reporter deposition (CARD-FISH; Pernthaler et al. 2002) to enhance sensitivity and improve detection of small cells with low rRNA content (Pernthaler et al. 2002). This method is particularly useful for characterizing bacterial community structure in planktonic samples, as these cells are often small, slow-growing, or starving (Morita 1997, Pernthaler et al. 2002). The collection, preparation, and storage of samples for CARD-FISH is essentially the same as for the classical FISH method, thus the effects of long-term sample storage on the detection of bacterial cells using CARD-FISH, as well as other FISH methods, should also be minimal.

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