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ORIGINAL ARTICLE

Effect of storage temperature on prokaryotic cell counts and community composition analysis from fixed and filtered seawater samples

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Abstract Marine, pelagic prokaryotes commonly are visualized and enumerated by epifluorescence microscopy after staining with fluorescent, DNA-binding dyes and sample preparation and storage has a major influence on obtaining reliable estimates. However, sampling often takes place in remote locations and the recommended continuous sample storage at –20°C until further sample evaluation is often logistically challenging or infeasible. We investigated the effect of storage temperature on fixed and filtered seawater samples for subsequent enumeration of total prokaryotic cells and community composition analysis by fluorescence in situ hybridization (FISH). Prokaryotic abundance in surface seawater was not significantly different after 99 days when filters were stored either at room temperature (RT) or at –20°C. Furthermore, there was no loss in detection rates of phylotypes by FISH from filters stored at RT or –20°C for 28–30 days. We conclude that fixed and filtered seawater samples intended for total prokaryote counts or for FISH may be maintained long-term at room temperature, and this should logistically facilitate diverse studies of prokaryote ecology, biogeography, and the occurrence of human and fish/shellfish pathogens.

Keywords Marine bacteria · Storage temperature · Prokaryotic abundance · Microbial community composition · Fluorescence in situ hybridization

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Introduction

Direct enumeration of prokaryotes from seawater samples using DNA-binding stains and epifluorescence microscopy (Zimmermann and Meyer-Reil 1974; Daley and Hobbie 1975) revolutionized the field of marine microbial ecology. Because of these advances, it was recognized that the world's oceans harbor microbes at much higher abundances than had been estimated from culture-based methods. Staining protocols and detection methods have been modified since their first introduction (Jones and Simon 1975; Hobbie et al. 1977; Porter and Feig 1980; Monger and Landry 1993) and DAPI counts, by microscopy or flow cytometry, are now accepted as reliable estimates of in situ abundances. In combination with microbial production measurements, abundance estimates have been indispensable for our understanding of carbon and nutrient fluxes in the ocean. Furthermore, the discovery of the importance of marine bacteria and archaea has driven methodological advances, especially in the field of molecular microbiology, resulting in the development and application of powerful tools such as gene sequencing, fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (tRFLP), and microautoradiography combined with

fluorescence in situ hybridization (MAR-FISH) for the detection of hitherto uncultured taxa, their diversity and functions in the ocean (DeLong et al. 1989; Amann et al. 1990; Muyzer et al. 1993; Moeseneder et al. 1999; Lee et al. 1999; Ouverney and Fuhrman 1999).

To obtain accurate estimates of prokaryotic abundance, the preservation and processing of seawater samples is critical. Turley and Hughes (1992, 1994) reported cell losses of 7–75, 65, and 56% from glutaraldehyde- or formaldehyde-fixed seawater samples stored for ∼1.5–3.5 month at 17–22°C, 10°C, and 6°C, respectively. The cause of apparent cell loss is unclear, but it is thought that cell attachment to container surfaces and cell clumping may be involved. Turley and Hughes (1992, 1994) also found that cell loss could be avoided by fixing, staining, filtering, and mounting the samples within a few hours of collection and storing them in slide boxes at –20°C until counted. Hyuan and Yang (2003) obtained highest prokaryotic cell counts when formaldehyde-fixed water samples were stored at –20°C and thawed directly before slide preparation and microscopic evaluation. For the analyses of microbial communities by FISH and their activity by microautoradiography, it is also common to filter fixed samples and store them in petridishes at –20°C until further processing (Fuchs et al. 2005; Elifantz et al. 2005; Teira et al. 2006).

However, keeping samples continuously at –20°C during field work, at sea, and especially during transport back into the home laboratory from remote places is not always possible or practical and thus samples are sometimes thawed intermittently. In this study, we investigated the influence of storage temperature on fixed, filtered seawater samples for subsequent analyses of total prokaryotic abundance and community composition.

Methods

Sampling

Surface seawater was collected in an acid-rinsed polycarbonate bottle of the pier of Scripps Institution of Oceanography (32°52′N, 117°15′W) on 22 February 2006. The sample was immediately transported to the adjacent laboratory and fixed with 0.2 μm filtered formaldehyde (final concentration, 1.8%, Sigma-Aldrich Inc., MO, USA, cat. no. F8775) for 1 h at 4°C. Well-mixed 1 ml sub-samples were filtered onto black polycarbonate membranes (0.2 μm pore size, 25 mm diameter, Millipore, Billerica, MA, USA), rinsed with ultra-pure water and air-dried. Filters were placed in petridishes of 35 mm in diameter (three filter each, interspersed by the filter separators from the filter box). Twenty-seven filters were stored at –20°C in the dark and an additional 27 filters were stored at room temperature (RT, 22–24°C) in the dark. A second sample of seawater was collected and fixed (same location and procedure) on 18 July 2006 for bacterial community analyses by FISH. Well-mixed 10 ml sub-samples were filtered onto white polycarbonate membranes (0.2 μm pore size, 47 mm diameter, Millipore, Billerica, MA, USA), rinsed with ultra-pure water and air-dried. Two filters each were stored for 1 month in petridishes (50 mm in diameter) at –20°C and RT in the dark.

Bacterial abundance

Within less than 24 h (=day 1) triplicate filters from each treatment (i.e. –20°C versus RT) were simultaneously stained and mounted on microscopic slides using a DAPI-containing mounting medium (DAPI-Vectashield, Vecta Laboratories, Burlingame, CA, USA) and cells were counted via epifluorescence microscopy (Olympus BX51, 1,000× magnification, filter set U-MNU 2, Olympus, Tokyo, Japan). Because the filters from day 1 were not treated differently, mean cell counts included counts from all six filters. Mean cell counts were also estimated from triplicate filters of each treatment 8, 15, 22, 29, 43, 57, 78, and 99 days after collection.

Bacterial community composition

Bacterial community structure was analyzed by FISH as described previously (Pernthaler et al. 2001). Hybridizations were performed on triplicate filter sections which were stored for 28–30 days at RT or –20°C. Fluorescently labeled (Cy3) oligonucleotide probes (Thermo Fisher Scientific, Ulm, Germany) were targeted to most organisms of the domain Bacteria (EUB338, EUB338-II, EUB338-III; Daims et al. 1999), Gammaproteobacteria (GAM42a and cGAM42a) and Bacteroidetes (CF319a; both Manz et al. 1992). The EUB338 antisense probe NON338 (Wallner et al. 1995) served as a negative control. Air-dried filter sections were counter-stained and mounted with DAPI-Vectashield. Cells were visualized by epifluorescence microscopy (see above, filter set U-MNG 2 for Cy3-labeled cells) and phylogenetic groups were enumerated as percentages of all DAPI-stained cells in identical microscopic fields.

Statistical analysis

A 2-sided Student's t test for paired samples was used to test for differences in cell counts between filtered samples

stored at RT or –20°C. Endpoint comparisons to test for differences in cell counts between treatments on day 1 and day 99 were conducted with 2-sided Student's *t* tests.

Results and discussion

Bacterial abundance in surface seawater collected from Scripps Pier in February was $2.34 \times 10^6 \pm 1.74 \times 10^5$ cells ml^{-1} (day 1, $n = 6$, Fig. 1). Bacterial abundance was not significantly different between treatments (RT versus –20°C) at any time point (*t* test for paired samples, $P = 0.24$). Furthermore, cells counts from stored filters of the same water sample stained, mounted, and counted after 99 days were not significantly different from the filters counted at day 1, whether they were stored at RT or –20°C (*t* tests for endpoints, $P_{RT} = 0.73$ and $P_{-20°C} = 0.77$). Bacterial abundance from seawater sampled, fixed and filtered in July was also not different between immediate counting and counting after 1 month of storage at RT or –20°C.

The detection rate of bacteria by FISH with Cy3-labeled oligonucleotide probes was 70 ± 6 and 72 ± 1% of total DAPI counts when stored at RT or –20°C, respectively (Fig. 2). Bacteroidetes and gammaproteobacteria accounted for 29 ± 3 and 10 ± 1% of total DAPI-counts, respectively, when filters were stored at RT and for 30 ± 2 and 10 ± 1%, respectively, when filters were stored at –20°C (Fig. 2). No statistical test was necessary to show that there was no significant difference in relative abundance of the three tested phylotypes with respect to storage temperature. These data provide experimental evidence for the statement by Glöckner et al. (1996) that dried filters can be stored for several weeks at RT without apparent changes in FISH results. Furthermore, we detected no difference in perceived staining intensity and overall image quality between the two treatments upon microscopic visualization (Fig. 3) even though the less sensitive FISH technique with monolabeled fluorescent probes and not the highly sensitive TSA-FISH technique was applied. This indicated that cellular ribosomes were not significantly degraded within 1 month of storage at either RT or –20°C and we see no reason why the more sensitive TSA-FISH method should work less well.

Often freshly prepared paraformaldehyde (PFA) is used as fixative for microbial cell counts and FISH analysis (Glöckner et al. 1996), but in our experience fresh formaldehyde gives equally good results for these purposes. Although both substances are potential carcinogens, the use of liquid formaldehyde eliminates the need for weighing and heating of the finely powdered PFA. However, the duration of fixation is crucial for optimal FISH results (Yang et al. 1999; Braissant and Wahli 1998) and fixed samples should be filtered and diligently rinsed within 24 h (Pernthaler et al. 2001). In conclusion, formaldehyde-fixed, filtered and air-dried seawater samples do not need to be kept frozen but can be stored at room temperature without cell loss (up to 99 days) or decrease in detection rates of phylotypes by FISH (up to 28–30 days). These time periods should be adequate for sample return to the laboratory even from remote sampling sites. We further note that the fixed and filtered but unstained samples can be stored at RT without a reduction in cell count. This simplifies the field processing of samples, and the unstained samples can be mounted with a DAPI-containing mounting medium and counted when it is logistically more practical to do so. This approach saves valuable time for sample processing in the field. Furthermore, it means that samples can be prepared with minimal resources, requiring only filters, a filter rig, and a hand-operated pump. Although we only analyzed samples from one coastal environment during two seasons, it is most likely that fixed and filtered samples from other habitats, e.g., freshwater or eutrophic, organically rich waters, can be stored at RT for later analyses of prokaryotic abundance and community composition as well. However, verification of this assumption is recommended for fundamentally different environments such as ultra-oligotrophic or deep-sea waters, in which microbial DNA- or ribosomal content might be extremely low.

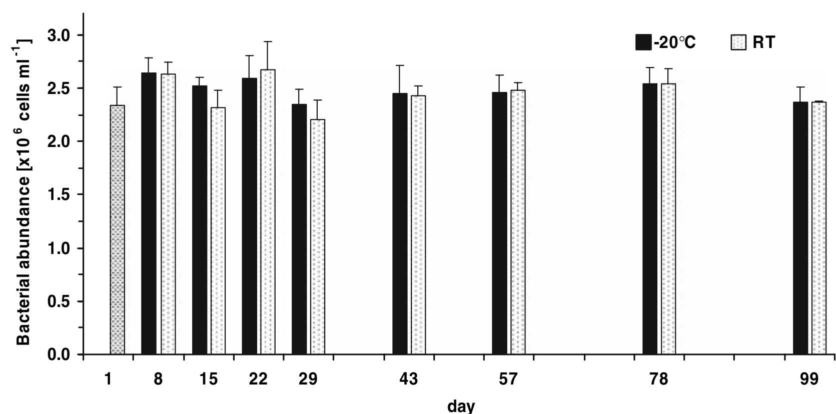

Fig. 1 Prokaryotic cell counts from sub-samples of fixed and filtered coastal seawater stored at room temperature (*RT*, 22–24°C) and *–20°C* for up to 99 days and DAPI-stained prior to microscopic enumeration (mean ± standard deviation; day 1, $n = 6$; all other, $n = 3$)

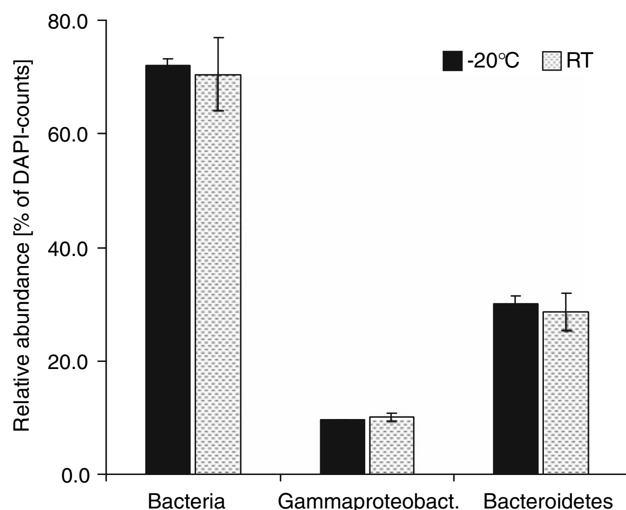

Fig. 2 Relative abundance of *Bacteria* (EUB338 I–III), *Gammaproteobacteria* (Gam42a), and *Bacteroidetes* (CF319a) of fixed and filtered coastal seawater stored at *RT* and *–20°C* for 1 month

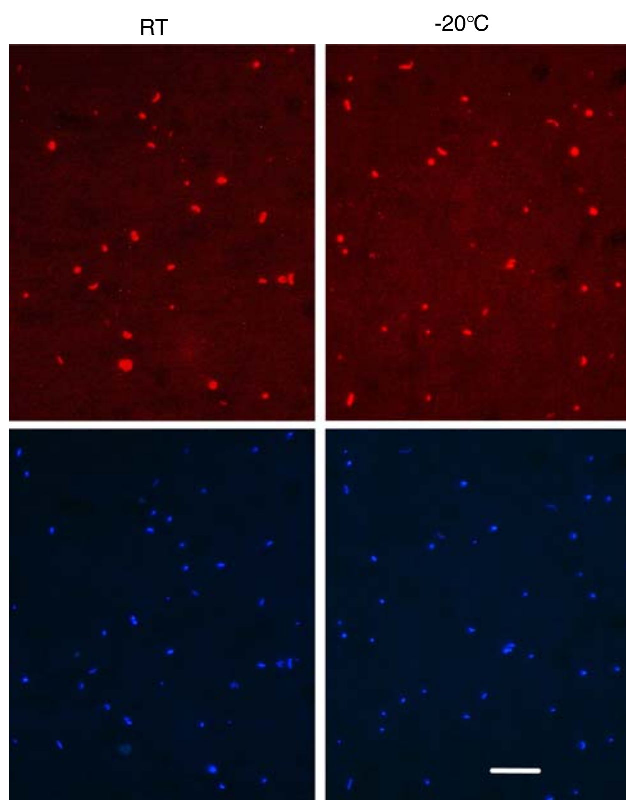

Fig. 3 Epifluorescence microscope images of fixed and filtered coastal seawater stored at room temperature (*RT*; left panel) and *–20°C* (right panel) for 1 month. Bacteria labeled with *Cy3-EUB338 I–III* (upper panel) and stained with DAPI (lower panel) in identical microscopic fields. *Scale bar*: 10 μm

Many types of investigations around the world that could benefit from the evaluation of microbial assemblages and dynamics currently are constrained because of a lack of facilities for advanced optical or molecular analyses. For instance, fish/shellfish pathogens can devastate aquaculture crops but farmers are rarely equipped to detect their occurrence or proliferation. They could easily filter, fix, and store water samples for subsequent analyses. Human pathogens in coastal and estuarine waters, e.g., *Vibrio cholerae*, could be monitored even in logistically challenging settings, by filtration and subsequent analysis in central laboratories. Given the breadth and potential value of resultant coverage, filters might be archived for future molecular studies, e.g., to detect emerging pathogens. Finally, studies of microbial ecology would benefit from setting up a network of sampling and analysis sites through collaborations for testing hypotheses on microbial diversity, biogeography and long-term changes in patterns of distribution of prokaryotes.

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