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Direct Counting of Bacteria Preserved with Lugol Iodine Solution

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Lugol iodine solution was compared with glutaraldehyde as a preservative for marine bacteria. Direct counts with the fluorochrome 4′,6-diamidino-2-phenylindole show no significant difference between the preservatives, but the use of Lugol solution has several advantages over glutaraldehyde, especially in the handling and storage of samples. Bacteria were counted in water samples that were preserved with Lugol iodine solution and stored at room temperature for 4 years. Microprotozoa were also counted in samples preserved with Lugol iodine solution by using the fluorochrome fluorescein isothiocyanate.

Direct counting by epifluorescence microscopy has emerged as a rapid and accurate method for enumerating aquatic bacteria. Various procedures recommend that samples be either counted immediately after collection or preserved, usually with formaldehyde or glutaraldehyde solutions, for subsequent analysis. Fixation with an aldehyde does not affect the nucleic acids, to which the most commonly used fluorescent dyes, acridine orange and 4′,6-diamidino-2-phenylindole (DAPI), bind (1). Formaldehyde in aqueous solution, however, will polymerize to form paraformaldehyde (6), and I have also found that glutaraldehyde precipitates, particularly if the temperature rises above 15°C. These precipitates can ruin samples because the white material formed by both aldehydes blocks filters and, more seriously, "scavenges" bacteria from suspension, resulting in an uneven distribution of bacteria across the filter surface. Storage of stock preservatives and samples at low temperature reduces the problem, but refrigerated storage space in the field or at sea is not always available, and the transport of samples at low temperature between remote field sites and the laboratory may prove impracticable.

Lugol iodine solution has been widely used for the fixation and preservation of phytoplankton and aquatic protozoans (8), and samples keep for up to 10 years if sealed and stored in darkness (7). This study demonstrates that Lugol iodine solution also preserves marine bacteria. Storage at low temperature is not essential, and although iodine may be oxidized and lost from solution by contact with air and light, it has several advantages over glutaraldehyde. Specifically, less stringent storage conditions are required and no precipitate is formed. Recent concern about the possible carcinogenicity of formaldehyde together with the irritant effect of its fumes and those of glutaraldehyde suggest that the use of Lugol iodine solution may have additional advantages.

Water samples were collected from various depths at station CS2 in the Celtic Sea (50°30′ N, 07°00′ W) during July, August, and October 1982 (3) and from station E5 in the Western Approaches of the English Channel (49°06′ N, 06°32′ W) during August 1983. Replicate 100-ml samples were preserved with 10 ml of 25% electron microscope-grade glutaraldehyde solution (prefiltered through 0.2-μm Nuclepore filters) and with 1 ml of acidic Lugol iodine solution (100 g of KI dissolved in 1 liter of 0.2-μm-filtered distilled water, plus 50 g of crystalline iodine and 100 ml of glacial acetic acid). Glutaraldehyde samples were stored in the dark at 4°C and Lugol samples were stored in the dark at room temperature.

Bacteria were counted in the laboratory by using DAPI and the epifluorescence technique of Porter and Feig (4). The color of the Lugol iodine solution was removed with sodium thiosulfate before staining with DAPI, since iodine in untreated samples discolors Nuclepore filters and masks bacterial fluorescence; 25 μl of sodium thiosulfate solution (3 g of $Na_2S_2O_3$ in 100 ml of 0.2-μm-filtered distilled water) will clear 1 ml of sample. Counts were made with a Leitz Ortholux microscope fitted with 50-W HBO light source and equipped with a Ploemopak 2.2 fluorescence vertical illuminator containing a BP340-380 exciting filter, an RKP400 beam-splitting mirror, an LP430 suppression filter (filter block A), and an NPL Fluorotar 100/1.32 oil objective; 15 fields of view and at least 300 cells were counted per slide. The results (Fig. 1) were treated by a standard fixed-effect two-way analysis of variance. There was no suggestion of an interaction effect ($F = 1.076$ on 24,700 df) or of a treatment main effect ($F = 0.567$ on 1,700 df), demonstrating that there was no significant difference in bacterial numbers between samples preserved with Lugol iodine solution or glutaraldehyde. A similar comparison with the fluorochrome acridine orange (1) was unsuccessful because the stain precipitated on contact with the sodium thiosulfate solution. However, comparable counts of marine microprotozoans were obtained from samples preserved with Lugol iodine solution or glutaraldehyde by using the fluorescein isothiocyanate method of Sherr and Sherr (5).

Having demonstrated that Lugol iodine solution preserves marine bacteria as effectively as glutaraldehyde, I examined the efficiency of long-term storage. The oldest samples available were collected 4 years ago at station E5 during September 1979. The eight samples were kept in the dark at room temperature for 48 months; two had no color left from the iodine, but the numbers of bacteria present in these samples were not significantly different from those in which excess iodine was still present. The mean number (and standard deviation) of bacteria in these 4-year-old samples was $4.31 \times 10^5 \pm 0.83 \times 10^5$ cells ml^{-1}. This compares with a mean of $3.00 \times 10^5 \pm 0.56 \times 10^5$ cells ml^{-1} reported by Holligan et al. (2) for samples taken at the same site in July 1981. Although conditions in July 1981 are unlikely to have been similar to those in September 1979, the similarity in bacterial numbers supports the idea that bacteria can be counted in samples which have been stored with Lugol iodine solution for at least 48 months. Various marine flagellates have been preserved effectively for 8 to 10 years or more with Lugol iodine solution (6), and it may now be

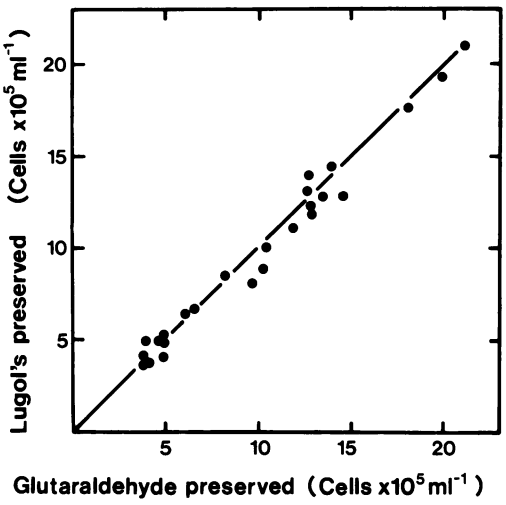

FIG. 1. Comparison of bacterial numbers in replication water samples preserved with Lugol iodine solution or 2.5% (vol/vol) glutaraldehyde.

possible to count bacteria retrospectively in water samples preserved with Lugol iodine solution which were collected before epifluorescence techniques were developed.

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