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Direct Acridine Orange Counting of Bacteria Preserved with Acidified Lugol Iodine

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Acidified and nonacidified Lugol iodine solution was tested under several storage temperatures and at several times as a preservative for marine bacteria. Direct counts with acridine orange showed no significant difference between glutaraldehyde- and Lugol iodine solution-preserved samples under any storage temperature when samples were counted within 1 week of collection. Specimens in long-term (up to 6 months) storage required refrigeration and treatment with acidified Lugol iodine solution for adequate preservation. Lugol iodine solution-preserved bacteria appeared intact under scanning electron microscopy. Lugol iodine solution did not preserve chlorophyll autofluorescence in phytoplankton.

Preservation of seawater samples with glutaraldehyde for direct counting of bacteria stained with various fluorochromes is a standard technique with several drawbacks. Pomroy (3) discussed these in detail and presented evidence suggesting that the classic Lugol iodine solution adequately preserves marine bacteria for both short- and long-term storage at ambient temperatures before counts are made on preparations stained with 4′,6-diaminidino-2-phenylindole (DAPI). Acridine orange (AO) is another fluorochrome widely used for direct bacterial counts (1); however, Pomroy (3) reported precipitation of AO upon contact with the sodium thiosulfate solution used to decolor the Lugol iodine solution. Pomroy (3) also reported that it may be possible to use DAPI to accurately count samples that have lost all iodine color during a storage period of 48 months.

The prospect of a bacterial preservative not subject to precipitation when stored and handled under less-than-ideal conditions warranted further investigation, especially if a method could be devised to make Lugol iodine solution compatible with AO. For many samples, AO is the fluorochrome of choice. AO stains a wider variety of materials than DAPI, and it also does not require the stringent handling conditions necessary for a successful DAPI-stained slide. A rinse with filtered artificial seawater (ASW) allowed AO staining of seawater samples preserved with Lugol iodine solution and led us to compare several different treatments of the samples. These included comparing acidified and nonacidified Lugol iodine solution with glutaraldehyde as a preservative for bacteria to be AO stained, as well as subjecting samples to various temperature treatments over time.

Five seawater samples were collected in Niskin bottles from four stations at various depths off the coast of Georgia, near the Savannah River mouth, on 12 February 1985. Seven subsamples that were collected from each Niskin bottle received the following treatments (Table 1). A 10-ml amount of seawater was preserved with 10 ml of 4% electron microscopy-grade glutaraldehyde buffered with sodium cacodylate in ASW. Three 20-ml portions were preserved with Lugol iodine solution, and three 20-ml portions were preserved with acidified Lugol iodine solution (6). The glutaraldehyde-preserved subsample and one each of the Lugol iodine solution-preserved and acidified Lugol iodine solution-preserved subsamples were immediately refrigerated and subsequently transported on ice to the laboratory at Athens, Ga., where they were again refrigerated. The remaining subsamples were stored in a light-tight box and transported to Athens at ambient temperature. In the laboratory, one Lugol iodine solution-preserved subsample and one acidified Lugol iodine solution-preserved subsample were stored at room temperature, which ranged from 25 to 30°C. The remaining two subsamples were heated to 50°C for 24 h and then stored at room temperature.

Bacteria were counted in the laboratory within 1 week of collection of the water samples and were counted again 1 and 6 months after collection (Table 2). All samples were stored in a clean room, and all slides were prepared in a clean hood located within the clean room. Aliquots were decolorized with sodium thiosulfate (3) and then filtered onto the smooth surface of a 0.22-μm-pore-size black Sartorius filter. To avoid AO precipitation, sodium thiosulfate was washed away with two rinses with 2 ml of 0.22-μm-pore-filtered ASW. Bacteria were stained with a final concentration of 50 mg of AO per liter of ASW solution and left in contact for 2 min. After two final rinses with filtered ASW, the damp filters were placed in immersion oil (Cargille's type A) on a microscope slide and covered with immersion oil, and the edges of the cover slip were sealed with fingernail polish. The use of Sartorius filters differs from the common practice of filtering through Nuclepore filters (1). Pomeroy et al. (2) have previously shown that counts on black Sartorius filters are not significantly different from counts on Nuclepore filters stained with Irgalan black. The thickness of the Sartorius filter facilitates handling and eliminates the irregular depth of field of thin Nuclepore filters.

At the time of the initial counts, a set of sodium thiosulfate-treated but unstained slides was prepared from the refrigerated subsamples to test for differences in chlorophyll autofluorescence from phytoplankton. At 6 months only, a set of DAPI-stained slides was prepared by the technique of Porter and Feig (4) with the Pomroy (3) modification. In addition, one more slide was prepared from a refrigerated acidified Lugol iodine solution-preserved subsample and double stained with AO and DAPI. Counts were made with a Zeiss Universal microscope fitted with a 75-W W2 DC Xenon lamp and vertical illuminator. For AO-stained slides,

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TABLE 1. Sampling and counting scheme

Sample no.	Fixative	Storage temp	Fluorochrome or other treatment[a] at:	
			1 Mo	6 Mo
1	Glutaraldehyde	Refrigerated	AO, SEM[b]	AO
2	Lugol iodine solution	Refrigerated	AO	AO
3	Lugol iodine solution	Ambient	AO	AO
4	Lugol iodine solution	50°C, then ambient[c]	AO	AO
5	Acidified Lugol iodine solution	Refrigerated	AO, SEM	AO, DAPI, AO-DAPI
6	Acidified Lugol iodine solution	Ambient	AO	AO
7	Acidified Lugol iodine solution	50°C, then ambient	AO	AO

[a] For initial counts, AO-stained and unstained slides were prepared for each sample.
[b] SEM, Scanning electron microscopy.
[c] Samples were heated to 50°C for 24 h and then stored at room temperature.

the Zeiss 48 77 09 filter set (BP 450-490 exciter, FT 510 dichroic beam splitter, and LP 520 barrier filter) was used with the Planapochromat 100/1.3 oil objective. For DAPI-stained slides, the Zeiss 48 77 01 filter set (BP 365/11 exciter, FT 395 dichroic beam splitter, and LP 397 barrier filter) was used with the Neofluor 100/1.3 oil objective. All bacterial cells within a Whipple grid field, 70 μm on a side, were counted at a magnification of ×1,250. A total of 1,000 cells or 40 fields, whichever was less, were counted. Mean counts per milliliter were analyzed by using two- and three-way analysis of variance (ANOVA) (5).

Both acidified and nonacidified Lugol iodine solution proved to be inadequate for preserving chlorophyll autofluorescence in unstained samples. Glutaraldehyde-preserved subsamples averaged 52.2 phytoplankton per 100 162-μm-diameter fields counted. Lugol iodine solution-preserved and acidified Lugol iodine solution-preserved subsamples averaged 11.6 and 13 cells per 100 fields, respectively.

To assess the performance of Lugol iodine solution and acidified Lugol iodine solution relative to our standard glutaraldehyde preservative, ANOVA was performed on refrigerated subsamples only. Three-way ANOVA (Table 3) showed significant effects for fixative and time, but not for stations. Two-way ANOVA, performed for station and fixative for each counting period (Table 3), showed significant station and fixative effects for initial counts and significant fixative effects at 1 month but no significant effects at 6 months, indicating that as samples aged initial station and fixative differences were lost because of increasing sample variability. Two-way ANOVA performed for station and time effects (Table 3) showed no significant effects for glutaraldehyde-preserved or acidified Lugol iodine-preserved subsamples but significant time effects for Lugol iodine solution-preserved subsamples, indicating that of the three preservatives the acidified Lugol iodine solution was the only preservative that maintained station differences over an extended time in storage.

Another consideration of this study was the degree of care necessary during storage and transportation of samples. Subsamples preserved with Lugol and acidified Lugol iodine solution were stored at ambient temperatures along with subsamples preserved with Lugol and acidified Lugol iodine solution that had first been stored at 50°C for 24 h to simulate storage in a hot van during transport. Glutaraldehyde-preserved subsamples were not included in this portion of the study because previous experience has shown that storage of samples preserved with glutaraldehyde for 24 h at room temperature will render them useless for fluorescence counting procedures. When the subsamples were taken from the Niskin bottles, samples from early stations were stored in glass, whereas samples from later stations were stored in polyethylene. Polyethylene-stored samples lost all or most of the iodine color after 6 months at room temperature. All refrigerated samples retained good color for the duration of this study.

Three-way ANOVA (Table 3) showed no significant effects of station, fixative, or temperature on initial counts. Counts at 1 and 6 months showed very significant fixative and temperature effects. Subsamples stored at ambient temperatures had lower counts than refrigerated subsamples, and Lugol iodine solution-preserved subsamples had lower counts than acidified Lugol iodine solution-preserved subsamples. The loss of color and consequent lower counts in polyethylene-stored subsamples at 6 months probably contributed to the significant station effects.

Since nonrefrigerated polyethylene-stored subsamples lost iodine color during 6 months of storage and subsequent AO counts were lower, we stained acidified Lugol iodine solution-preserved subsamples with DAPI to see whether DAPI counts would differ from 6-month AO counts and would be similar to initial AO counts. A three-way ANOVA on 6-month counts comparing station, stain, and temperature effects showed no significant differences between AO and DAPI (Table 3). In addition, identical fields were counted on the double-stained slide; AO counts had a mean and 95% confidence limit of $3.97 \times 10^5 \pm 0.44 \times 10^5$ cells per ml, and DAPI counts indicated $4.04 \times 10^5 \pm 0.42 \times 10^5$ cells per ml. Initial counts on this sample indicated $8.64 \times 10^5 \pm 0.75 \times 10^5$ cells per ml. This contrasts with Pomroy's report (3) of no apparent loss of counts in samples that had lost all iodine color during a 48-month storage period.

In a separate trial, filters were prepared for scanning

TABLE 2. Mean counts for all stations for each fixative and temperature treatment

Time of count/ fixative temp	Mean count (10^5)/ml with:			
	Glutaraldehyde[a]	Lugol iodine solution[a]	Acidified Lugol iodine solution[a]	Acidified Lugol iodine solution (DAPI)
Initial				
Refrigerated	6.29	7.50	9.24	
Ambient		6.72	6.25	
50°C		6.98	6.31	
1 Mo				
Refrigerated	3.46	5.01	7.37	
Ambient		2.14	4.81	
50°C		2.75	5.37	
6 Mo				
Refrigerated	1.94	2.73	3.86	5.21
Ambient		0.32	2.44	2.75
50°C		0.37	1.80	1.70

[a] All counts for AO-stained slides.

TABLE 3. ANOVA *F* values

ANOVA/parameters	Factorless *F* value	*F* value for the following factor or time of determination:						Critical value[a]
		Initial	1 Mo	6 Mo	Glutaraldehyde	Lugol iodine solution	Acidified Lugol iodine solution	
Two way[b]								
Station		5.05[c]	0.10	2.70				3.84
Fixative		6.03[c]	6.32[c]	1.88				4.46
Two way[b]								
Station					0.05	0.07	7.01[c]	3.84
Time					3.70	7.76[c]	4.46	4.46
Three way[b]								
Station	3.04							3.01
Fixative	9.92[d]							3.63
Time	26.68[e]							3.63
Three way								
Station		1.26	1.18	6.80[c]				3.84
Fixative		0.09	59.98[e]	18.03[d]				5.32
Temp[f]		3.28	25.11[d]	14.22[d,g]				4.46
Three way								
Station				7.15[c]				3.84
Stain				1.12				5.32
Temp[f]				11.19[d]				4.46

[a] At 0.05 confidence level.
[b] Refrigerated samples.
[c] *F* value exceeds critical value at 0.05 confidence level.
[d] *F* value exceeds critical value at 0.01 confidence level.
[e] *F* value exceeds critical value at 0.001 confidence level.
[f] Glutaraldehyde samples were not included in these data.
[g] Ambient temperature samples stored in polyethylene had lost all iodine coloration at 6 months.

electron microscopy from glutaraldehyde- and acidified Lugol iodine solution-preserved samples taken at the same station. More bacteria were seen on the Lugol iodine solution filter than on the glutaraldehyde filter, with a much cleaner background free of the glutaraldehyde precipitate often seen in these preparations. The Lugol iodine solution-preserved bacteria appeared to be in good condition, and the same shapes and varieties were seen on both filters.

In summary, it appears that acidified Lugol iodine solution is comparable to glutaraldehyde for preserving marine bacteria for direct AO counting. Although high ambient temperatures may not affect counts done soon after sample collection, counts after long-term storage at high temperatures need to be viewed with caution. AO counts were identical to DAPI counts; both were low after prolonged storage at elevated temperatures. Reliable counts cannot be performed on samples that have lost all iodine color.

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