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Preservation of Estuarine Sediments for Lipid Analysis of Biomass and Community Structure of Microbiota

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Various methods were tested for preserving estuarine sediments in the field before biochemical analysis of the microbiota. Total microbial biomass was determined as lipid phosphate (LP), and the fatty acids of the microbial lipids were used as indicators of community structure. Control samples were sieved to remove macroinvertebrates and plant materials and were extracted immediately in the field. Other samples were preserved both before and after sieving and stored for 5 days before analysis. Freezing resulted in a 50% decline in LP and significant decreases in many fatty acids. Refrigeration resulted in a 19% decrease in LP but no change in the fatty acids. Samples preserved with Formalin before sieving exhibited no significant change in LP but substantial increases in many fatty acids, which were probably derived from the macroinvertebrates. Sieved samples preserved with Formalin showed a 17 to 18% decline in LP but no change in the fatty acids. Ideally, samples should be sieved and extracted immediately in the field. However, short-term refrigeration and longer-term preservation of sieved samples with Formalin may be acceptable compromises.

An important but often-ignored question in microbial ecology is the effect of handling and storage on environmental samples. Many experiments require taking large numbers of samples in a short time. The short time frame and the necessary logistic support make it impossible to analyze the samples without some period of storage. The indiscriminate selection of methods for preservation and storage could result in the invalidation of an experiment. Methods of preservation and storage should, ideally, maintain the microbiota in the state in which it existed at the moment of sampling, or should have minimal or documented effects.

Many methods of preservation have been investigated for the storage of bacterial monocultures (6). These methods ensure that the organisms remain viable, but none are able to maintain a constant population size. Less work has been done on the preservation of environmental samples, with only a few reports existing on the preservation of water samples for water quality assessment by enumeration (2, 4) and total direct counts by using epifluorescent microscopy (3).

Analysis of the microbiota of sediments by these methods presents a variety of problems related to the complexity of the environment and the difficulties of quantitatively recovering the organisms from the sediment particles. Detailed examination of the biomass and microbiota of sediments has been achieved by the biochemical analysis of the lipids and lipid components, which can be quantitatively extracted from the sediments (1, 9, 11). Phospholipid is an excellent indicator of microbial biomass (11). Phospholipids are found in all biological membranes in a relative constant proportion to cellular biomass (9) and have a short half-life in living and dead microorganisms in the environment (10). The fatty acids from a lipid sample can give additional insight into the community structure of the microbiota (1). Many organisms contain unique fatty acids that have been used in the taxonomic study of bacteria (7) and microeucaryotic organisms (5).

This paper reports on the effect of various methods of preservation and storage on the lipids and lipid components extracted from the microbiota of estuarine sediments. The purpose of this study was to identify a method of preservation and storage that had the least effect on the phospholipids and fatty acids compared with samples extracted immediately in the field.

MATERIALS AND METHODS

Materials. Nanograde solvents (Burdick & Jackson Laboratories Inc., Muskegon, Mich.) and freshly distilled chloroform (Mallinckrodt Inc., St. Louis, Mo.) were used. Fatty acid standards were obtained from Supelco, Inc. (Bellefonte, Pa.) and Applied Science Laboratories (State College, Pa.). Analytical grade formaldehyde solution (37%) was purchased from Mallinckrodt, and [^{14}C]formaldehyde was obtained from New England Nuclear Corp. (Boston, Mass.).

TABLE 1. Effect of various methods of preservation on the lipid phosphate of estuarine sediments after 5 days of storage

Method of preservation	Lipid phosphate[a] (nmol/g [dry wt])		Mean % change in lipid PO_4 (relative to control)
	Control	Treatment	
Unsieved samples			
Refrigeration (5°C)	70.3 (7.9)	56.7 (2.9)[b]	−19.3
Freezing (−70°C)	85.7 (10.0)	43.3 (8.8)[b]	−50.6
Freezing under argon	121.1 (15.4)	59.9 (8.5)[b]	−50.5
Freezing with 5% MeOH:$CHCl_3$	107.0 (10.0)	83.5 (30.0)	−22.0
5% Formalin	107.0 (10.0)	78.3 (11.2)[b]	−26.8
10% Formalin	116.1 (20.5)	107.5 (23.0)	−0.1
20% Formalin	116.1 (20.5)	87.6 (15.4)	−24.6
Sieved samples			
Freezing (−70°C)	85.7 (10.1)	70.8 (10.4)	−17.4
10% Formalin	69.3 (4.1)	57.5 (2.9)[b]	−17.0
	93.6 (10.4)	76.2 (4.2)[b]	−18.6

[a] Expressed as mean ± (standard deviation) ($n = 5$).
[b] Significantly different from control ($P \leq 0.05$).

Samples. Cores (2.54 cm in diameter) were taken on a mud flat near the Florida State University Marine Laboratory (29°54.0'N; 84°37.8'W). Five replicate cores were taken for each control and preservation treatment. The top 2 cm of sediment were extruded from the core tubes with a plunger and analyzed. Control samples were sieved through a 500-μm screen with buffer (0.01 M KH_2PO_4; 5.2×10^{-4} M EDTA) directly into the first phase of a lipid extraction immediately in the field. In some experiments, the extruded sediment was immediately preserved, and in others it was preserved after sieving. Differences between the control and preserved sediment samples were determined with a pooled *t*-test (8).

Preservation of unsieved samples. (i) Refrigeration. Unsieved samples were placed into plastic Whirlpaks (Nasco), transported in melting ice, and stored for 5 days in a 5°C cold room. After storage, the samples were sieved (500 μm) with 2.5% saline into a 250-ml centrifuge bottle, centrifuged at 9,000 rpm for 20 min, and decanted, and the sediment was extracted.

(ii) Freezing. Unsieved sediments were placed into Whirlpaks (Nasco), transported in dry ice, and stored for 5 days at −70°C. They were thawed and processed as previously described. This same experiment was repeated with the samples purged with argon or mixed with methanol-chloroform (2:1) to give a final concentration of 5% before freezing.

(iii) Formalin. Unsieved samples were placed in wide-mouth jars (250 ml) and fixed with Formalin to give final concentrations of 5, 10, and 20%. They were stored at room temperature for 5 days and processed as described above.

Preservation of sieved samples. (i) Freezing. Extruded sediments were sieved with 2.5% saline into 250-ml plastic centrifuge tubes (Nalgene) in the field, transported in dry ice, and stored at −70°C for 5 days. The samples were thawed and centrifuged, and the sediment was extracted.

(ii) Formalin. Sediments were sieved in the field as before and mixed with Formalin to give a final concentration of 10%. After storage, the samples were centrifuged, and the sediment was suspended in saline and recentrifuged to remove residual Formalin before extraction.

Lipid extraction. Lipids were extracted from the sediments by using modified Bligh Dyer extractions (11).

(i) Field extractions. Sediments were sieved with buffer into a jar containing 112.5 ml of $CHCl_3$ and 225 ml of methanol. Additional methanol was added as necessary to ensure a single phase. The samples were returned to the laboratory, extracted for at least 2 h, and transferred into 500-ml separatory funnels with 112.5 ml of water and 112.5 ml of $CHCl_3$.

(ii) Laboratory extractions. Centrifuged sediments were transferred to 250-ml separatory funnels with 25 ml of buffer, 75 ml of methanol, and 37.5 ml of $CHCl_3$. The mixtures were allowed to stand for at least 2 h in a single phase and then were separated into two phases with 37.5 ml of water and 37.5 ml of $CHCl_3$.

Lipid phosphate. Aliquots of the $CHCl_3$ layer were dried and digested with perchloric acid, and phosphate was determined colorimetrically as described by White et al. (11).

Fatty acids. Fatty acids were measured as their methyl esters as described by Bobbie and White (1). The $CHCl_3$ layer was dried and subjected to acid methanolysis, and the resulting fatty acid methyl esters were purified by thin-layer chromatography and separated, identified, and quantified by high-resolution gas capillary chromatography. The fatty acids are designated as number of carbon atoms:number of double bonds. With polyenoic acids, the position of the ultimate double bond is designated ω3, ω6, etc.

Formaldehyde removal experiment. Replicate sediment samples were sieved and preserved with 10% Formalin as described previously. We added 5 μCi of [^{14}C]formaldehyde (47 mCi/mmol) to each sample, and the samples were stored for 5 days. The samples were centrifuged, washed, and extracted, and the lipid was prepared for gas capillary chromatography. Radioactivity was quantified at each step by suspending the aqueous or lipid fraction in Aquasol (New England

TABLE 2. Effect of various methods of preservation on the fatty acids of estuarine sediments after 5 days of storage[a]

Fatty acid	Unsieved samples (nmol/g [dry wt])				Sieved samples (nmol/g [dry wt])			
	Control	Refrigerated	Control	Preserved with 10% Formalin	Control	Preserved with 10% Formalin	Control	Frozen
Iso plus anteiso								
15:0	1.4 (0.7)	1.8 (0.5)	8.9 (1.3)	12.7 (2.6)[b]	16.2 (3.4)	18.8 (2.1)	6.6 (0.7)	5.8 (0.5)
15:0	1.3 (0.7)	1.7 (0.5)	19.0 (1.8)	30.9 (4.6)[b]	23.1 (3.9)	25.3 (5.9)	5.4 (0.7)	7.1 (0.9)
16:0	9.2 (3.8)	10.2 (2.8)	43.2 (6.5)	57.9 (8.6)[b]	102.8 (21.3)	92.1 (21.1)	33.0 (3.0)	33.6 (2.7)
18:1ω7	3.0 (1.3)	4.2 (0.6)	11.7 (1.6)	15.8 (3.6)	28.6 (5.3)	27.4 (6.3)	12.1 (1.0)	11.0 (1.2)
18:1ω9	0.8 (0.3)	0.9 (0.2)	7.0 (0.8)	6.8 (1.0)	8.2 (2.3)	8.0 (0.5)	2.5 (0.1)	2.7 (0.3)
18:2ω6	0.6 (0.2)	0.6 (0.2)	2.6 (0.4)	3.2 (0.4)	4.3 (0.7)	4.0 (0.5)	2.1 (0.3)	1.2 (0.1)[b]
20:4ω6	1.0 (0.3)	1.2 (0.2)	1.3 (0.2)	2.6 (0.5)[b]	1.3 (0.2)	1.6 (0.3)	3.8 (0.4)	1.7 (0.3)[b]
20:5ω3	1.5 (0.4)	1.1 (0.3)	2.7 (0.9)	6.1 (0.9)[b]	2.4 (0.8)	2.8 (0.8)	4.5 (0.7)	1.8 (0.3)[b]
22:5ω3	0.02 (0.01)	0.02 (0.01)	0.09 (0.07)	0.16 (0.09)	0.30 (0.14)	0.17 (0.17)	0.08 (0.01)	0.05 (0.03)
22:6ω3	0.11 (0.03)	0.06 (0.02)	0.24 (0.16)	0.35 (0.08)	0.24 (0.13)	0.26 (0.25)	0.38 (0.03)	0.21 (0.05)[b]

[a] Expressed as mean ± (standard deviation).
[b] Significantly different from control ($P \leq 0.05$).

Nuclear) and counting the sample with a liquid scintillation counter.

DISCUSSION

No method of preservation was totally effective. Although a priori one might select freezing as an excellent method of preservation, it was one of the least effective, for it resulted in substantial decreases in the concentrations of phospholipid and some fatty acids. Only one method, preservation of unsieved samples with Formalin, did not result in a significant decrease in lipid phosphate. However, significant increases in many fatty acids were observed. Sieving the samples before Formalin fixation eliminated this change in the fatty acids but resulted in a 17% decline in lipid phosphate. The observed increase in fatty acids probably resulted from the release of lipids and lipid components from macroinvertebrates and plant materials, verifying the importance of sieving.

Ideally, one should sieve and extract samples immediately in the field. Samples remain stable for as long as 12 days when stored in the first phase of the lipid extraction (B. Baird, personal communication). Unfortunately, this procedure requires at least three times more solvent than does laboratory extraction and is impractical for a large number of samples. As a compromise, samples could be stored at 5°C. Although lipid phosphate declined minimally and fatty acids were not significantly altered in refrigerated unsieved samples after 5 days, these samples potentially remain biologically active. With longer storage, cold-induced successional changes in the microbiota could occur.

Samples preserved with Formalin also exhibited minimal declines in lipid phosphate and no changes in fatty acids, and they remained stable for up to 2.5 months. The decrease in lipid phosphate was reproducible. Two samples with quite different initial lipid phosphates showed similar percentages of decline. In addition, preservation did not increase the variance among samples. Our experience indicates that the decrease in lipid phosphate occurs during the first 24 h after preservation and is probably related to the lag in the inhibition of lipid-associated degradative enzymes. No additional changes in lipid phosphate were observed after this initial decline. The experiment with radiolabeled formaldehyde indicated that formaldehyde was removed during the preparation of the samples and should not have interfered with the biochemical analysis. Thus, Formalin represents an inexpensive way to preserve sediment samples as long as (i) care is taken to remove the Formalin from the samples and (ii) one measures the percent decline in lipid phosphate and verifies its reproducibility.

TABLE 3. Fate of radiolabeled formaldehyde during the preparation of Formalin-preserved estuarine sediments for gas capillary chromatography analysis of the fatty acid methyl esters

Purification stage	dpm[a]
Formaldehyde added to sample	11.0×10^6
Wash 1	9.6×10^6 (0.4×10^6)
Wash 2	7.0×10^5 (0.1×10^4)
Aqueous fraction of lipid extraction	6.2×10^4 (4.5×10^4)
$CHCl_3$ fraction of lipid extraction	1.5×10^2 (1.1×10^2)
$CHCl_3$ fraction after acid methanolysis	ND[b]
Fatty acid methyl esters purified by thin-layer chromatography	ND[b]

[a] Expressed as mean ± (standard deviation).
[b] ND, Not detected.

RESULTS

The effect of various methods of preservation on the microbial biomass, as measured as lipid phosphate, is shown in Table 1. Large variation was observed among different sampling locations and dates, so comparisons were limited to those between control and preserved samples taken at the same location at the same time. Changes in lipid phosphate, relative to the control, ranged from no change with unsieved samples preserved with 10% Formalin to a 50% reduction with unsieved samples preserved by freezing after 5 days of storage. The worst methods were those that involved the freezing of unsieved samples. Freezing with 5% methanol-$CHCl_3$ decreased this reduction in lipid phosphate but increased the variance threefold. The smallest decreases in lipid phosphate occurred with unsieved samples preserved by refrigeration and with Formalin and with sieved samples preserved by freezing and Formalin. The lipid phosphate of sieved samples preserved with 10% Formalin remained constant after this initial reduction for as long as 2.5 months. The effects of these preservation methods on the fatty acids from the samples were further evaluated.

Table 2 shows the concentration of fatty acids in preserved samples relative to samples extracted in the field. Unsieved samples preserved by refrigeration exhibited no significant difference in concentration relative to the control after 5 days of storage. Unsieved samples preserved with 10% Formalin exhibited significant increases in a number of fatty acids compared with the control. The fatty acids iso and anteiso 15:0 increased by 50%; palmitic acid (16:0) increased by 20%, and the long-chain polyenoic fatty acids 20:4ω6 and 20:5ω3 increased by a factor of 2. In contrast, sieved samples preserved with Formalin showed no change in the concentration of any fatty acid. Frozen sieved samples exhibited approximately 50% reductions in the concentrations of 18:2ω6, 20:4ω6, 20:5ω3, and 22:6ω3.

Since Formalin preservation showed promise, we examined the fate of radiolabeled formaldehyde during the analysis. There was concern that formaldehyde might interfere with the analysis and damage capillary columns. Table 3 shows the amount of carry-over of radiolabeled formaldehyde into the lipid at various stages in the purification. Almost all of the formaldehyde was removed with the washes and aqueous phase of the lipid extraction. Only 0.01% of the radiolabel was in the $CHCl_3$ fraction of the lipid extraction. After acid methanolysis, no radioactivity was detected in the lipid fraction.

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