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Efficacy of Phospholipid Analysis in Determining Microbial Biomass in Sediments†

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Improvements in the analysis of lipid-bound phosphates resulted in a simplified and sensitive method for determining microbial biomass in sediments. Sensitivity was enhanced over previous methods by use of a dye, malachite green, which when complexed with phosphomolybdate at low pH has a high extinction coefficient (at 610 nm). The use of a persulfate oxidation technique to liberate phosphate from lipids increased the simplicity and safety of the method relative to the traditional perchloric acid digestions. The modified method was both accurate (yielding quantitative recoveries of cells added to sediments) and precise (coefficient of variation of less than 5% for cells and sediments). A comparison with an epifluorescence technique indicated that the analysis of lipid-bound phosphate was more rapid and less tedious and could be successfully applied to a wider variety of sediment types. An estimate of the lipid-bound phosphate-to-carbon conversion factor based on a diverse enrichment culture from sediments suggested that previous factors for pure cultures may have been too low.

Several different research groups have documented the dynamic nature of benthic microbial biomass (12, 13, 29, 34) and have shown that biomass can account for a significant fraction of sedimentary organic matter (1, 20, 31). In addition, other studies have shown that microbial biomass is an important component of the total living benthic biomass (16, 19, 25) and that microbial biosynthesis can account for a significant fraction of total carbon flow (15). These observations suggest that the accurate measurement of biomass is a critical factor for understanding the trophic interactions of bacteria. The extent to which bacteria mineralize organic matter as opposed to synthesizing biomass is important for determining the role of bacteria as a food source of meio- and macrofauna and for understanding the kinetics of detritus mineralization. Similar issues and questions regarding the water column have been the subject of earlier controversy and debate (18, 23).

To date, the primary methods for measuring bacterial biomass in sediments have been similar to those used for the water column: epifluorescence microscopy (EFM) or a limited number of techniques based on the biochemical components of cells. The former has been criticized because of problems with quantitative or uncertain recoveries of attached bacteria from particles (3, 35), because of limitations on accurately measuring biovolumes (4, 8), and because of uncertainties in the factors used to convert either cell numbers or biovolumes to biomass (4, 8, 17, 28, 33). The various biochemical methods (e.g., ATP or total adenylates, muramic acid, phospholipid phosphate, etc.) also suffer from uncertainties in conversion factors. In addition, these methods can be technically cumbersome and time-consuming and in some cases can require relatively expensive instrumentation.

As a result of our own needs for a more convenient and reliable assay for biomass, we modified a biochemical technique and compared this method with EFM. We have significantly streamlined the extraction, digestion, and colorimetric procedures for measuring lipid-bound phosphates. These modifications have resulted in a method that is easier to use and potentially accessible to a wide variety of investigators. This method provided greater sample throughput than the microscopic technique and yielded more quantitative recoveries with an internal standard. In addition, biomass could be more accurately estimated by lipid-bound phosphates in a variety of sediments that were not amenable to analysis by microscopy.

MATERIALS AND METHODS

Sediments. Sediments for lipid-bound phosphate and microscopic analyses were collected from several sites. Intertidal sediments were collected by hand coring a mudflat in the lower intertidal zone of Lowes Cove, Walpole, Maine (for a description of the sediments, see reference 2), by using 10-cm (inner diameter) polycarbonate corers. Cores were returned to the laboratory for further processing. Subtidal sediments were collected with a Smith-MacIntyre grab at approximately 30 m of water depth in the outer Sheepscot Bay off Seguin Island, Maine. Two sediment types were collected at this site; the first was a well-sorted sand, and the second was a bimodal sediment composed of a mixture of fine gravel and silts. Subtidal sediments were fixed on board ship in Formalin (for microscopy) or chloroform-methanol (for lipid analysis) and returned to the laboratory for further processing.

Phospholipid analysis. Lipids were recovered from all samples by chloroform-methanol extraction (6, 42–44). Briefly, chloroform, methanol, and phosphate buffer (50 mM, pH 7.4) were added to samples in separatory funnels in a ratio of 1:2:0.8 such that the volume of chloroform in milliliters was 7 or more times the fresh weight of sediment in grams (or 50 mg [fresh weight] of cells; see below). The extraction mixture was allowed to stand for 2 to 24 h. The lipid-containing solvent (chloroform) was partitioned from the other solvents by the addition of chloroform and water such that the final ratio of chloroform-methanol-buffer was 1:1:0.9. The extraction mixture, now separated into lipid (lower) and aqueous (upper) phases, was allowed to stand for 24 h. The chloroform was filtered through Whatman 2V filter paper and recovered in a round-bottom flask, and the

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solvent was removed in vacuo. Some sediments retained significant but variable amounts of chloroform, introducing a source of error into the procedure. This source of variation was minimized by measuring the amount of chloroform recovered in a graduated cylinder and then quantitatively transferring the solvent to the round-bottom flask. If dry weights of sediment were desired, the filters were tarred and the sediment was recovered after removal of the chloroform. The lipid was transferred in chloroform to a screw-cap test tube, and the solvent was removed under a stream of nitrogen. The lipids were stored at −20°C in fresh chloroform at this point with no losses.

An alternative extraction procedure utilizing 50-ml screw-cap test tubes was compared with the procedure above. This alternative was based on the addition of 2-ml samples of a sediment slurry into test tubes and the addition of 28.5 ml of extraction mixture (as described above). The test tubes were shaken and allowed to stand for 2 h. Solvents were partitioned by adding 7.5 ml of chloroform and 7.5 ml of water. The tubes were shaken, centrifuged at low speed for 10 min, and allowed to stand overnight. To facilitate recovery of the chloroform, the aqueous (upper) phase was aspirated from the test tubes. A subsample of chloroform (10 of the 15 ml was convenient) was filtered as described above and collected in screw-cap test tubes. The chloroform was removed under a stream of nitrogen. Total lipid-bound phosphate was determined as described below. Four samples from the same sediment slurry were extracted in separatory funnels as described above for use as controls.

Phosphate was liberated from lipids recovered by either extraction method by potassium persulfate digestion (26, 38). The lipid samples were diluted in chloroform (usually 2 to 3 ml) such that a 100-μl subsample would contain between 1 and 20 nmol of phosphate. Two replicate 100-μl portions per sample were each placed in a 2-ml glass ampoule (Wheaton Scientific), and the solvent was removed under a stream of nitrogen. A saturated solution of potassium persulfate (0.45 ml; 5 g added to 100 ml of 0.36 N H_2SO_4) was added, and the ampoules were heat sealed and incubated at 95°C overnight.

The effectiveness of the persulfate liberation of lipid-bound phosphate was evaluated with three experiments. First, the release of phosphate from 10 nmol of glycerol phosphate and phosphatidylethanolamine was estimated relative to 10 nmol of P_i. Second, 5 nmol of phosphatidylethanolamine was added to an environmental lipid extract to determine recovery efficiencies of lipid in a natural sample matrix. Finally, increasing amounts of an environmental lipid extract were digested in a persulfate solution to determine the linearity of phosphate liberation over a range of lipid concentrations.

P_i released by digestion was determined by the method of Van Veldhoven and Mannaerts (39). A solution of 0.1 ml of ammonium molybdate [2.5% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 5.72 N H_2SO_4] was added to the ampoules, and the mixture was allowed to stand for 10 min. A solution of malachite green and polyvinyl alcohol in water (0.45 ml; 0.111% polyvinyl alcohol dissolved in water at 80°C was allowed to cool, and 0.011% malachite green was added) was added and allowed to stand for 30 min. The A_{610} was then read (LKB Ultrospec 4050; LKB Biochrom Ltd.; zero set with a cuvette filled with deionized water), and concentrations of phosphate were calculated by using the regression line from a standard curve prepared by digesting glycerol phosphate as described above.

EFM. Samples of sediment for epifluorescence bacterial enumeration were preserved in 10% Formalin and stored for no more than 2 weeks. The preserved sample was placed into a beaker and brought up to 100 ml with filtered (0.02-μm pore size) seawater and stirred for about 3 min; then a 3-ml subsample of this suspension was removed to a 15-ml disposable screw-cap centrifuge tube. The remaining 97 ml of sample was dried at 60°C for 24 h after removal of the seawater by centrifugation and three washes with fresh water. The dried sediment was weighed to determine the grams (dry weight) of sediment analyzed. Three milliliters of filtered seawater was added to the 3-ml subsample, and the mixture was vigorously stirred in a blender by the method of Montagna (30). Under low light, 0.125 ml of 4,6-diamidino-2-phenylindole (0.5% in deionized water; Sigma Chemical Co., St. Louis, Mo.) was added and allowed to stand for 15 min. While the mixture was stirred, a 1-ml subsample was removed, placed on a 0.2-μm-pore-size Irgalan black-stained Nuclepore filter, and evacuated at 7 lb/in^2. The filter was immersed in Cargill type B oil, and the stained cells were enumerated at ×1,200 with a Zeiss Laborlux microscope equipped with a Ploemopak 2.4 system, a HBO 50W burner, a 340- to 380-nm excitation filter, a 400-nm reflection short pass filter, and a 430-nm long pass suppression filter. At least 25 fields from duplicate filters were counted, and the results were extrapolated by using statistical techniques (30).

Enrichment culture. An enrichment culture was obtained from Lowes Cove as reported earlier (24). Surface sediments (1 g) were placed into 100 ml of MB 2216 broth (Difco Laboratories, Detroit, Mich.) amended with 0.01% tryptone (Difco). The initial culture was incubated aerobically for 24 h on a reciprocating shaker at room temperature. Approximately 90 ml of this starter culture was then transferred to 900 ml of the same medium, and the culture was incubated until maximum optical density was achieved approximately 24 h later. Then 200 ml of this secondary culture was transferred to 800 ml of medium, and the culture was incubated until maximum optical density was achieved. Cells were harvested by centrifugation and washed twice by centrifugation and suspension in phosphate buffer. These cells were then utilized in a recovery experiment (see below) as well as for determination of cell number, dry weight, total carbon, total nitrogen, and lipid-bound phosphate. Cells used for total phosphate determinations were washed with Tris buffer before lyophilization. Total carbon and nitrogen were analyzed by using an elemental analyzer (Carol Erba model 1106), and total phosphate was analyzed by digesting cells with potassium persulfate followed by analysis of the phosphate released as described above.

Cell recovery. To determine recovery efficiencies, cells from the enrichment culture were added to a sediment slurry. The slurry was prepared by using subsurface (0.5 to 1.5 cm deep) intertidal sediments. The sediment was stirred without dilution for several minutes to produce a homogeneous mixture. Five 1-g (lipid analysis) and five 0.1-g (direct count) subsamples were removed. Concurrently, a 10-fold concentration of the enrichment culture was prepared by centrifugation; this concentrate was also sampled for analysis. Sediment slurry (12 g) was mixed with 1.2 ml of the cell suspension, and five 1-g (lipid analysis) and five 0.1-g (direct count) subsamples were removed again. All samples were analyzed as described above for lipid-bound phosphates and epifluorescence counts.

Materials. All solvents used for lipid extractions were pesticide residue grade or higher quality (Fisher Scientific Co., Pittsburgh, Pa., or J. T. Baker Chemical Co., Phillipsburg, N.J.). Lipid standards and malachite green were of

TABLE 1. Color development in response to P_i, glycerol phosphate, and phosphatidylethanolamine before and after hydrolysis with persulfate

Compound	A_{610} (10 nmol; mean ± SD; n = 3)	
	Without persulfate digestion	After persulfate digestion
P_i	1.026 ± 0.013	0.987 ± 0.024
Glycerol phosphate	0.043 ± 0.002	1.009 ± 0.013
Phosphatidylethanolamine	0.042 ± 0.009	1.004 ± 0.006
Distilled water	0.041 ± 0.001	0.043 ± 0.001

reagent quality (Sigma). Polyvinyl alcohol was 100% hydrolyzed (average molecular weight, 14,000; Aldrich Chemical Co., Inc., Milwaukee, Wis. [no longer available]; Janssan Chemica [alternate source]; for discussion of need for 100% hydrolyzed polyvinyl alcohol, see reference 9). Glassware was washed with phosphate-free detergent, rinsed 5 times with tap water and 10 times with 16.7 "megaohm" deionized water, and air dried. Glassware was rinsed with chloroform just before use.

RESULTS AND DISCUSSION

Improvements in the phospholipid analysis involved modifications of the technique used to determine P_i. The method proposed by Van Veldhoven and Mannaerts (39) was found to be the most satisfactory for quantification of P_i liberated from lipids by persulfate digestion when compared with other methods (9–11, 21, 22, 27, 32, 37), which were also based on the formation of a complex between phosphomolybdate and malachite green (or other basic dyes) at low pH. The low absorbance of the reagent blank (A_{610} of about 0.040) along with high sensitivity (calculated molar absorption coefficient, 110,833 ± 1,643; n = 6), stability of the color complex, linearity (from 1 to 20 nmol of phosphate [$y = 0.01 + 0.103x$; $r^2 = 0.998$]), and the high permissible acidity in the assay (including the use of sulfuric acid) all contributed to the utility of the method chosen. With the conditions reported here, lower limits of detection were about 0.1 nmol of phosphate, which corresponds to approximately 3.4×10^6 cells. White et al. (44) reported a detection limit of 10 nmol of phosphate with a less sensitive method.

Additional improvements in methods were based on the digestion of lipid extracts with potassium persulfate. This reagent has been used frequently in the past for a variety of organic carbon, nitrogen, and phosphorus analyses (14, 26, 36, 38). The suitability of persulfate as a hydrolytic agent for lipids was demonstrated by its lack of inhibition of color development for P_i (Table 1) and by recoveries of a variety of standards and samples. Without digestion, glycerol phosphate and phosphatidylethanolamine gave little to no response, whereas after digestion, both gave equimolar responses relative to P_i. Phosphatidylethanolamine was also quantitatively recovered in the presence of lipid extracted from marine sediments (Table 2). In addition, color development was proportional to the amount of environmental lipid subjected to persulfate digestion (Fig. 1). These results suggested that a variety of organophosphates could be recovered quantitatively in a complex matrix and at concentrations typical of organic-rich marine sediments. Earlier lipid digestions were also quantitative but used perchloric acid as an oxidant (44). Although effective, perchloric acid presents safety hazards and requires the use of a stainless steel fume hood. In this regard, persulfate is to be recommended, since it can be used safely with standard laboratory apparatus. Waste problems are also minimal, since the final solution is dilute sulfuric acid.

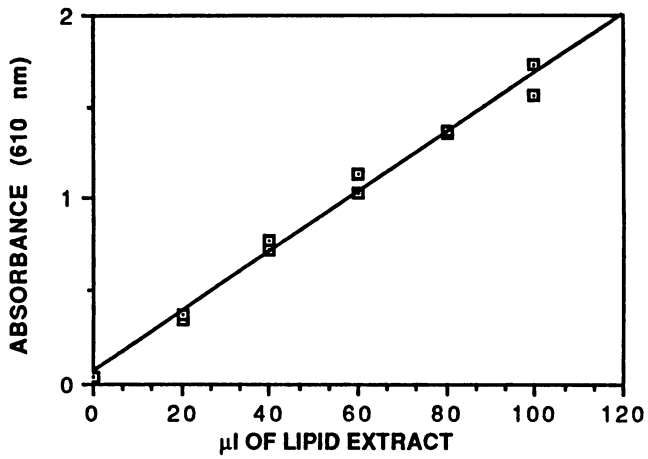

FIG. 1. Regression line ($y = 0.0625 + 0.0162x$; $r^2 = 0.991$) of microliters of lipid extract digested with persulfate versus A_{610} of malachite green-phosphomolybdate complex.

The analysis of lipid-bound phosphates in sediments can also be facilitated by eliminating the need for separatory funnels, round-bottom flasks, and removal of the lipid solvent in a rotary evaporator. In this study, extraction of sediment has proven equally efficient when conducted in either separatory funnels or 50-ml test tubes (Table 3). Depending on the volume of sediment extracted, smaller tubes can also be used. Test tubes provide several advantages: lower costs, ease of handling, fewer liquid transfers, and increased sample numbers. The use of convenient and economical glassware, an improved lipid digestion, and a more sensitive method of phosphate detection should avail to a wider group of investigators the use of phospholipids as a measure of microbial biomass.

TABLE 2. Efficiency of phosphate recovery from phosphatidylethanolamine in the presence of environmental lipids[a]

Prepn	A_{610} (mean ± SD, n = 5)	nmol of phosphate
Phosphatidylethanolamine	0.533 ± 0.016	5.01 ± 0.16
Environmental lipid extract	0.577 ± 0.010	5.43 ± 0.09
Environmental lipid plus phosphatidylethanolamine mixture	1.074 ± 0.018	10.2 ± 0.17

[a] The expected recovery of phosphate from phosphatidylethanolamine was 5.0 nmol. The amount recovered, calculated as (environmental lipid + phosphatidylethanolamine mixture) − environmental lipid, was 4.81 ± 0.09. The recovery efficiency, calculated as (recovered phosphate/expected phosphate) × 100, was 96.0%.

TABLE 3. Comparison of lipid-bound phosphate recovery with separatory funnels and test tubes

Apparatus	Mean nmol ± SD (% SD) (n = 4) of lipid-bound phosphate recovered per:	
	ml of sediment	g (dry wt) of sediment
Separatory funnels	65.37 ± 2.9 (4.4)	221.5 ± 9.6 (4.4)
Test tubes	65.71 ± 3.4 (5.2)	223.1 ± 3.4 (5.8)

TABLE 4. Recovery of cells added to marine sediments[a]

Prepn	Recovery by the following method:	
	Lipid analysis[b]	Direct counts[c]
Sediment	74.6 ± 2.50	9.1 ± 5.8
Culture	107.6 ± 4.5	36.8 ± 3.6
Sediment plus culture	166.4 ± 19.9	34.0 ± 9.1

[a] Recovery efficiencies, calculated as (recovered phosphate or cells/expected recovery of phosphate or cells) × 100, were 102.2 ± 20.5% for the lipid analysis and 77.9 ± 27.3% for direct counts. The expected recovery of phosphate or cells was the value obtained from the culture multiplied by 90% (a correction for dilution during the preparation of the sediment-plus-culture slurry). The recovered phosphate or cells value was calculated as sediment plus culture − (sediment × 90%).

[b] Nanomoles of lipid-bound phosphate recovered per gram (wet weight) of sediment or 100 μl of culture (mean ± standard deviation; $n = 5$).

[c] Cells (10^8) per gram (wet weight) of sediment or 100 μl of culture (mean ± standard deviation; $n = 5$, except for culture, where $n = 4$).

Additional advantages of the phospholipid analysis for biomass are its relative accuracy and precision. Recovery of lipid-bound phosphate from cells added to marine sediments was quantitative (Table 4), and replicate analyses of both sediments and cells were highly reproducible, with coefficients of variation of 3.4 and 4.2%, respectively. The coefficient of variation for the sediment-cell mixture was somewhat higher (11.9%), possibly due to incomplete mixing. In contrast, recovery of added cells by EFM was only about 75%. Although the reproducibility of the EFM method was acceptable for the enrichment culture (coefficient of variation, 9.8%), replicate analyses of sediment were quite variable, with a coefficient of variation of 64%. This comparison is even more striking if one considers that the added cells had distinct, easily recognizable morphologies and were not likely bound in biofilms to the sediment grains. Thus it is likely that the recoveries for the EFM method are upper limits.

Inaccuracies in the EFM method have been reported by others. The problems have been attributed by some (7, 40) to incomplete removal of bacteria from sediment. Schallenberg et al. (35) emphasized problems with masking which resulted in estimates of cell numbers that may have been low by a factor of 2 to 10. Bott and Kaplan (7) and Balkwill et al. (5) have also reported lower estimates of biomass from 4,6-diamidino-2-phenylindole and acridine orange, respectively, relative to estimates from phospholipids. Bott and Kaplan (7) attributed the differences to detrital or nonliving phospholipids. However, this explanation is inconsistent with the reported rapid turnover of phospholipids in sediments (41, 44). Although part of the discrepancy may be an artifact of incorrect biomass conversion factors for both biovolume and phospholipids, it is likely that a real discrepancy does exist due to limitations of the EFM method. Since underestimates of even a factor of 2 have substantial ramifications for interpreting the dynamics of microbial biomass, a resolution of the discrepancies between biochemical versus EFM methods is imperative.

Schallenberg et al. (35) have proposed modifications of the EFM method that reduce its errors, but these modifications make a somewhat tedious technique even more so. As a result, it may be desirable to assess the absolute accuracy of the phospholipid approach. This approach is simple and quantitative, without the need for complicated recovery factors. In addition, it is free from the subjective biases that can be introduced by different microscopists. Its accuracy appears primarily limited by the conversion factor used to calculate cell biomass from phospholipids. Of course, analogous conversion factors also limit the EFM method.

TABLE 5. Elemental composition of an enrichment culture from a marine sediment[a]

Element or compound	mmol/g of cells	μg/100 nmol of lipid-bound phosphate
Carbon	33.4	191.7
Nitrogen	7.4	49.6
Phosphate	0.350	15.9
Lipid-bound phosphate	0.209	

[a] Numbers of cells were 7.17×10^{12} per g and 3.43×10^9 for 100 nmol of lipid-bound phosphate.

Based on data from the mixed enrichment culture, it is possible to generate both cell number and biomass conversion factors for lipid-bound phosphate. These conversion factors (3.4×10^9 cells or 191.7 μg of C per 100 nmol of phospholipid; Table 5) differ from values used in most of the existing ecological literature (4×10^9 to 2×10^{11} cells or 1,000 μg of C per 100 nmol of phospholipid; 5, 41). In part, the differences may reflect the use of a naturally occurring, mixed population in this study versus pure *Escherichia coli* cultures in others. A more detailed analysis of the variability in phospholipid conversion factors is currently under way. However, it is advisable that individual investigators establish appropriate conversion factors for the particular system being examined until (if ever) a more universal factor is accepted.

By using the cell number conversion factor established here, it is possible to make additional comparisons of results from the EFM and phospholipid methods. Lipid analyses of the silty clays in Lowes Cove indicate that the EFM estimates are low by a factor of 2.8-fold. In sandy sediments, numbers from EFM are 3 to 3.5 times lower than those from phospholipids (Table 6). A greater discrepancy is calculated for the bimodal gravel-silt sediments, where lipid-based values are 22 to 31 times higher than those from EFM. These observations are consistent with the trends noted by Schallenberg et al. (35) for decreased cell recovery as a function of decreasing water content and increasing grain size. Given the inherent difficulties in using sediments with large grains for EFM techniques, it is perhaps preferable to use a technique such as the phospholipid analysis even if discrepancies among techniques are minimal.

In conclusion, the analysis of phospholipids has been modified to provide a relatively simple and convenient

TABLE 6. Comparison of estimated microbial cell numbers: lipid-bound phosphate and direct counts

Sediment type (site)	Cell no. estimates, 10^9[a]		Difference (fold)
	Lipid-bound phosphate	Direct counts	
Intertidal mud	2.5	0.93	2.7
Subtidal sand (A)	1.0	0.33	3.0
Subtidal sand (B)	0.61	0.17	3.6
Subtidal sand (C)	1.3	0.43	3.0
Subtidal gravel-silt (D)	2.0	0.064	31
Subtidal gravel-silt (E)	2.7	0.097	28
Subtidal gravel-silt (F)	1.7	0.078	22

[a] Cell numbers were estimated by using a conversion factor of 100 nmol of lipid-bound phosphate = 3.43×10^9 cells. Data for the intertidal site are means of five analyses, given as 10^9 cells per g (wet weight) of sediment. Data from the subtidal sites are from single analyses, given as 10^9 cells per g (dry weight) of sediment.

method for estimating microbial biomass. The modifications increase sensitivity, sample throughput, and ease of handling. The method is more rapid and less tedious than epifluorescence techniques and appears to be more accurate and precise in terms of generating the primary data (e.g., phospholipid concentrations). In addition, a greater variety of samples is amenable to phospholipid extractions than to EFM. The major limitation at present concerns the conversion factor used to calculate biomass in terms of dry weight or, preferably, carbon. This limitation may be overcome by using conversion factors generated from mixed populations of bacteria present within the sample matrix.

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