for chloroform in regions of high primary production. Fresenius Z. Anal. Chem. **327:** 39–40.
———, AND ———. 1987*b*. Global baseline pollution studies. Fresenius Z. Anal. Chem. **327:** 198–204.
COLLÉN, J., A. EKDAHL, K. ABRAHAMSSON, AND M. PEDERSÉN. 1994. The involvement of hydrogen peroxide in the production of volatile halogenated compounds by *Meristiella gelidium*. Phytochemistry **36:** 1197–1202.
FAULKNER, D. J. 1977. Interesting aspects of marine natural products chemistry. Tetrahedron Rep. **28,** p. 1421–1442.
GRIBBLE, G. W. 1992. Naturally occurring organohalogen compounds—A survey. J. Nat. Products **55:** 1353–1395.
GSCHWEND, P. M., J. K. MACFARLANE, AND K. NEWMAN. 1985. Volatile halogenated organic compounds released from temperate marine macroalgae. Science **227:** 1033–1035.
KLICK, S., AND K. ABRAHAMSSON. 1992. Biogenic volatile iodated hydrocarbons in the ocean. J. Geophys. Res. **97:** 12,683–12,687.
LOVELOCK, J. E. 1975. Natural halocarbons in the air and over the Atlantic. Nature **256:** 193–194.
MCCARTY, P. L., AND M. REINHARD. 1993. Biological and chemical transformations of halogenated aliphatic compounds in aquatic and terrestrial environments, p. 839–852. *In* R. S. Oremland [ed.], Biogeochemistry of global change. Chapman and Hall.
MANLEY, S. L., AND M. N. DASTOOR. 1987. Methyl halide (CH_3X) production from the giant kelp, *Macrocystis* and estimates of global (CH_3X) production by kelp. Mar. Biol. **98:** 477–482.
———, K. GOODWIN, AND W. J. NORTH. 1992. Laboratory production of bromoform, methylene bromide, and methyl iodide by macroalgae and distribution in nearshore southern California waters. Limnol. Oceanogr. **37:** 1652–1659.
MOORE, R. E. 1977. Volatile compounds from marine algae. Acc. Chem. Ref. Data **10:** 1175–1199.
———, R. TOKARCZYK, V. TAIT, M. POULIN, AND C. GREEN. 1995. Marine phytoplankton as a natural source of volatile organohalogens, p. 283–294. *In* A. Grimvall and E. W. B. de Leer [eds.], Naturally-Grimvall produced organohalogens. Kluwer.
NEWMAN, K. A., P. M. GSCHWEND, AND J. K. MACFARLANE. 1987. A method for quantitative determination of volatile organic compounds in macroalgae. Limnol. Oceanogr. **32:** 702–708.
SCHALL, C., F. LATURNUS, AND K. G. HEUMANN. 1994. Biogenic volatile organoiodine and organobromine compounds released from polar macroalgae. Chemosphere **28:** 1315–1324.
SIUDA, J. F., AND J. F. DEBERNARDIS. 1973. Naturally occurring halogenated organic compounds. Lloydia **36:** 107–143.
STURGES, W. T., G. F. COTA, AND P. T. BUCKLEY. 1992. Bromoform emission from Arctic ice algae. Nature **358:** 660–662.
———, C. W. SULLIVAN, R. C. SCHNELL, L. E. HEIDT, AND W. H. POLLOCK. 1993. Bromoalkane production by Antarctic ice algae. Tellus **45B:** 120–126.
VANDEN DRIESSCHE, T., C. KEVERS, M. COLLET, AND T. GASPER. 1988. *Acetabularia mediterranea* and ethylene: Production in relation with development, circadian rhythms in emission, and response to external application. J. Plant Physiol. **133:** 635–639.
WALTER B., AND K. BALLSCHMITTER. 1992. Formation of C_1/C_2-bromo-/chloro-hydrocarbons by haloperoxidase reactions. Fresenius Z. Anal. Chem. **342:** 827–833.
ZAREMBINSKI, T. I., AND A. THEOLOGIS. 1994. Ethylene biosynthesis and action: A case of conservation. Plant Mol. Biol. **26:** 1579–1597.

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A dye-binding assay for the spectrophotometric measurement of transparent exopolymer particles (TEP)

Abstract—Large transparent exopolymer particles (TEP) are found abundantly in the ocean and play an important role in many fields of marine ecology. Quantification of TEP by light microscopy, however, is labor-intensive and slow. Here we introduce a simple, semiquantitative method to determine the concentration of TEP colorimetrically. In this method TEP are first stained with alcian blue. The dye complexed with TEP is then redissolved and measured spectrophotometrically. Several independent tests of the method show that the concentration of TEP measured spectrophotometrically compares well with parallel light microscope counts. Fractionation experiments confirm that TEP are not generated as an artifact of filtration. Field data show that the concentration of TEP in different oceanic environments ranges from 10 to 500 μg liter^{-1} xanthan equivalent depending on season, depth, and plankton community composition.

Transparent exopolymer particles (TEP) have recently been discovered to be abundant (up to 10^4 ml^{-1}) both in the ocean and in algae cultures (Alldredge et al. 1993; Passow et al. 1994; Passow and Alldredge 1994). After they are stained, these transparent polysaccharide particles can be observed on filters as discrete deformable strings, disks, or films up to several 100 μm long. Although similar particles have been described before (Gordon 1970; Wiebe and Pomeroy 1972; Emery et al. 1984), their impact on different pelagic processes has never been investigated because these particles are hard to visualize, identify, and quantify.

However, the existence of exuded exopolymers as discrete gellike particles, rather than as dissolved molecules or as particle coatings, has far-reaching implications not

only for the aggregation of diatom blooms (Passow et al. 1994) but also for many fields of oceanography (Alldredge et al. 1993). For example, chemical reactivity of many substances will change in the presence of polysaccharide surfaces; TEP may be used as food by zooplankton; and TEP act as a substrate for bacteria (Passow and Alldredge 1994), changing interactions between bacteria and their environment. Because TEP may be formed abiotically from dissolved excretion products of phyto- and bacterioplankton (Passow et al. 1994; Passow and Wassmann 1994; Mopper et al. 1995), the presence of these particles also suggests an alternative route (to bacterial uptake) for the conversion of dissolved to particulate organic carbon. However, little is presently known about the natural distribution or abundance of these particles because methods to quantify TEP are still in their infancy.

TEP are made visible by staining them with alcian blue (copper-phtalocyanin with four methylene-tetramethyl-cisothiouronium-chloride sidechains), a hydrophilic cationic dye that complexes with anionic carboxyl or halfester-sulfate groups of acidic polysaccharides. The alcian blue sidechains react with the acidic groups of polysaccharides yielding an insoluble non-ionic pigment. The selectivity of the stain can be controlled by varying either the pH or the salt content of the dye solution (Horobin 1988). An aqueous dye solution at a pH of 2.5 without extra electrolyte will stain both carboxyl and sulfated polysaccharides instantaneously, but not neutral sugars. The actual mechanism of the reaction between the dye and the substrate, however, is not well understood (Horobin 1988).

Alcian blue has been used in medical and biological research to stain internal or external mucous layers, glycosamines in blood or urine, intracellular polysaccharides in seaweed and extracellular polysaccharides in colony matrices or capsules of algae and bacteria (e.g. Parker and Diboll 1966; Whiteman 1973; Ramus 1977; Vandevivere and Kirchman 1993). Applicability in marine systems, however, has been limited because artifacts are formed in the presence of salts.

Recently, a protocol that stains TEP, but not cell interiors or colony matrices, has been introduced for enumeration with the microscope (Alldredge et al. 1993) and described in detail (Passow and Alldredge 1994). The microscopic determination, however, is labor-intensive especially since stained particles often do not have high enough contrast to use image analysis systems. In this note we introduce a semiquantitative method to determine the amount of TEP present in seawater or freshwater. Our method is based on the colorimetric determination of the amount of dye complexed with extracellular particles present and therefore measures staining capability of TEP. The method is easy to use and will permit routine measurements of TEP, thus increasing our knowledge of their abundance, distribution, and role in the ecology of the ocean.

TEP is stained by gently filtering the sample (4–6 replicates) at low, constant vacuum (150 mm of Hg) onto polycarbonate filters (0.4-μm pore-size) and staining particles on the filter for <2 s with 500 μl of a 0.02% aqueous solution of alcian blue (8GX) in 0.06% acetic acid (pH 2.5). The stain should never be added to the aqueous sample before filtration because it forms an insoluble pigment with dissolved substrates (Horobin 1988). Because the dye coagulates, the staining solution should be prefiltered (0.2 μm) before use. After being stained, filters are rinsed once with distilled water to remove excess dye. Rinsing will not wash off dye bound to substrates. Filters are then transferred into 25-ml beakers. Six milliliters of 80% H_2SO_4 are added and filters soaked for 2 h. The beakers should be gently agitated 3–5 times over this period. The absorption maximum of the solution lies at 787 nm (Fig. 1) and absorption is measured in a 1-cm cuvette against distilled water as a reference.

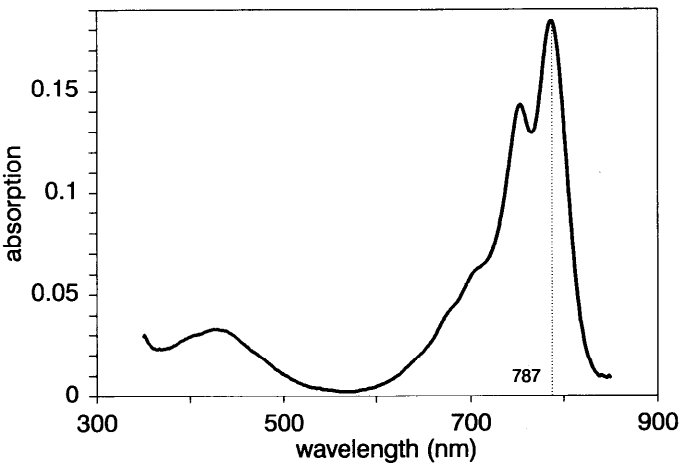

Fig. 1. The continuous absorption spectrum of alcian blue in 80% sulfuric acid. Spectra were measured with a UV-vis spectrophotometer (SLM-Aminco DW/200). The absorption maximum lies at 787 nm.

Absorption is linearly related to the amount of stainable material present in the sample (Fig. 2), unless the filters are clogged. It is critical to filter a sample volume small enough to avoid clogging of filters. Samples of 1–20 ml in volume for phytoplankton cultures and of 30–500 ml in volume for natural seawater usually yield good absorbance. Absorbance should lie above 0.15 and below 0.40 with standard deviations of replicates of <5–10%.

Concentration of TEP (C_{TEP}) is expressed as gum xanthan equivalent (μg liter^{-1}) and can be determined from

$$C_{TEP} = (E_{787} - C_{787}) \times (V_f)^{-1} \times f_x. \quad (1)$$

E_{787} is the absorption of the sample, C_{787} is the absorption of the blank, V_f is the volume filtered in liters, and f_x is the calibration factor in micrograms.

Filter blanks, which vary with filter type and stain batch, consist of stained blank filters. Average absorptions of filter blanks range between 0.07 and 0.09 for 0.4-μm polycarbonate filters and are subtracted from the absorptions of samples. Sample blanks (measuring the unstained sample) are generally not necessary. Sample blanks for seawater, batch cultures of diatoms, and marine snow were always zero, with one exception. One type of marine snow we collected showed an appreciable sample blank. Culture

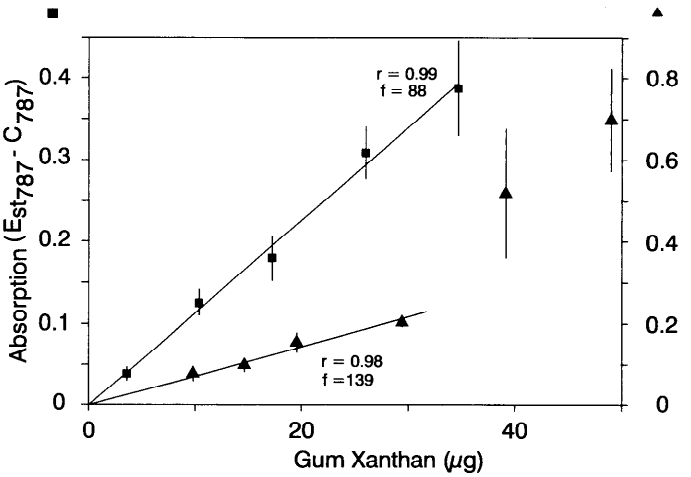

Fig. 2. Two standard curves made with gum xanthan for two batches of alcian blue. The absorptions corrected for blanks vs. dry weights are depicted. Below an absorption of ~0.4, the absorption is linearly related to the amount of xanthan. The slopes (f^{-1}) of the regressions vary for different batches of alcian blue. When filtered concentrations of gum xanthan are too high, the filter clogs and absorption increases nonlinearly.

media blanks (staining and analyzing sterile media) need to be determined for each batch of media for culture work because stainable artifacts may form from precipitates in the media. Media blanks should be only slightly higher than filter blanks, if media are prepared precipitate-free.

Alcian blue shows marked batch variation in both purity and solubility, and even if the staining solution is prepared very carefully, the dye content and subsequent measured absorption of a sample varies. Furthermore, the dye content of the staining solution decreases with age because reaggregated dye particles are removed during prefiltration. Therefore, calibrating the staining solution is necessary so that samples measured with different batches of staining solution can be compared.

The calibration standard is prepared by mixing ~15 mg of gum xanthan into 200 ml of distilled water. The solution is ground with a tissue grinder, mixed for 30 min, and ground again to break apart the gellike particles that form. Dry weights of the standard solution are determined by filtering 0.5–3-ml aliquots onto preweighed filters as described by Sharp (1992). Alcian blue stainable particles are measured by filtering 0.5–3 ml of the calibration standard and following the protocol described above. The calibration factor f_x is calculated by relating dry weight measurements of gum xanthan particles retained on the filters to their staining capacity according to

$$f_x = W \times [(\text{est}_{787} - C_{787}) \times V_{st}^{-1}]^{-1}. \quad (2)$$

W is the dry weight of the standard (μg liter^{-1}), est_{787} its average absorption, C_{787} the absorption of the blank, and V_{st} the volume (in liters) filtered for staining.

Two different calibration curves of gum xanthan (Fig. 2) show the linear relationship between weight and absorption below an absorption of ~0.4, where clogging of the filter occurs. The calibration factors vary according to the batch of staining solution.

We tested 10 different commercially available substances for their suitability as standards (Table 1). The ideal standard substance "dissolves" in water but forms small gellike particles, similar to TEP, that are retained by a 0.4-μm filter. Ideally, the staining capacity of such particles must be high enough so that the amount of dye bound is measurable by our method. Furthermore, the particles must be abundant enough that dry weight determinations are reliable and the standard regression must be easily replicated.

Table 1. Staining of various substances with our colorimetric TEP assay. Column 1 gives concentrations (mg ml^{-1}) of the different test solutions. Column 2 shows the volume filtered (ml) for weight and TEP determinations. The weight of particles (μg ml^{-1}) retained on a 0.4-μm filter is depicted in column 3. Staining capacity (μg) as measured with the colorimetric assay is given in column 4 (—: staining below detection; number: calibration factor = f-factor). Results from the microscopical observations are shown in column 5 (0: no stained material present; +: stained particles had TEP-like appearance; —: stained material did not have TEP-like appearance). Column 6 gives the percentage of material in each test solution that was retained on filters.

Substance	1	2	3	4	5	6
Carbohydrates						
Agarose	0.16	4	112.9	—	0	71
Amylose	0.75	6	465.5	—	0	62
Chitin	0.06	6	33.9	—	0	57
Laminarin	1.00	8	4.1	—	(0/+)	0.4
Alginic acid-free acid	0.09	6	58.2	6,539	—	62
Alginic acid Na-salt	2.07	6	5.0	357	+	0.2
Gum xanthan	0.08	1.5	12.6	341	+	16
D-glucuronic acid	25.00	8	3.0	—	+	0.01
Carrageenin	0.16	4	30.8	1,962	+	19
Proteins						
Bovine serum albumen	0.08	10	18.1	—	0	23
Carboxylase	0.15	8	6.8	—	0	5

We made aqueous solutions of the different substances as described for gum xanthan, and by trial and error found the respective maximum concentrations that could be filtered without clogging the filter. Concentrations of the different solutions and volumes filtered for the tests are given in Table 1. We determined the dry weight of the particles retained by the filters and measured alcian blue staining capacity on 4 replicates for each solution as described for gum xanthan. Furthermore, we prepared slides for microscopic investigations (Passow and Alldredge 1994) by staining particles as described for the colorimetric method and transferring stained particles from filters onto a glass slide according to the filter-transfer-freeze technique (Hewes and Holm-Hansen 1983).

The percentage of material retained on the filter ranged between 0.01 and 71% for the different substances (Table 1). Agarose, amylose, chitin, and laminarin do not contain either carboxyl or sulfate groups, which explains why these substances did not stain with alcian blue (measured colorimetrically). Staining of some TEP-like particles was observed microscopically for laminarin, but most of the particles present on the filter did not stain. No stained particles were generated by the other three substances. Because staining of laminarin particles was rare, we assumed that it was caused by contaminants. Alginic acid, xanthan, and D-glucuronic acid contain carboxyl groups and should stain with alcian blue. The sodium salt of alginic acid and xanthan generated stained TEP-like particles and gave good colorimetric results. Microscopical observations revealed that D-glucuronic acid generated stained TEP-like particles; however, the solubility of D-glucuronic acid was so high (only 0.01% of the solution was retained on the filter) that overall staining capacity was below the detection limit of the colorimetric method. Alginic acid in its free-acid form gave measurable results on the colorimetric method but did not form TEP-like particles. Instead, a layer of material covered the whole surface area of the filter. Carrageenin contains no carboxylated groups, but does contain sulfated groups, to which alcian blue also binds well (Table 1). The number of sulfated sites of carrageenin can vary considerably resulting in variable staining capacity (Collin 1987). As expected, the two proteins tested (albumen and carboxylase) did not stain.

Of the three substances that showed appreciable staining, alginic acid and gum xanthan fulfilled the conditions concerning both colorimetric and dry weight measurements best. We choose gum xanthan (Sigma G-1253) as a standard because its replicability was better than that for alginic acid (Sigma A-7003), but we have used both successfully.

The sensitivity of the colorimetric method to variations of specific parts of the protocol was tested. Seawater samples and marine snow collected in the Santa Barbara Channel, California, on several occasions were used for these tests. Some tests were also conducted with *Chaetoceros gracilis* batch cultures or with gum xanthan. In each experiment an aliquot of the sample was measured according to the standard protocol (4–6 replicates) and other aliquots were measured varying one part of the standard protocol at a time (4–6 replicates each). Averages of measured absorption were normalized to the average absorption measured according to the standard protocol.

Test 1: The impact of the concentration of the staining solution on the quantification of TEP was investigated in four independent experiments using seawater, marine snow (twice), and gum xanthan. For each experiment, six different staining solutions were prepared, two with an alcian blue concentration below the standard 0.02% and three with higher concentrations of alcian blue (Table 2, A). Normalized absorption was <1 when the concentration of the dye was lower than the standard 0.02%. Normalized absorption did not differ significantly from 1 when aliquots were stained with the more concentrated dye solution; however, the calibration factors measured for the respective staining solutions varied according to the staining capability of the dye, and the concentration of TEP expressed as xanthan equivalents was independent of the dye concentration of the staining solutions. This experiment indicates that the dye solution as used in our standard protocol is concentrated enough to avoid understaining. With concentrations of alcian blue that are $<0.02\%$, understaining might occur; however, potential variations in the staining of samples due to different concentrations of alcian blue are corrected for by calibrating each batch of alcian blue solution.

Test 2: The effect of pH of the dye solution on the staining capability of alcian blue was determined (one experiment) by adding 1–2 drops of HCl or NaOH to dye solutions (Table 2, B). Staining capability of the dye solutions decreased if the pH of the dye solution was lower or higher than that of the standard dye solution (pH 2.6). At pH 1, only sulfated polysaccharides stain fully and polysaccharides with carboxyl groups do not stain at low pH (Horobin 1988).

Test 3: We also investigated whether a staining time of >2 s would increase the amount of staining of fresh seawater, Formalin-preserved seawater, marine snow, or gum xanthan (six different experiments, Table 2, C). A staining time of >2 s does not result in higher absorption if gum xanthan, Formalin-preserved samples, or cell-poor marine snow are used (Table 2, C). Phytoplankton cells, however, will lyse if subjected to the fresh staining solution for >5 s, and products of lysis often stain with alcian blue. Thus, keeping the staining time short is important if live cells are present.

Test 4: According to the standard protocol, stained filters are soaked for 2 h in H_2SO_4 to decompose polysaccharides and redissolve the dye before measuring absorption. We conducted six experiments measuring absorption after shorter and longer soaking periods (Table 2, D). Although the dye redissolves in <0.5 h, small bubbles that form during decomposition of the organic material impede reading absorption for ~ 2 h. These bubbles disappear after 2 h if the samples are mixed intermittently. The color of the sample solution remains stable for >20 h. Samples, however, need to be carefully covered if they are stored for long periods because H_2SO_4 is hygroscopic.

Table 2. Variations of the standard protocol and their impact on the staining assay: A—dye concentration (% of alcian blue); B—staining time (s); C—pH of the dye solution; D—soaking time of the filters in H_2SO_4 (h). Results were normalized to results of the standard protocol (shown bold) and given as average relative absorption ± SD (n—number of experiments, 4–6 replicates each). Results of one of the four experiments (A1) where dye concentration was varied are also depicted in xanthan equiv. ($\mu g\ ml^{-1}$) because differences in the calibration factors between the different dye solutions corrected for different absorptions. (NA—not available.)

Treatment	n	Avg relative absorption	Xanthan equiv.
A. Dye concn			
0.010	4	0.50±0.10	5.2±0.36
0.015	2	0.63±0.05	5.2±0.47
0.020	**4**	**1.00**	5.5±0.28
0.025	2	1.01±0.01	NA
0.030	2	0.87±0.02	5.2±0.16
0.040	4	1.09±0.17	5.4±0.11
B. Stain time*			
2-5	**6**	**1.00**	
10–15	5	1.11±0.06	
30	2	1.03±0.11	
60	2	1.14±0.07	
C. pH			
1.03	1	0.41±0.03	
1.50	1	0.91±0.04	
2.61	**1**	**1.00**	
3.29	1	0.79±0.02	
4.55	1	0.73±0.03	
D. Soak time			
0.25	2	0.94±0.05	
0.5	6	0.92±0.08	
1.0	6	0.98±0.03	
1.5	2	1.00±0.01	
2.5	**6**	**1.00**	
4.5	6	1.00±0.01	
11.0	6	0.97±0.02	
20.0	4	1.00±0.03	

* Formalin-fixed samples or artificial substances were used to avoid lysing of cells.

Test 5–6: The standard protocol suggests one rinse after staining, but no measurable difference was observed when samples were not rinsed or rinsed twice at this time (Fig. 3). The surface area of our filters is covered by 0.5 ml of the staining solution, but using a larger quantity of staining solution does not lead to overstaining (Fig. 3).

Test 7: Modifications in filtration pressure produced the greatest variations on the measured absorption of samples of any of the variables investigated. Keeping filtration pressure low and at a constant magnitude are critical because TEP are very flexible gellike particles and can easily be pulled through the pores of the filter at high pressure (Fig. 3).

Test 8: TEP can be measured from Formalin-preserved samples because Formalin does not interfere with the staining procedure itself (Passow and Alldredge 1994).

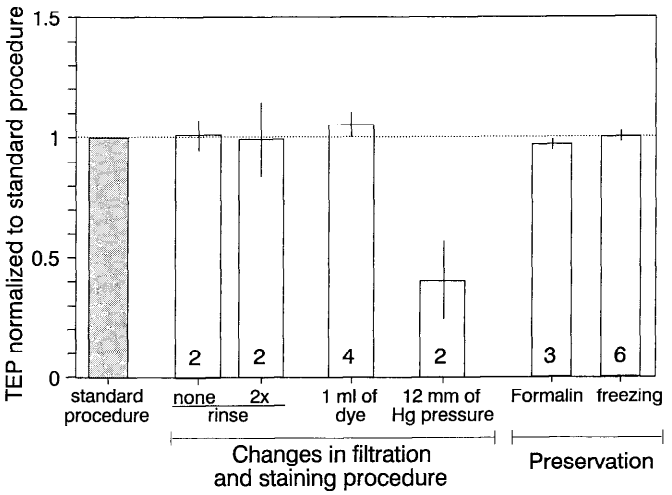

Fig. 3. Tests of sensitivity to variations of the standard protocol. Columns depict averages and standard deviations of TEP normalized to the standard procedure. Numbers in columns indicate the number of experiments conducted for each kind of test. Neither the degree of rinsing nor the amount of dye impacted the results significantly; however, filtration pressure was critical because TEP is very flexible and can be sucked through the filter at high pressures. Freezing stained filters for 1–24 weeks or measuring TEP from Formalin-preserved samples did not alter measurements of TEP.

However, if organisms that disintegrate in Formalin are present, the concentration of TEP may be overestimated in Formalin-preserved samples compared to fresh samples because disintegrated material may stain. The intact colony matrix of *Phaeocystis*, for example, does not stain with our method, but shreds from disrupted colonies stain well. Thus, TEP measured in Formalin-fixed samples that contain *Phaeocystis* may originate from disintegrating senescent colonies or from originally intact colonies that disintegrated due to the impact of Formalin. Measuring TEP in Formalin-preserved samples may be useful for sediment trap samples that often are preserved in situ. For routine measurements of seawater, however, where large sample volumes are needed for TEP measurements, Formalin preservation would not be the method of choice.

Test 9: We tested whether filtered and stained samples can be frozen before measurements. Seawater samples were collected off Santa Barbara in June and July 1993. Several replicate samples were filtered and stained according to the standard protocol of the TEP assay. Six replicates were measured immediately according to the standard method; the other stained filters were transferred into Petri dishes and frozen. Dye blanks were also prepared and frozen. Frozen samples were later measured (6 replicates) by the same method. We compared the quantity of TEP measured immediately after sampling with values obtained after measuring stained filters frozen for 1, 4, and 24 weeks. No statistically significant differences were observed (Fig. 3).

As an alternative to the colorimetric method, the quantity of TEP can also be estimated from total area calculated from microscopical counts (Passow et al. 1994). We

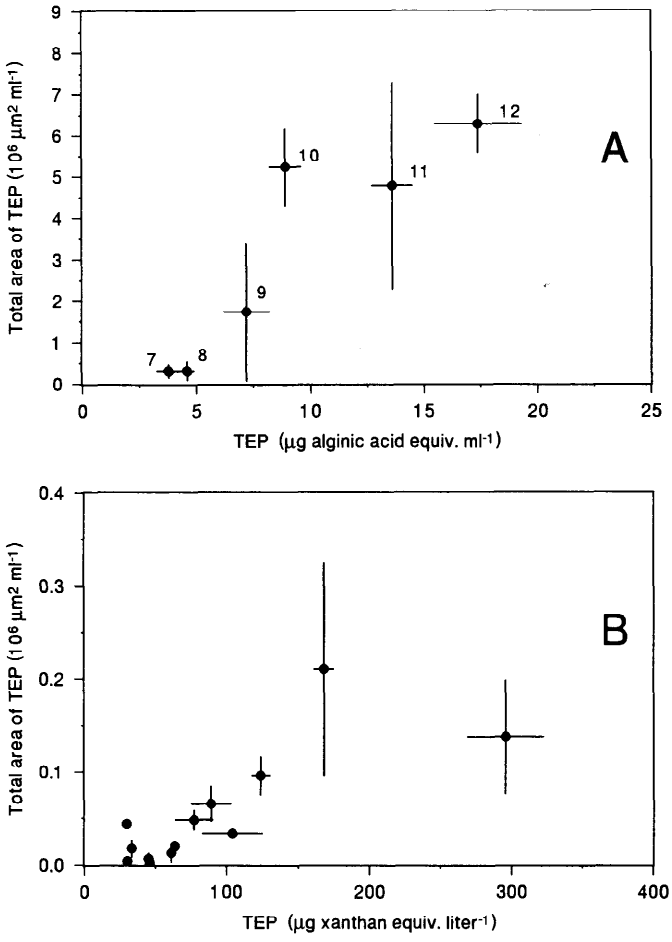

Fig. 4. Relationship between total area of TEP (microscopical determination) and the quantity of TEP determined colorimetrically from samples collected during a diatom bloom grown in a mesocosm (A) and from representative in situ samples collected between January and June 1994 in the Santa Barbara Channel (B). (Some error bars are too small to be resolved graphically.)

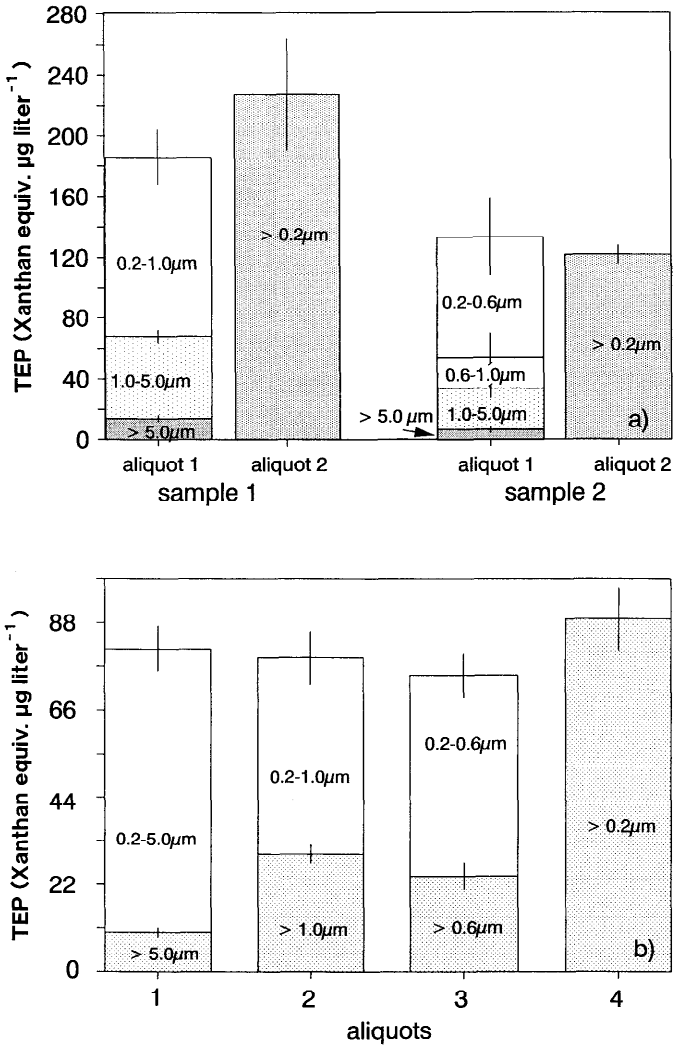

Fig. 5. Results of two types of fractionation experiments. Aliquots of two samples for experiment A (a) and one sample for experiment B (b) collected in the Santa Barbara Channel in June 1993 were passed sequentially through filters of different pore sizes. In all experiments the total amount of TEP in size-fractionated aliquots equaled that in unfractionated aliquots.

measured TEP with both methods during the development of a diatom bloom grown in a 1,400-liter mesocosm over a period of 13 d (Alldredge et al. 1995; Passow and Alldredge 1995) and in 14 field samples collected between January and July 1994 at a fixed station in the Santa Barbara Channel.

Filtered seawater in the mesocosm was inoculated on day 0 with 50 liters of raw seawater and nutrients were added to mimic a rich upwelling. The tank was kept in a temperature-controlled chamber and light (9×10^{20} quanta m^{-2} s^{-1}) was provided on a 14 : 10 h L/D cycle. Mesocosm samples were collected daily between day 7 and 12. Field samples were chosen for microscopy based on colorimetric measurements from samples collected every 2 weeks from six depths between 0 and 75 m.

Two replicate slides of 5–40 ml each were prepared for the microscopic enumeration of TEP from mesocosm and field samples as described above. TEP were quantified by counting 20 fields per slide at a magnification of 200× with a compound microscope. TEP were classified according to their maximal length into nine different size classes between 4.5 and 1,152 µm with each size class double the length of the previous one. Particles within each size class were counted in two groups according to their shape (round or rectangular). Total area of TEP was calculated from the average diameter of each size class for round particles and from the product of their average length and width, assuming width was 0.1 of length for each rectangular particle. This number was obtained as an average.

The quantity of TEP in mesocosm samples was measured spectrophotometrically on 6 replicates of 10–200 ml each following an earlier, slightly different procedure than is described here. Stained particles were transferred from filters to glass slides by the filter-transfer-freeze technique (Hewes and Holm-Hansen 1983) and then washed off the glass slide with 80% H_2SO_4. This method was later

Table 3. Average concentration of TEP in the ocean and phytoplankton cultures of pooled samples in xanthan equiv. (μg liter^{-1}); n—number of samples; SD—standard deviation between different samples (depth or days). SD between replicates of one sample were usually $\leq$5%.

Sample	Depth (m)	n	TEP Mean±SD	TEP Range
Santa Barbara Channel (34°N, 120°W)				
26 Jun–1 Jul 93*	1–10	5	147±63	85–252
	50–500	16	24±8	14–44
5 Jan–2 Feb 94†	0–75	11	47±12	29–68
Monterey Bay (36°50′N, 121°55′W)				
23–28 Jul 93‡	1–5	7	129±41	85–191
	7–12	8	76±19	50–102
29–30 Jul 93§	0–10	3	271±30	81–310
	10–50	6	59±12	46–63
Marine snow‡§	0–25	12	6±2‖	3–12‖
Norwegian fjord (69°21′N, 19°06′E)				
7–29 May 92#	0–18	8	190±53	100–255
7–29 May 92#	21–63	8	191±45	125–250
Laboratory samples				
Mesocosm¶		11		21–363**
Chaetoceros gracilis††		1	1,000	
Thalassiosira rotula††		1	706	
Emiliania huxleyi††		1	920	

* Summer assemblage: flagellates, small diatoms, small dinoflagellates.
† Winter assemblage: phytoplankton scarce.
‡ Summer assemblage: *Distephanus* sp., coccolithophorids, dinoflagellates.
§ Upwelling: floccing *Chaetoceros* bloom.
‖ Concentration of TEP in marine snow in μg per floc.
\# Senescent *Phaeocystis*-diatom bloom.
¶ Development of a mixed diatom bloom during 13 d in 1,400-liter tank. No average is given because TEP increased exponentially with time (Fig. 3).
** Values were calibrated with alginic acid and converted to xanthan equiv. based on average calibration factors of xanthan (1 mg liter^{-1} alginic acid = 9.87 μg liter^{-1} xanthan).
†† Late exponential phase of batch cultures.

abandoned because standard deviations between replicates tended to be high. Field samples were measured following the superior standard procedure described here. Alginic acid was used as a standard for the mesocosm samples and gum xanthan for the field samples. Standard curves indicated that ~10 μg liter^{-1} equiv. gum xanthan yielded the same dye-binding absorbance as 1 mg liter^{-1} equiv. alginic acid.

Total area of TEP (μm^2 ml^{-1}) during the mesocosm study was linearly related to colorimetric determinations of TEP (Fig. 4). Concentrations of TEP in the field samples were low compared to those of the mesocosm study and large TEP typical during diatom blooms were rare in field samples. The linear relationship between the colorimetric method and the microscopic enumeration, however, is also visible in the field samples, although there is more scatter. The field samples were collected over a 6-month period, spanning a wide variety of situations and types of TEP. Microscopical determinations of the total area of TEP are not accurate enough to avoid errors due to changes in the shape of TEP. Furthermore, total abundance and average size of TEP were smaller in field samples, making TEP harder to measure and count.

We tested whether TEP are generated as an artifact during filtration by conducting two types of fractionation experiments using seawater from the Santa Barbara Channel. TEP were size-fractionated for experiment A by passing an aliquot successively through 3–4 filters (5.0, 1.0, 0.6, and 0.2 μm) and filtering another aliquot directly onto 0.2-μm filters. In experiment B, four aliquots were filtered onto 5.0$^-$, 1.0$^-$, 0.6$^-$, and 0.2-μm filters, respectively. The filtrates of the first three aliquots were then filtered onto 0.2-μm filters. TEP were measured colorimetrically on all filters (4 replicates per set). No significant difference in the total quantity of TEP was observed between the fractionated and the unfractionated aliquots, confirming that TEP were not created as a filtering artifact and indicating good replicability of the method (Fig. 5).

In the following section, we present examples of the concentration and variability of TEP in the ocean. Table 3 summarizes average concentrations of TEP and the variability of TEP at several stations in the Pacific and

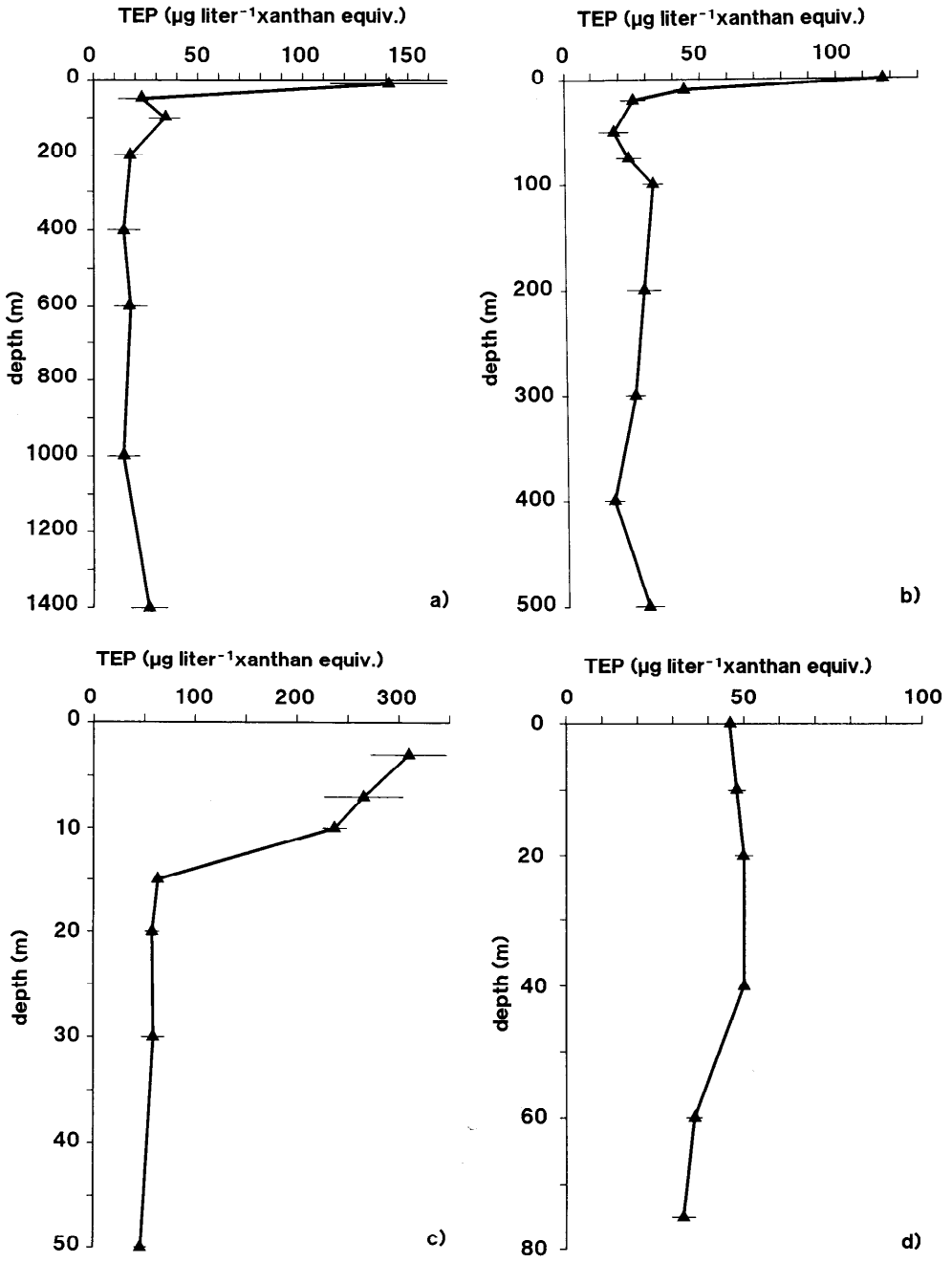

Fig. 6. Vertical distribution of TEP during summer in the Santa Barbara Channel, June 1993 (a, b), during upwelling in Monterey Bay, July 1993 (c), and during winter in the Santa Barbara Channel, March 1994 (d).

Atlantic Oceans and in phytoplankton cultures. Water samples were collected with water bottles or by divers, and marine snow was collected by divers (Alldredge 1992). The quantity of TEP was analyzed according to the standard protocol from fresh samples with the exception of the samples from Norway, which were preserved in Formalin.

The highest concentrations of TEP were observed in batch cultures of phytoplankton (Table 3). The concentration of TEP in the ocean varied by <2 orders of magnitude (between 10 and 500 μg liter^{-1} xanthan equiv.), whereas number abundance of TEP has been found to vary by 4 orders of magnitude (Passow and Alldredge 1994). The concentration of TEP depended on season, depth, and plankton composition (Table 3). Maximal concentrations of TEP were observed during floccing diatom blooms both in the field and in the mesocosm. Concentrations of TEP were also high during the declining phase of a bloom of *Phaeocystis* and diatoms observed in a Norwegian fjord (*see* Riebesell et al. 1995). Concentrations of TEP during marine oligotrophic summer conditions were appreciably lower than during blooms (Table

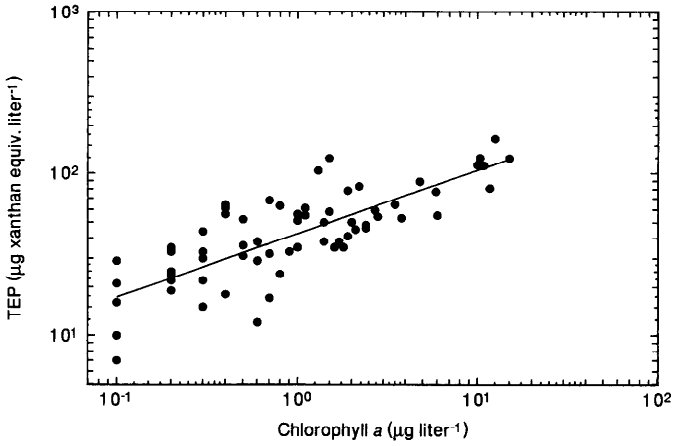

Fig. 7. Relationship between Chl *a* and TEP concentrations. Samples were taken at a fixed station in the Santa Barbara Channel. Regression: log(TEP) = 0.39 log(Chl *a*) + 1.63; r^2 = 0.62, df = 88.

3). Lowest concentrations of TEP in surface waters were observed in winter, when phytoplankton was scarce. All marine snow analyzed contained abundant TEP.

Depth profiles of TEP were measured on several occasions off Santa Barbara. The concentration of TEP below the phytoplankton-rich layer was always ~50 µg liter^{-1} xanthan equiv. (Fig. 6). Elevated concentrations (140 µg liter^{-1} xanthan equiv.) were measured off Santa Barbara at the surface in summer (Fig. 6a). A similar distribution was observed a few days later at a shallower station (Fig. 6b). High concentrations (>300 µg liter^{-1} xanthan equiv.) of TEP observed during a floccing bloom of *Chaetoceros* spp. were also restricted to the upper layers (Fig. 6c). The concentration of TEP was low at all depths in winter (Fig. 6d).

TEP are generated from excretion products of diatoms, and general distribution patterns of TEP appear similar to those of chlorophyll. Between January and July 1994, Chl *a* and TEP concentrations were analyzed in samples collected every 2 weeks at six depths between 0 and 75 m at a fixed station in the Santa Barbara Channel. Figure 7 shows the relationship between Chl *a* and TEP during the study period. The correlation between TEP and Chl *a* strongly suggests that phytoplankton were a main source of TEP. A correlation between concentration of TEP and Chl *a* also was observed during the mesocosm study (Passow and Alldredge 1995). A relationship between Chl *a* and concentration of TEP is consistent with previous findings of TEP formation in diatom cultures (Passow and Alldredge 1994).

The role of TEP in pelagic processes, until recently, has been ignored, although the presence of a high abundance of TEP in the ocean has implications not only for the aggregation of diatom blooms (Passow et al. 1994), but also for food-web structure, microbial processes, carbon cycling, and the chemistry of the ocean (Alldredge et al. 1993).

The chemical composition of TEP is largely unknown. Evidence, however, is accumulating that TEP generated by diatoms contain large quantities of sulfated polysaccharides (Mopper et al. 1995). TEP are believed to be a mixture of several acidic polysaccharides and their chemical composition presumably varies.

Under these circumstances it is both important and difficult to measure the concentration of TEP. The semiquantitative assay introduced here is based on the definition of TEP. TEP are defined as discrete particles that stain with alcian blue. Our method measures the amount of stain bonded to particles present in a sample.

Alcian blue is a large and slow-diffusing molecule and therefore enters only more permeable sites during the short staining interval used in our procedure. Thus, the dye would not penetrate organisms or colonies.

The biggest disadvantage of the method is that it is not truly quantitative because it measures staining capacity. Furthermore, because TEP can be very flexible, high filtration pressures may result in underestimating TEP, and filtration pressure must be kept low and constant. Because TEP have gellike properties, filter pores clog easily. Therefore, the dye and subsequent rinse water must filter easily to ensure that no clogging occurs. However, if high filtration pressure and filter clogging are avoided, our semiquantitative assay of TEP works reliably and appears to give a good estimate of the amount of TEP. Replicability is good compared to the changes encountered both in the field and in cultures.

We have used the assay in several studies and our data are self-consistent as well as consistent with auxiliary data (Riebesell et al. 1995; Passow and Alldredge 1995). We believe that distribution patterns of TEP and changes of such patterns need to be studied routinely to understand the role TEP play in marine ecosystems. The colorimetric method introduced here provides the means for such routine measurements until a fully quantitative method can be developed.

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References

ALLDREDGE, A. L. 1992. In situ collection and laboratory analysis of marine snow and large fecal pellets, p. 43–46. *In* D. C. Hurd and D. W. Spencer [eds.], Marine particles: Analysis and characterization. Geophys. Monogr. 63. AGU.

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———, C. GOTSCHALK, U. PASSOW, AND U. RIEBESELL. 1995. Mass aggregation of diatom blooms: Insights from a mesocosm study. Deep-Sea Res. Part 2 **42:** 9–27.

———, U. PASSOW, AND B. LOGAN. 1993. The abundance and significance of a class of large transparent organic particles in the ocean. Deep-Sea Res. **40:** 1131–1140.

COLLIN, P. M. [ED.] 1987. Carbohydrates. Chapman & Hall.

EMERY, K. O., I. A. JOHNS, AND S. HONJO. 1984. Organic films on particulate matter in surface waters off eastern Asia. Sedimentology **31:** 503–514.

GORDON, D. C. 1970. A microscopic study of organic particles in the North Atlantic Ocean. Deep-Sea Res. **17:** 175–185.

HEWES, C. D., AND O. HOLM-HANSEN. 1983. A method for recovering nanoplankton from filters for identification with the microscope: The filter-transfer-freeze (FTF) technique. Limnol. Oceanogr. **28:** 389–394.

HOROBIN, R. W. 1988. Understanding histochemistry. Selection, evaluation and design of biological stains. Wiley.

MOPPER, K., AND OTHERS. 1995. The role of surface-active carbohydrates in the flocculation of a diatom bloom in a mesocosm. Deep-Sea Res. Part 2 **42:** 47–73.

PARKER, B. C., AND A. G. DIBOLL. 1966. Alcian stains for histochemical localization of acid and sulfated polysaccharides in algae. Phycology **6:** 36–46.

PASSOW, U., AND A. L. ALLDREDGE. 1994. Distribution, size and bacterial colonization of transparent exopolymer particles (TEP) in the ocean. Mar. Ecol. Prog. Ser. **113:** 185–198.

———, AND ———. 1995. Aggregation of a diatom bloom in a mesocosm: The role of transparent exopolymer particles (TEP). Deep-Sea Res. Part 2 **42:** 99–109.

———, ———, AND B. E. LOGAN. 1994. The role of particulate carbohydrate exudates in the flocculation of diatom blooms. Deep-Sea Res. **41:** 335–357.

———, AND P. WASSMANN. 1994. On the trophic fate of *Phaeocystis pouchetii* (Hariot): 4. The formation of marine snow by *P. pouchetii*. Mar. Ecol. Prog. Ser. **104:** 153–161.

RAMUS, J. 1977. Alcian blue: A quantitative aqueous assay for algal acid and sulfated polysaccharides. J. Phycol. **13:** 345–348.

RIEBESELL, U., M. REIGSTAD, P. WASSMANN, T. NOJI, AND U. PASSOW. 1995. On the trophic fate of *Phaeocystis pouchetii* (Hariot): 6. Significance of *Phaeocystis*-derived mucus for vertical flux. Neth. J. Sea Res. **33:** 193–203.

SHARP, J. H. 1992. Total mass and particulate carbon, nitrogen, and phosphorus in marine particles: Analysis and characterization, p. 87–91. *In* D. C. Hurd and D. W. Spencer [eds.], Marine particles: Analysis and characterization. Geophys. Monogr. 63. AGU.

VANDEVIVERE, P., AND D. L. KIRCHMAN. 1993. Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl. Environ. Microbiol. **59:** 3280–3286.

WHITEMAN, P. 1973. The quantitative measurement of alcian blue–glycosaminoglycan complexes. Biochem. J. **131:** 343–350.

WIEBE, J. W., AND L. R. POMEROY. 1972. Microorganisms and their association with aggregates and detritus in the sea: A microscopic study. Mem. Ist. Ital. Idrobiol. **29:** 325–352.

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