

Production, concentration, and isolation of transparent exopolymeric particles using paramagnetic functionalized microspheres

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Abstract

We describe a new method to isolate and concentrate transparent exopolymeric particles (TEP), either naturally occurring or produced by phytoplankton in cultures. The principles of this method are to use TEP-sticking properties coupled with magnetic properties of paramagnetic microspheres to magnetically and selectively concentrate TEP. First, we studied the formation of mixed aggregates of TEP and 1- μm microspheres using the bubbling method. TEP precursors were obtained from diatom cultures and from natural seawater. Mixed aggregates of TEP–microspheres were efficiently formed—all TEP formed were associated with microspheres, and no clusters of microspheres alone were observed. The number of attached microspheres scaled with TEP size raised to an exponent of 1.53. The second step was to combine TEP with paramagnetic microspheres and isolate and concentrate the TEP by flowing out the solution containing mixed aggregates inside a strong magnetic field. About 90% of the magnetically labeled TEP were trapped inside the magnetic field. TEP volume concentration measured inside the magnetic field after isolation was about 5 times the initial TEP volume concentration. However, the predicted concentration factor, estimated from the relationship between number of attached microspheres versus TEP size, and the TEP size spectra in the initial solution was ~ 9 . This suggests that TEP observed after isolation inside the magnetic field may be compacted and/or that a fraction of the TEP pool inside the magnetic field may remain glued to the inner walls of the culture flask and, thus, the observed TEP size spectra could be underestimated.

A decade ago Alldredge et al. (1993) described a new class of particles, transparent exopolymeric particles (TEP). Transparent in seawater, they are visualized using a stain specific for polysaccharides or mucoid matter, the Alcian blue (Gordon 1970; Wiebe and Pomeroy 1972). TEP range in size from 1 to 100 μm or more in diameter and have been found in high concentration in different regions of the oceans (Alldredge et al. 1993; Passow and Alldredge 1994; Schuster and Herndl 1995; Mari and Kiørboe 1996; Niven et al. 1997; Kiørboe et al. 1998; Engel

2000; Mari et al. 2001; Beauvais et al. 2003) as well as in freshwater (Worm and Søndergaard 1998; Berman and Viner-Mozzini 2001). They form by coagulation of dissolved or colloidal organic matter (Mari and Burd 1998; Mari 1999; Passow 2000). The discovery of TEP has challenged our understanding of biogeochemical processes in the pelagic zone. Owing to their physicochemical properties, dynamics, and characteristic of distribution, TEP can affect trophic web structure and flux processes in the ocean. There is both direct and indirect (i.e., extrapolated from studies on colloids) evidence that TEP can act as sorption sites for dissolved organic (Schuster et al. 1998) and inorganic (Niven et al. 1997; Hirose and Tanoue 1998; Buffle et al. 1998; Schlekot et al. 1998; Schoemann et al. 1998; Schoemann et al. 2001) matter; attachment sites for bacteria (Alldredge et al. 1993; Mari and Kiørboe 1996); and a food source for microphageous protozoans (Shimeta 1993; Tranvik et al. 1993), particle grazers such as copepods (Carman 1990; Decho and Moriarty 1990; Ling and Alldredge 2003), or passive filter feeders such as tunicates (Flood et al. 1992). TEP can also aggregate with other particles (Kiørboe and Hansen 1993; Passow et al. 1994; Jackson 1995; Logan et al. 1995;

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Jackson and Burd 1998; Mari and Burd 1998) into marine snow flocs and thus enhance the vertical flux of major elements, such as carbon, and of adsorbed trace elements (Fowler and Knauer 1986; Alldredge and Silver 1988; Burd et al. 2000). Finally, TEP can modify the physical properties of seawater, such as viscoelasticity (Jenkinson et al. 1998; Wyatt et al. 1998).

Two methods have been used so far to measure TEP, both primarily designed to observe TEP and describe their abundance and distribution using Alcian blue. First, the microscopic method (Alldredge et al. 1993; Passow and Alldredge 1994) allows one to observe TEP directly under a compound light microscope, either by transferring the stained and retained TEP onto 0.2- μm polycarbonate filters onto microscope slides using the Filter-Transfer-Freeze technique (Hewes and Holm-Hansen 1983), or without transfer onto a slide using brightfield microscopy (Logan et al. 1994). The microscopic method has mainly been applied to determine TEP size spectra and to calculate TEP abundance and volume fraction. Second, the spectrophotometric method (Passow and Alldredge 1995) allows estimation of TEP abundance by quantifying Alcian blue staining. Both methods have advantages and disadvantages (*see* review in Passow 2002), but the main disadvantage for both is that neither one allows the study of TEP without staining and, thus, the possibility to work directly with them.

A common feature of the two existing methods is the close and intrinsic relationship between TEP and Alcian blue. Although no work has been published contesting the existence of TEP, the use of this exclusive staining approach to define a class of particles, which cannot be observed otherwise, could be criticized. Another property should be used not only to detect and eventually quantify this pool of organic matter, but also to isolate and concentrate them for further analysis. TEP have high sticking properties (Kjørboe and Hansen 1993; Dam and Drapeau 1995; Hansen and Kjørboe 1997), i.e., high probability of adhesion upon collision. The polysaccharides that compose TEP are highly surface-active (high stickiness) because of a high fraction of sulfate half-ester groups (Mopper et al. 1995) that promote the formation of divalent cations bridging. This high sticking property confers to TEP a major role for aggregation/sedimentation processes, because the stickiness TEP is much higher than that of cells (Kjørboe and Hansen 1993; Passow et al. 1994; Logan et al. 1995), with values generally 2 to 4 orders of magnitude higher than most marine particles. Because of their high stickiness, we reasoned that TEP could be detected by tagging them with small inorganic particles, such as micrometer-size microspheres. The first step for such an approach would be to associate TEP and microspheres. Since the first description of the bubbling method by Baylor and Sutcliffe (1963), this approach has been extensively used to produce aggregates from dissolved organic matter (Johnson et al. 1986; Kepkay and Johnson 1988; Kepkay 1991; Alber and Valiada 1994; Mopper et al. 1995; Mari 1999), as well as

producing mixed aggregates with bacteria, algae, and other particulate material (Blanchard 1989; Chen et al. 1998; Monahan and Dam 2001). During the present study, the bubbling method was used to produce mixed aggregates of TEP–microspheres.

In the present study we describe a new method: (1) to detect TEP without Alcian blue staining and (2) to isolate and concentrate TEP.

Materials and procedures

Production of mixed aggregates of TEP–microspheres—Two sets of experiments were conducted. First, we studied the ability of TEP to form mixed aggregates with 1- μm Polybead™ Polystyrene Microspheres (Polysciences). To determine if the interactions between micrometer size particles and TEP depend on the chemical surface properties of particles, the following 1- μm microspheres were used: (1) Polybead Amino Microspheres, (2) Polybead Carboxylate Microspheres, and (3) Polybead Carboxy-Sulfate Microspheres. This study was first conducted using TEP produced in the laboratory from exudates of diatom species (*Thalassiosira weissflogii* and *Skeletonema costatum*) known to release large amounts of extracellular polymeric substances. Diatoms were grown in eight l-batch cultures on *f/2* media with silica (Guillard 1975) at constant illumination (ca. 150 $\mu\text{E m}^{-2} \text{s}^{-1}$). Diatom cultures in stationary growth phase (ca. 10-d-old culture) were filtered at low and constant vacuum pressure (<100 mbar) through a 47-mm-diameter GF/C Whatman filter (nominal pore size = 1.2 μm). Second, because the physicochemical properties of TEP produced from diatoms grown in cultures might differ from naturally occurring TEP, the formation of mixed aggregates was also investigated during a spring cruise in three subarctic Norwegian fjords (Malangen fjord, Balsfjord, and Ullsfjord). TEP were produced from natural seawater collected using a 5-L Niskin bottle at the maximum of fluorescence depth on each sampling occasion. Seawater samples were directly filtered at low and constant vacuum pressure (<100 mbar) through a 47-mm-diameter GF/C Whatman filter, and the filtrate was placed in a bubble adsorption column. This experiment was conducted once for each station.

For the first set of experiments (diatom cultures), a 4-L bubble adsorption column made of borosilicate glass was used to produce the mixed aggregates of TEP–microspheres. For the second set of experiments (natural seawater collected in Norwegian fjords), we used a 2.5-L bubble adsorption column made of borosilicate glass. This difference in column size was dictated by restriction of bench space on board.

The microspheres were added to the bubbling column to obtain a final concentration (C_f) of 2.5×10^5 particles mL^{-1} (ca. 25% of bacterial concentration). The volume of microspheres solution (V_i) to add was calculated as:

$$V_i = (C_f V_f) / C_i$$

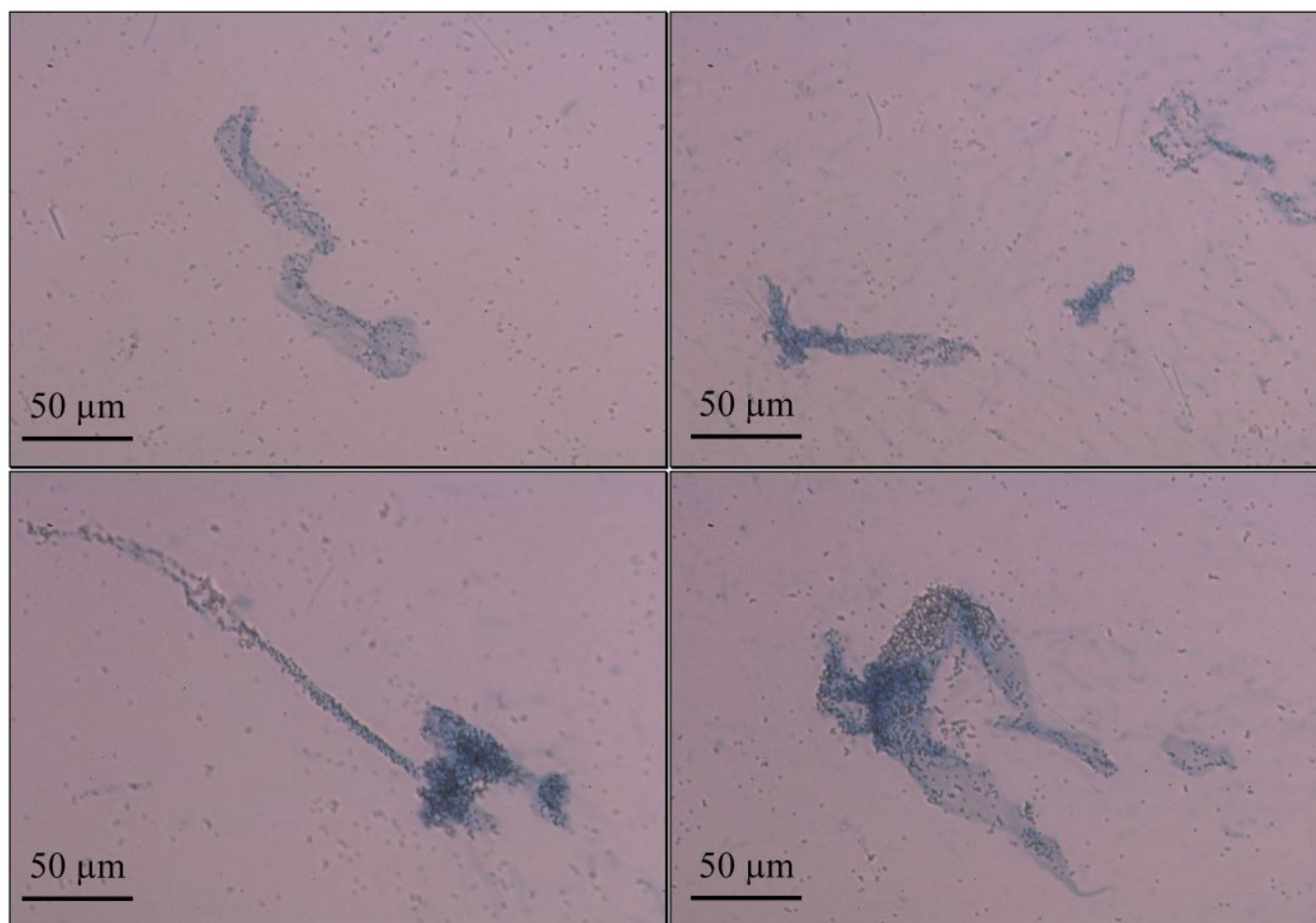


Fig. 1. Examples of TEP produced by bubbling, dissolved, and colloidal organic matter in the presence of 1- μm microspheres. The black dots associated with TEP are the microspheres. The pictures were taken under a compound light microscope at 200 \times magnification.

where C_i is the initial concentration of microspheres and V_f is the volume of the bubbling column. The initial concentration, C_i , was calculated from:

$$C_i = 6W10^{12}/\rho\pi d^3$$

where W is the mass of polymer per mL of latex (0.025 g mL⁻¹ for 2.5% solid volume according to manufacturer), ρ is the density of polymer in grams per mL (1.05 for polystyrene), and d is the microspheres' diameter in micrometers. To avoid clumping prior to bubbling, the volume of microspheres to add in the column (V_f) was diluted in 50 mL of 0.22 μm filtered seawater and sonicated for 5 min. The solutions containing the TEP precursors and the microspheres were mixed and then added to the column. The solutions were bubbled with air at a constant gas flow rate of 100 mL min⁻¹ (Kepkay 1991). Bubbles were produced by a 10- to 20- μm pore size glass frit. The air for bubbling passed through a 0.1- μm air filter (Polycap TF, Whatman) and a trap containing molecular sieves (4 \AA , 45/60 mesh) for removal of impurities and moisture. The

solutions were bubbled for 90 min at room temperature (ca. 20°C), and samples were collected in the middle of the column at 10, 20, 30, 45, 60 and 90 min and immediately filtered for TEP determination and microspheres enumeration. During bubbling the pH remained constant. Between each experiment, the column and the glass frit were soaked for 2 h with 10% HCl and then rinsed three times with Milli-Q water.

TEP and microspheres determination—The microscopic method was used for quantifying TEP within the bubbling column. TEP were stained with Alcian Blue and slides prepared following the method of Passow et al. (1994). TEP size spectra were determined by counting and sizing TEP at three successive magnifications (Mari and Burd 1998). At least 10 images were taken per slide and for each magnification, and the TEP size spectra were compiled by combining the size distributions obtained at each magnification. The images were analyzed using an image analyzing system (ImagePro Plus, MediaCybernetics). The number of 1- μm microspheres attached to TEP was determined under visible light at 400 \times magnification, and individual TEP were sized (Fig. 1). As a result, for the 9 exper-

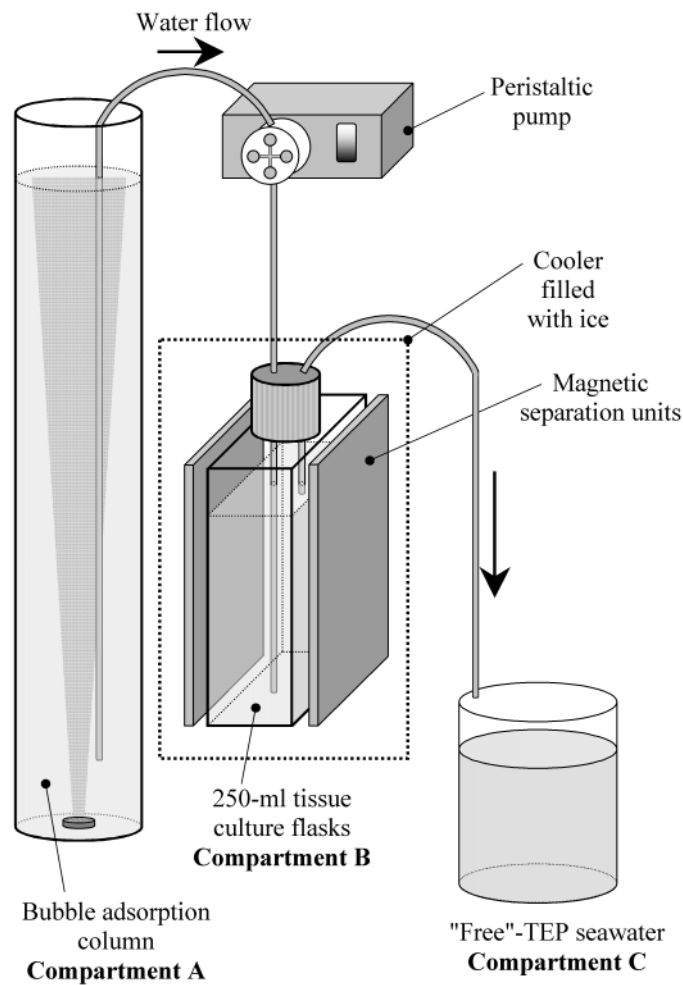


Fig. 2. Experimental setup for isolation and concentration of TEP produced by surface coagulation in the presence of PCM of 1 μm diameter

iments conducted (equivalent to 48 sampling times), a total of 1885 TEP were sized, and their attached microspheres counted. A relationship between TEP size and number of attached microspheres was determined for each sample to estimate the fraction of total microspheres that was attached to TEP. The number of attached microspheres (n) per TEP was fitted to the power-law relationship:

$$n = ad^b$$

where d is the equivalent spherical TEP-diameter (ESD; μm), and a and b are constants for a given sample determined from linear regression of log-log transformed numbers of associated microspheres versus TEP diameter (Mari and Kjørboe 1996). The fraction of attached microspheres was calculated by combining (i) the above relationship, (ii) the TEP size distribution, and (iii) the total concentration of microspheres in seawater.

TEP isolation and concentration using paramagnetic microspheres—

The possibility of isolating and concentrating TEP using the

sticking properties of TEP (which allows the formation of mixed aggregates of TEP–microspheres) coupled with the magnetic properties of 1- μm paramagnetic carboxylated microspheres (PCM) was investigated. The principle of this methodological approach is, first, to form mixed aggregates of TEP and magnetic microspheres and, second, to collect these mixed aggregates using a magnetic field. We used encapsulated super-paramagnetic carboxylate microspheres (Estapor® Microspheres) of 0.95 μm diameter with high ferrite content (magnetic pigment content = 48%) and with high carboxyl group density at the surface ($\text{COOH} = 114 \mu\text{eq g}^{-1}$). These paramagnetic microspheres are characterized by a core-shell structure (Polystyrene), where the iron oxide material does not interfere with the surface components. They are colloidally stable in the absence of a magnetic field and total separation can be achieved when PCM are exposed to a magnetic field, and they are easily resuspended once the magnetic field is removed. These microspheres are used in many biotechnological applications, such as cell separation and nucleic acid isolation. To our knowledge this is the first time that PCM were used for oceanographic applications.

An experiment was conducted using TEP colloidal precursors originating from two diatom cultures (*Thalassiosira weissflogii* and *Skeletonema costatum*). Diatoms were grown in 8-L batch cultures on f/2 media with silica (Guillard 1975) at constant illumination (ca. $150 \mu\text{E m}^{-2} \text{s}^{-1}$). Diatom cultures in stationary growth phase (ca. 10-d-old culture) were filtered at low and constant vacuum pressure ($< 100 \text{ mbar}$) onto a 47 mm diameter GF/C Whatman filter (nominal pore size = 1.2 μm). To obtain a full range of TEP volume concentrations, filtrates from *S. costatum* and *T. weissflogii* cultures were diluted with artificial seawater. The filtrate from *S. costatum* was diluted 2- and 4-fold and the filtrate from *T. weissflogii* was diluted 2-fold. As a result, five magnetic isolation experiments were conducted (*S. costatum*, *S. costatum* 1:2, *S. costatum* 1:4, *T. weissflogii*, and *T. weissflogii* 1:2). TEP–PCM aggregates were created by bubbling in a 2.5-L adsorption column (as above). PCM were added in the bubbling column to obtain a final concentration (C_f) of $5 \times 10^5 \text{ PCM mL}^{-1}$, to reach optimum microspheres “demand” (100% attachment) and according to prediction (see Assessment, Fig. 5). During bubbling, the pH remained constant.

TEP were isolated and concentrated using the magnetic properties of the PCM. After 1 h of bubbling, mixed aggregates of TEP–PCM were gradually concentrated by placing the seawater from the bubbling column inside a magnetic field as follows. A tissue culture flask was disposed in between two Flask Magnets (Qiagen) held together by a rubber band (Fig. 2). Flask Magnets are 12.5 cm \times 6 cm rectangular magnetic separation units specially designed for use with 250-mL tissue culture flasks. Each unit consists of permanent magnets encased in a plastic frame. Seawater from the bubbling column passed inside the tissue culture flask by means of a peristaltic pump whose optimal flow rate (25 mL min^{-1}) depended on the flask

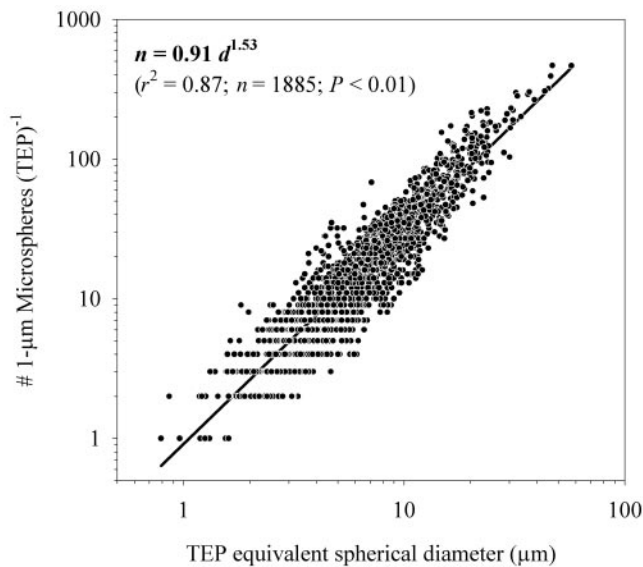


Fig. 3. Number of 1- μm microspheres attached to a TEP (n) as a function of its size (d , μm). Regression line was fitted to the observations ($n = ad^b$)

volume and the time necessary for complete separation of the PCM from the water. The seawater inlet was placed at the bottom of the flask while the seawater outlet was placed at the top. Samples for microscopic TEP determination were collected in the initial solution (i.e., before magnetic separation), inside the tissue culture flask (i.e., after complete magnetic separation and resuspension of the trapped mixed aggregates), and in the final solution (i.e., solution supposedly “free” of TEP), thereafter respectively called compartments A, B, and C. Because processing the solution takes about 100 min, the system was kept in a cooler filled with ice to minimize bacterial degradation of TEP, once the mixed aggregates of TEP-PCM were trapped within the magnetic field.

An experiment was conducted to verify the magnetic properties of the PCM by running the same procedure, but diluting PCM into Milli-Q water (final concentration = 2.5×10^5 PCM mL^{-1}). A 2.5-L solution was processed through the magnetic field and the number of PCM in each compartment (A, B, and C) was enumerated. As a result, about 90% of the PCM were magnetically retained in compartment B, which implies that about 10% of the PCM were not magnetizable.

Assessment

Relationship between TEP size and number of attached microspheres—No difference (ANCOVA, $P > 0.05$) was found between treatments and between time points either in TEP formation or in abundance of microspheres per TEP size. That is, the relationship between the number of attached 1- μm microspheres and TEP size was independent of the TEP source (either *Thalassiosira weissflogii* and *Skeletonema costatum* cultures or natural seawater), bubbling time, or chemical properties of the microspheres (Amino, Carboxylate, or Carboxy-Sulfate). The

number of 1- μm microspheres per TEP (n) scaled with TEP diameter (d ; μm) was

$$n = 0.91 d^{1.53}$$

which indicates that the number of microspheres attached to TEP increases out of proportion to size (Fig. 3). As an example, 10 microspheres of 1- μm should be attached to a TEP with an ESD of 4.8 μm , whereas 100 microspheres should be attached to a TEP with an ESD of 21.6 μm . However, because volume scales to L^3 , the volume-specific ($n L^{-3}$) number of microspheres (n') decreases with TEP size; that is, $n' = aL^{-1.57}$, where the value of the coefficient a depends on the shape used for calculation volume. The independence of the scaling relationship from TEP source and microsphere surface property group suggests that the formation of mixed aggregates of TEP and 1- μm microspheres is controlled by TEP sticking properties and size of the microspheres rather than the chemical characteristics of the microspheres. Additionally, mixed aggregates of TEP-microspheres were always formed very efficiently, and 100% of the TEP formed were associated with microspheres; no clusters of solely microspheres were observed.

Finally, the exponent of the relationship between the number of particles attached to TEP and TEP size can be considered as estimates of TEP fractal dimension (Mari and Kiørboe 1996), under the assumption that the number of attached particles is directly proportional to TEP porosity. An exponent of 1.53 for the number of microspheres per TEP is consistent with fractal dimensions estimated for marine snow particles and TEP aggregates (1.39 to 1.52) and is consistent with dimension of aggregates formed through shear coagulation (Logan and Wilkinson 1990; Logan and Kilps 1995). Therefore, this method of aggregates formation with microspheres of known size could be used to estimate fractal dimension of aggregates.

Optimal initial microspheres concentration—For all experiments, $\geq 95\%$ of the microspheres were attached to TEP after 10 min bubbling. In addition, the percentage of attached microspheres scaled with TEP volume concentration (Fig. 4) according to

$$\% \text{Microspheres}_{\text{attached}} = 9.8(\text{TEP volume}) + 95.5$$

This relationship implies that the theoretical percentage of attached microspheres increases when the TEP volume fraction increases. As the optimal percentage of attached microspheres equals 100%, higher values indicate that TEP present in the solution are undersaturated in microspheres or that a fraction of the TEP pool is not associated with microspheres. Because in all experiments, no TEP were observed without attached microspheres, it is possible that some TEP were undersaturated with respect to their microspheres' specific density. To reach the optimal $\% \text{Microspheres}_{\text{attached}} = 100\%$ according to TEP volume concentration, the initial concentration of microspheres has to be adjusted to the TEP

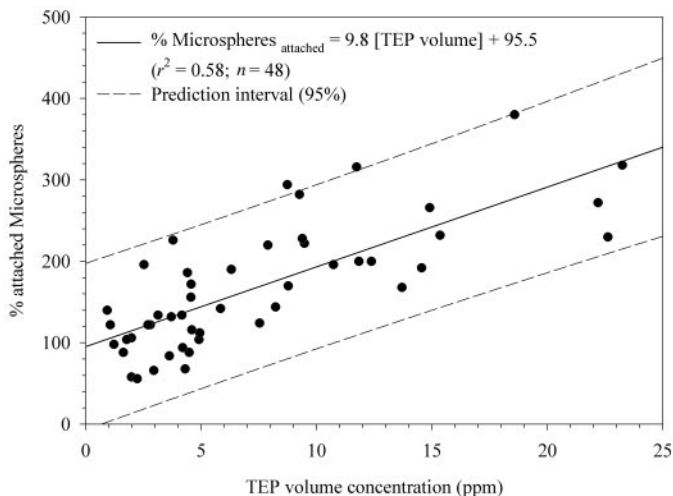


Fig. 4. Relationship between the percentages of attached 1- μm microspheres and TEP volume concentration in the bubbling column

volume. The microspheres demand to reach 100% attachment as a function of the TEP volume concentration was obtained from the above relationship, considering that an increase by factor x of the microspheres concentration amounts to dividing the $\% \text{Microspheres}_{\text{attached}}$ by the same factor. Over a TEP volume concentration range of 1 to 100 ppm, the optimal initial concentration of microspheres to target maximum attachment varied between ca. 2.5×10^5 and 27.0×10^5 microspheres mL^{-1} (Fig. 5). Although volume concentration of naturally occurring TEP can reach values higher than 100 ppm (Passow et al. 1994; Mari and Burd 1998; Berman and Viner-Mozzini 2001), they usually occurred at concentration ≤ 20 ppm (Passow and Alldredge 1994; Mari and Kiørboe 1996; Mari and Burd 1998; Kiørboe et al. 1998). Therefore, for an average TEP volume concentration of 20

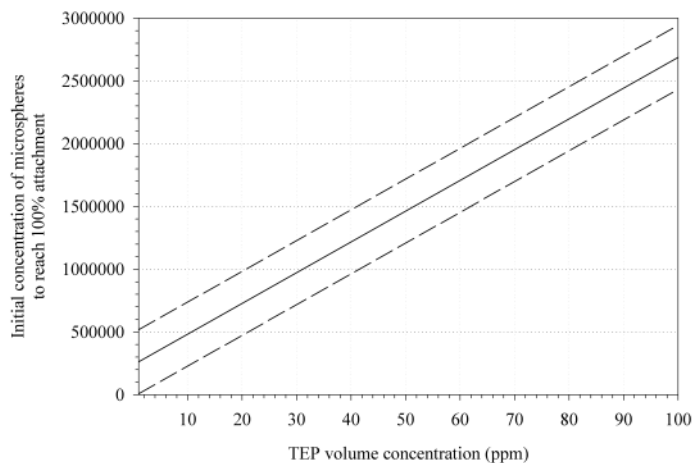


Fig. 5. Predicted optimal initial concentration of 1- μm microspheres as a function of initial TEP volume concentration

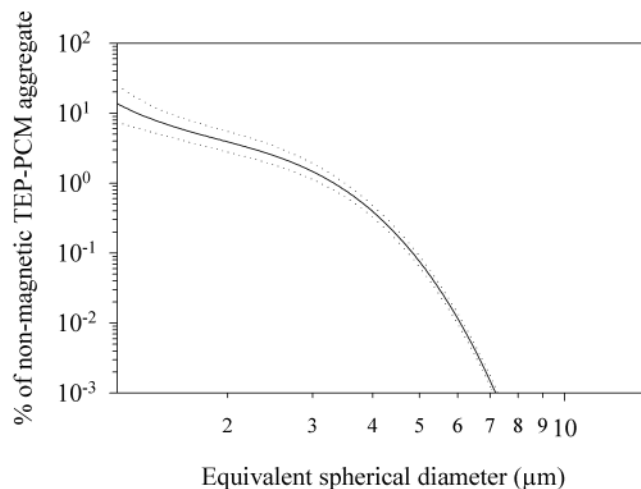


Fig. 6. Probability of a TEP-PCM aggregate not being magnetizable according to its size

ppm, an initial microspheres concentration of 5×10^5 to 10×10^5 PCM mL^{-1} (optimum 7.5×10^5 PCM mL^{-1}) is recommended (95% prediction interval).

TEP-PCM magnetizability—The probability of a mixed aggregate of TEP-PCM not being retained inside the magnetic field was estimated using the number of attached 1- μm microspheres versus TEP size relationship described above, and assuming that such an aggregate is not retained if $>50\%$ of the attached PCM are not magnetizable. Because only TEP-PCM aggregates between 1 and 3 μm diameter are potentially not retained due to the 10% nonmagnetizable PCM (Fig. 6), it is likely that the fraction of nonmagnetizable PCM does not significantly affect the overall magnetizability of the TEP-PCM pool. However, because a large fraction of the TEP pool is represented in the small size range, one would expect this isolation procedure to be inappropriate to remove all TEP from solution, as a full TEP-size spectrum should be rapidly reformed from remaining TEP precursors. Consequently, unless we are able to force aggregation of all TEP colloidal precursors toward large-size classes prior to flowing out the solution inside the magnetic field, compartment C should show a significant TEP pool, because TEP free of PCM will be reformed between compartments B and C.

TEP-PCM isolation and concentration—During the five magnetic isolation experiments, mixed aggregates of TEP-PCM present in the initial compartment (A) were efficiently retained inside the magnetic field (Fig. 7). However, a relatively high TEP volume concentration was also observed in compartment C, with an average of $13.6 \pm 10.3\%$ of the initial TEP volume concentration (Table 1). Between compartments A and B, observed TEP volume concentration increased by an average factor of 5.02 ± 1.14 . Expected TEP volume concentrations in compartments B and C were estimated from (i) the initial TEP size spectra in compartment A, (ii) the expected fraction of attached PCM per size class considering the initial

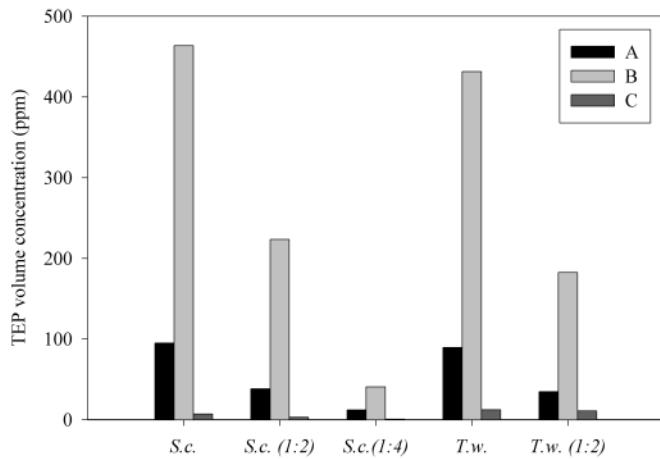


Fig. 7. Variations of the TEP volume concentrations during the magnetic isolation experiments conducted with *Skeletonema costatum* and *Thalassiosira weissflogii* exudates, in each compartment (A, B, and C)

concentration of PCM and the PCM number versus TEP size relationship obtained above, and (iii) the fraction of nonmagnetizable PCM. With the assumption that mixed aggregates of TEP-PCM should be retained if more than 50% of the attached PCM are magnetizable, the expected TEP volume concentration retained in B should be higher than what is observed with an average concentration factor of 8.77 ± 0.03 , whereas it should be lower in compartment C with values close to 0 ppm (Table 1). The discrepancy between observed and expected TEP volume concentrations in compartment B might be an artifact caused by the compression of the particles due to high physical forces generated by the magnetic field. In other words, TEP-PCM aggregates observed in compartment B could be compacted and, thus, more dense than those observed in A.

Such a process would lead to an underestimation of TEP volume concentration in B. Although compacting of TEP into more dense particles should not alter their chemical composition, it may modify their staining capacity by reducing their porosity. Therefore, such a modification of structure may alter the determination of TEP concentration using the spectrophotometric method (Passow and Alldredge 1995). This discrepancy may also be explained by the possibility that some TEP-PCM aggregates remained glued to the inner walls of the culture flask. Considering that TEP-PCM aggregates settle in successive coats and stick to the walls thereby creating a biofilm-like structure, one might expect that a significant TEP-PCM fraction will not resuspend and will be lost from microscopic counts.

The study of the spectral slopes, δ , of observed and expected TEP size spectra in the three compartments (Table 1) shows that δ_{observed} and δ_{expected} in compartment B do not differ statistically, whereas the spectral slopes in B are higher than the spectral slopes in C (either observed or expected) ($P < 5\%$). This suggests that the fraction of small TEP is higher in compartment C than in compartment B (i.e., large TEP-PCM aggregates are retained more efficiently inside the magnetic field than smaller ones). Additionally, δ_{observed} and δ_{expected} in compartment C were statistically different ($P < 5\%$)— δ_{observed} being larger than δ_{expected} —which suggests that the large TEP-PCM aggregates observed were probably formed between compartments B and C. These two hypotheses are supported by the discrepancies between observed and expected TEP volume concentrations in compartments B and C.

Furthermore, the microsphere density inside TEP-PCM aggregates was determined in compartment C (Fig. 8). PCM density inside mixed aggregates was much lower than for TEP-microspheres aggregates initially formed (Fig. 3), and no clear

Table 1. Observed and expected TEP volume concentrations and slopes, δ , of the TEP size spectra regression lines during the five magnetic purification experiments in different compartments^a

		<i>T. weissflogii</i>	<i>T. weissflogii</i> (1:2)	<i>S. costatum</i>	<i>S. costatum</i> (1:2)	<i>S. costatum</i> (1:4)	Average δ (\pm SD)
Observed							
A	δ	-2.29	-2.41	-1.88	-2.21	-2.46	-2.25 ± 0.23
	TEP volume (ppm)	89.7	34.8	95	38.4	12.3	
B	δ	-2.49	-2.58	-2.78	-2.55	-2.27	-2.53 ± 0.18
	TEP volume (ppm)	431.3	218	463.4	223.4	40.7	
C	δ	-3	-3.31	-3.34	-4.1	-3.53	-3.46 ± 0.41
	TEP volume (ppm)	12.4	10.9	7	3.1	0.9	
Expected							
B	δ	-2.26	-2.38	-1.84	-2.17	-2.41	-2.21 ± 0.23
	TEP volume (ppm)	788.3	305.6	834.4	336.4	107.3	
C	δ	-6.26	-6.41	-6.01	-6.22	-6.11	-6.20 ± 0.15
	TEP volume (ppm)	0.01	0.01	0.01	0.01	0.01	

^aExpected TEP size spectra in compartments B and C were estimated from (i) the initial TEP size spectra in compartment A, (ii) the expected fraction of attached PCM per size class considering the initial concentration of PCM and the PCM number versus TEP size relationship ($n = 0.91 d^{1.53}$), and (iii) the fraction of non-magnetizable PCM (i.e., 10%).

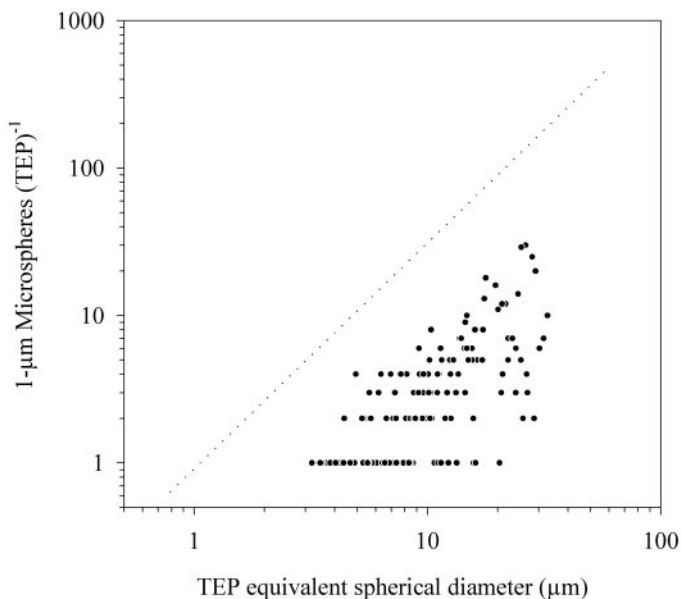


Fig. 8. Number of PCM attached to a TEP (n) as a function of its size (d , μm) in compartment C. The dotted line represents the regression line of Fig. 3

relationship between TEP size and number of attached PCM was observed. This suggests that the TEP-PCM aggregates occurring in compartment C were probably formed between compartment B and C and that the few PCM observed in compartment C and attached to TEP were the non-magnetizable ones.

Efficiency of the magnetic isolation procedure—The efficiency of the magnetic isolation procedure to concentrate TEP was estimated by calculating the fraction of the TEP pool magnetically retained as a function of the initial TEP volume in 1 mL (Fig. 9). For the five experiments conducted, the TEP volume trapped inside the magnetic field (calculated as the difference between TEP volumes in A and in C) averaged ca. 88% of the initial TEP volume. However, the observed TEP volume in B averaged $57.2 \pm 12.6\%$, which suggests that $30.9 \pm 19.7\%$ of the initial TEP volume observed in compartment A was missing. The same two processes detailed above could explain the missing TEP (i.e., a fraction lost via sticking on the walls of the culture flask and/or physical compression of the TEP-PCM aggregates).

Volumes for compartments A and B—The experiments were conducted with a volume of 2500 mL and of 250 mL, respectively for compartments A and B. Considering the efficiency of the magnetic procedure to concentrate TEP, this approach could be used to obtain a “pure” solution of TEP (i.e., 100% in vol/vol). A concentrated solution where TEP occupy the entire volume of compartment B solution is obtained when the ratio of expected TEP volume magnetically retained:volume of B equals 1. This can be achieved by either increasing the volume of compartment A or decreasing the volume of compartment B. A ratio of 1 was targeted by increasing the volume of com-

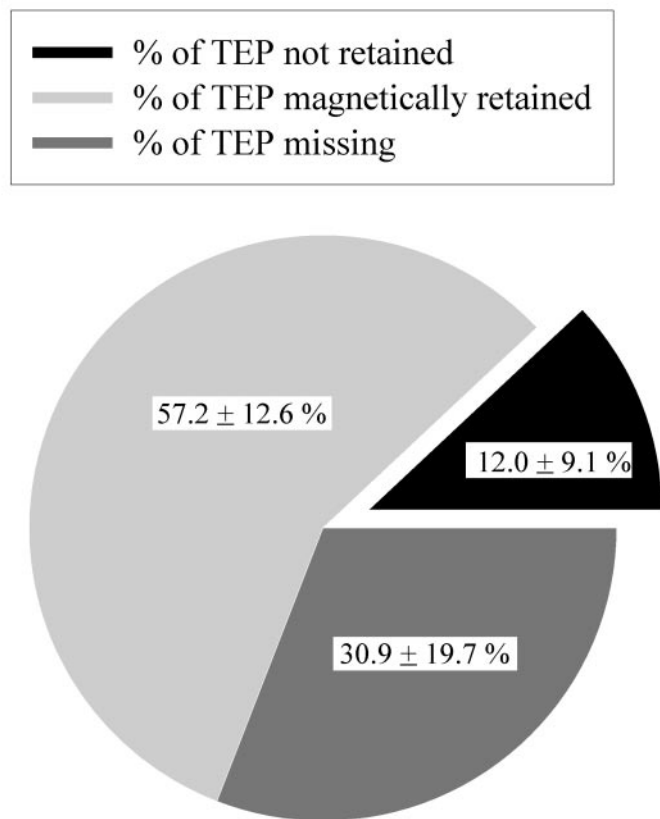


Fig. 9. Fraction of the TEP pool magnetically retained (in compartment B), not retained (in compartment C), and missing as a function of the initial TEP volume (mL)

partment A from 2500 to 100×10^6 mL or decreasing the volume of compartment B from 250 to 0.5×10^3 mL. Because the amount of TEP-PCM retained inside the magnetic field depends on the initial TEP concentration, the volume of the different compartments was predicted over a range of TEP volume concentrations from 5 to 200 ppm. The study of the surface plots of $\text{Log}(\text{Volume A})$ versus $\text{Log}(\text{TEP volume fraction})$ and of $\text{Log}(\text{Volume B})$ versus $\text{Log}(\text{TEP volume fraction})$ allows determining the volumes for A or B for which the ratio, expected TEP volume magnetically retained:volume of B equals 1 (i.e., $\text{Log}[\text{Ratio}] = 0$). For a fixed volume of 250 mL in compartment B, it would be necessary to flow out ca. 1.8×10^6 mL and 56.2×10^6 mL to reach 250 mL of pure TEP in compartment B for initial TEP volume concentrations of 200 and 5 ppm, respectively (Fig. 10a). For a fixed volume of 2500 mL in compartment A, it would be necessary to decrease the volume of compartment B to ca. 0.4 mL and 0.01 mL to obtain a pure TEP solution in compartment B for initial TEP volume concentrations of 200 and 5 ppm, respectively (Fig. 10b). Because complete separation of TEP-PCM aggregates from seawater is achieved for an optimal flow rate of 25 mL min^{-1} , it would take ≥ 50 days to obtain 250 mL of “pure” TEP. Therefore, such a

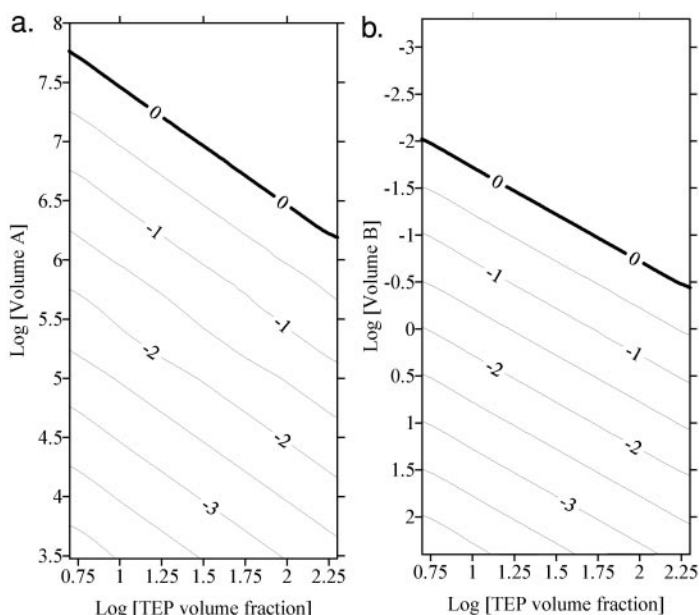


Fig. 10. Ratio of expected TEP volume magnetically retained to volume of B as a function of TEP volume concentration and (a) volume of compartment A for a fixed volume of B = 250 mL, and (b) volume of compartment B for a fixed volume of sample processed. (Volume of A) = 2500 mL.

“pure” TEP solution can only be obtained following this procedure by decreasing the volume of compartment B.

Discussion

Physical labeling of TEP—The first goal of this study was to design a procedure to detect TEP without intermediary Alcian blue staining. We demonstrated that the presence of 1- μm microspheres clusters was due to the existence of an unstained TEP matrix. Because all TEP formed in the presence of 1- μm microspheres were associated with microspheres and the 1- μm microspheres never aggregated by themselves by means of a biological glue insensitive to Alcian blue staining (no clusters of microspheres alone with a ghost matrix were observed), one could use the association between micrometer-size spheres and TEP to physically label TEP. Although a large variety of surface-active substances (i.e., not only polysaccharides) may be enclosed in the TEP formed, the systematic Alcian blue staining of the microspheres clusters matrix suggests that the stained matrix could be operationally defined as TEP. This finding has implications for TEP detection, because a cluster of microspheres observed following a similar methodological approach, but without using Alcian blue, can be defined as a TEP particle. Such an approach would free us from using Alcian blue staining as an exclusive means to detect TEP. As an example, a cluster of 100 1- μm microspheres should indicate the presence of a TEP particle of ca. 22 μm equivalent spherical diameter. This approach seems inappropriate to determine the size of the TEP pool because it will be more time-consuming to enumerate the number of 1- μm microspheres in each cluster than to determine the size via Alcian blue staining.

However, several other applications are possible. One example would be examination of TEP consumption by metazoans following the ingestion of 1- μm fluorescently labeled microspheres (the size distribution of microspheres being altered by the presence of TEP). Another is examination of TEP concentration on modification of trophic web structure.

Preparation prior to chemical analysis—The second goal of this study was to design a procedure to isolate and concentrate TEP. A current major limitation when working with TEP is the impossibility to separate them from other particulate material, such as phytoplankters, bacteria, fecal pellets, and other particles. We believe that the isolation procedure described here will prove useful in several respects: e.g., in studies of the characterization and chemical composition of TEP or in studies of the ability of TEP to adsorb organic solutes, such as amino acids (Schuster et al. 1998), and to complex with trace metals (e.g., Fe, Cd, and Mg; Schelkat et al. 1998; Schoemann et al. 2001) and radioisotopes (e.g., ^{234}Th ; Niven et al. 1997).

Removing TEP and adsorbed substances—Due to their physicochemical characteristics, TEP are suspected to adsorb efficiently many dissolved compounds (Logan and Hunt 1987) such as proteins, amino acids, fatty acids, and trace metals (Morel and Gschwend 1987; Decho 1990; Wells and Goldberg 1991; Niven et al. 1997; Schuster et al. 1998). Direct evidence for the adsorption of a specific dissolved substance onto TEP can be obtained by monitoring changes in TEP concentration as well as in the targeted solute concentration in the water column. This approach has been followed by Niven et al. (1997) in their study of TEP as a strong organic ligand for Thorium-234. They showed that ^{234}Th removal was closely linked to variations of the TEP pool and concluded that TEP were efficient scavengers for this radioisotope. We argue that the magnetic isolation procedure can be used to directly investigate TEP adsorptive properties. As an example, one could incubate a known concentration of the targeted dissolved substance (e.g., ^{234}Th) with TEP in compartment A and measure its concentration in compartment C after magnetic isolation; the missing quantity of solute being the amount lost via adsorption onto the TEP fraction that has been trapped inside the magnetic field.

Comments and recommendations

Potential improvements—One of the limitations of the magnetic isolation procedure is that it does not allow the removal of all TEP and TEP colloidal precursors from solution. Just as with icebergs, for which the emerged part represents only a minor fraction of the overall mass, a minor fraction of the overall surface-active polysaccharide material exists as TEP (Chin et al. 1998). And again as with icebergs, removing the emerged part of the surface-active polysaccharide material pool (i.e., TEP pool) will be rapidly followed by a replenishment of the removed part. A way to improve the transfer efficiency between TEP colloidal precursors and the particulate phase of the TEP pool would be to introduce an additional nucleus for aggregation. Glass fibers have been used to promote the for-

mation of marine snow from *Phaeocystis* mucilage (Passow and Wassmann 1994), and we hypothesize that adding such fibers during the formation of TEP-PCM aggregates would significantly enhance aggregation processes. Another improvement could come from the technique used to increase the frequency of collision necessary for coagulation. The bubbling method might not be the most appropriate or efficient way of producing TEP-PCM mixed aggregates (see Kepkay 1994 and Monahan and Dam 2001). Other means for abiotic TEP formation from precursors, such as laminar (Passow 2000) and turbulent shear (Engel and Passow 2001) could be used and may give better transfer rates as the shear rate can easily be controlled. Finally, although the role of bacteria during the production, concentration, and isolation procedure was not investigated, it is likely that bacterial activity could alter the formation of TEP-microspheres aggregates by degrading and solubilizing the aggregates (Smith et al. 1992), thereby diminishing the efficiency of this method for removing TEP from solution. Future research should consider combining adsorption onto particles (such as glass fibers) and shear coagulation to improve efficiency of the magnetic isolation to remove TEP and investigate the influence of bacteria on the isolation procedure.

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