

## Measurement of acid polysaccharides in marine and freshwater samples using alcian blue

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### Abstract

Extracellular polymeric substances (EPS), including transparent exopolymer particles (TEP), play an important role in ocean biogeochemistry. This paper describes a simple method for measuring acid polysaccharides (APS), which are the major components of TEP and other EPS. APS were stained and precipitated with alcian blue, the precipitate was retained on a 0.2  $\mu\text{m}$  pore size filter and the absorbance of the filtrate was measured at 610 nm using a spectrophotometer. The color of the filtrate was inversely proportional to the amount of APS in the sample. This relationship was linear and was calibrated using gum xanthan or alginic acid as a standard. Salts interfered with the assay; therefore samples from marine environments were dialysed (1000 Da molecular weight cut-off [MWCO]) before addition of alcian blue. The assay was applied to whole water samples to give a total concentration of APS (particulate plus dissolved) in freshwater ponds, and cultures of marine protists and seawater from the continental shelf of the northern Gulf of Mexico. The assay was effective in measuring dissolved extracellular APS after removal of particulates by filtration, although in marine samples APS molecules smaller than the MWCO of the dialysis tubing (1000 Da) were not measured. The new method has potential in studies on the biogeochemistry of particulate and dissolved APS, including TEP and its precursors.

Organic carbon exists in marine and freshwaters in three pools: dissolved organic carbon (DOC), colloidal organic carbon (COC), and particulate organic carbon (POC). The fate of organic carbon in the water column is affected by the relative size of these different pools and the rate of transformation between them. An important class of polymers that contribute to the DOC to POC continuum are extracellular polymeric substances (EPS). EPS are estimated to form an organic carbon pool of 70 Pg C in the ocean (Verdugo et al. 2004). This is a large carbon pool compared with the mass of organic carbon associated with living organisms (1–2 Pg C; Falkowski et al. 2000). A major component of EPS are acid polysaccharides (APS). Due to the central role of APS in water column biogeochemistry, there is a need for techniques that enable the measurement of APS in both the particulate and dissolved phase.

Transparent exopolymer particles (TEP) were first described by Alldredge et al. (1993) as discrete transparent gel particles that stained for APS and were formed from extracellular polymeric substances (EPS) exuded by microorganisms. TEP are operationally defined as particles that are retained on a 0.4- $\mu\text{m}$  polycarbonate filter under low vacuum (150 mm Hg) and stained with alcian blue 8GX at pH 2.5. At this pH, alcian blue stains acid (carboxylated and sulphated) polysaccharides (Passow and Alldredge 1995). TEP are both a ubiquitous and abundant pool of POC that has a profound affect on biogeochemical cycling and food web structure (see review by Passow 2002a). Since their initial description in Pacific coastal waters off California (Alldredge et al. 1993), TEP have been found in a wide variety of marine locations, including the Atlantic off Bermuda (Passow and Alldredge 1994), the Benguela upwelling (Kjørboe et al. 1998), Ross Sea (Hong et al. 1997), and Northern Adriatic (Schuster and Herndl 1995). TEP is also a feature of freshwater environments and has been observed in Lake Frederiksborg (Denmark) (Worm and Søndergaard 1998), Lake Constance (Germany) (Grossart et al. 1997), Lake Kinneret (Israel) (Grossart et al. 1998), and the River Danube (Berger et al. 1996). TEP are primarily associated with phytoplankton (particularly diatoms) and bacterioplankton (Passow 2002a, 2002b). However, they are also produced by larger organisms such as macroalgae (Ramaiah et al. 2001) and oysters (McKee

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et al. 2005), and during the degradation of macroalgal detritus (Thornton 2004).

Research over the last decade has shown that TEP are important in water column processes. TEP affect the aggregation of nonsticky particles and are the glue which hold marine snow aggregates together (Passow 2002a). Therefore, TEP dynamics are important to the downward flux of carbon and other elements from the surface ocean to depth as larger aggregates of the same density sink faster than smaller ones. TEP plays a significant role in the termination of diatom blooms by affecting aggregation (Passow et al. 1994; Engel 2000). Aggregation processes bring particles into and out of the size range consumed by organisms in the water column (Passow 2002a) and, therefore, affect foodweb dynamics. In addition, TEP creates microenvironments with very different physical and chemical conditions from the surrounding sea or freshwater. These may be significant refugia for microorganisms and hotspots of biogeochemical activity in the otherwise relatively homogenous environment of the water column. The majority of TEP are formed abiotically from dissolved precursors (Passow 2000, 2002a; Verdugo et al. 2004). For example, bubbling seawater containing EPS of diatom origin with air leads to the formation of TEP (Mopper et al. 1995; Mari 1999) and marine DOC spontaneously assembles into polymer gels (Chin et al. 1998).

Increasingly,  $^{234}\text{Th}$  is used to investigate the biological carbon pump as a tracer of upper ocean particle dynamics and export of POC from the euphotic zone (Buesseler et al. 1998, 2006) as thorium (IV) has a strong affinity for organic particles in seawater (Guo et al. 2002). The use of  $^{234}\text{Th}$  as a tracer of organic matter depends on an understanding of how  $^{234}\text{Th}$  interacts with organic matter and whether it preferentially binds to certain compounds (Guo et al. 2002). APS are surface active compounds, and recent work has shown that APS is very effective at scavenging  $^{234}\text{Th}$  (Quigley et al. 2002; Guo et al. 2002; Santschi et al. 2003).

The objective of this paper is to present a simple technique for the measurement of APS in water samples using alcian blue as an APS stain. The proposed method stains acid polysaccharides using alcian blue under the same conditions as used with the established methods for TEP (Alldredge et al. 1993; Passow and Alldredge 1995). However, these methods are limited to alcian blue staining material that is larger than the  $0.4\ \mu\text{m}$  pore size filter that is used to retain TEP. The proposed method precipitates APS with alcian blue and the resulting precipitate is retained on a  $0.2\ \mu\text{m}$  filter. As the dissolved APS is precipitated, the new method can be used to address the dynamics of both the particulate and dissolved pool of APS. Furthermore, different fractions of the APS pool may be isolated by filtration and dialysis. Unlike the method of Ramus (1977), this technique does not require high speed centrifugation, and the addition of a dialysis step enables the analysis of marine samples. The method will enable researchers to address a knowledge gap; although there are many measurements of TEP,

there are relatively few studies of the dissolved precursors of TEP (especially in the field) or measurement that relate TEP to the total APS pool. Secondly, the new technique will be useful in studies of POC flux based on  $^{234}\text{Th}$  as it will enable measurements of APS in both the dissolved and particulate pools.

### Materials and procedures

*General procedure*—Alcian blue stains and precipitates the APS that form TEP and its dissolved EPS precursors at a pH of 2.5. Alcian blue (1 mL) was added to a 5 mL water sample and concentrated acetic acid was added (approximately 7–9 drops, depending on the sample) to produce a final pH of 2.5 (checked using a Mettler Toledo seven easy pH meter). The sample was vigorously mixed and then filtered. Stained APS precipitates were retained on a syringe filter containing a surfactant free cellulose acetate (SFCA) membrane with a pore size of  $0.2\ \mu\text{m}$  (Nalgene). The color of the filtrate was inversely proportional to the concentration of APS in the sample. The final 1 mL filtrate was collected directly into a disposable 1 cm path semi-micro polystyrene cuvette and absorbance was measured against an ultra high purity (UHP) water blank in a spectrophotometer (Shimadzu UV mini 1240) at 610 nm (Ramus 1977). Our absorption maximum was lower than that of Passow and Alldredge (1995) as they extracted alcian blue from the filters using 80% sulphuric acid, which shifts the absorption maximum from 610 nm to 787 nm. The removal of color by APS in the samples was calibrated against color removal by gum xanthan, which is commonly used to calibrate TEP assays (Passow and Alldredge 1995; Corzo et al. 2000; Ramaiah et al. 2001; Thornton 2004). Data are presented as gum xanthan equivalents ( $\text{mg X eq. L}^{-1}$ ).

Salts interfere with the binding properties of alcian blue, therefore the technique cannot be directly applied to samples from estuarine or marine waters. To overcome this limitation, dialysis was used to de-salt marine samples (and standards made up in seawater) before analysis. Dialysis tubing with a relatively small molecular weight cut-off (MWCO) of 1000 d (Spectra/Por 7 regenerated cellulose, Spectrum Laboratories) was used to ensure minimum loss of APS. APS molecules smaller than the MWCO of the dialysis tubing would have been lost during dialysis. Samples were analyzed immediately after dialysis.

*Alcian blue solution*—Stocks of alcian blue (Sigma) were made up to a concentration of 0.02% in 0.06% acetic acid (Passow and Alldredge 1995). Final pH was adjusted to 2.5. Working stocks of alcian blue solution were prepared once a week by filtering the stock solution through a  $0.2\ \mu\text{m}$  pore size SFCA syringe filter (Nalgene) to remove alcian blue precipitates (Passow and Alldredge 1995). Alcian blue solutions were stored refrigerated ( $4^\circ\text{C}$ ) in the dark.

*Standards*—Gum xanthan was used as the standard for the assay. Gum xanthan dissolves to form gel-like particles and is commonly used to calibrate TEP assays (Passow and Alldredge 1995; Corzo et al. 2000; Ramaiah et al. 2001; Thornton 2004). A gum xanthan stock solution was made by placing 0.1 g gum

xanthan in a flask with 100 mL of either UHP water, artificial seawater salts (ESAW, according to the recipe of Harrison et al. 1980), or natural seawater, depending on the assay conditions. The stock solution was vigorously stirred for at least 2 h until the large gel particles were broken up and no gum xanthan was visibly stuck to the inside of the flask. A manual tissue homogenizer (Kontes Glass Company) was used to break up the small gel particles before the standards were made up by dilution of the stock solution. Tests were carried out with alternative standards (alginic acid, D-glucose, and D-glucuronic acid), which were made up in the same way.

*APS free seawater*—If natural seawater is used as the solvent for the standard, then there will be a background concentration of APS as both EPS and TEP. To reduce the effect of this interference, blue water from the Gulf of Mexico was filtered through a glass fiber filter (GF/C, Whatman) followed by a 0.2  $\mu\text{m}$  pore sized polycarbonate filter (Nuclepore, Whatman) under low vacuum. The filtered seawater was then placed in a stirred ultrafiltration cell (Amicon 8200, Millipore) at room temperature with the gas pressure ( $<3.9 \text{ kg cm}^{-2}$ ) supplied by ultra-pure nitrogen (Matheson Tri-Gas). The ultrafiltration cell was fitted with a regenerated cellulose acetate ultrafiltration membrane (Amicon YM1, Millipore) with a 1000 Da MWCO. The filtrate passing through the membrane was collected and stored in an amber bottle at 4°C. This filtrate was used to make standards in natural seawater. Although the filtrate would have contained molecules  $< 1000 \text{ Da}$ , these were lost during the subsequent dialysis of the standards in tubing with a 1000 MWCO.

*Effect of alcian blue concentration on absorbance*—The alcian blue solution was diluted over a range of 15 concentrations from 0% to 100% to determine the concentration at which light absorbance is no longer linear. Solutions were placed in 1 cm path polystyrene cuvettes (VWR International) and absorbance was measured at 610 nm (Ramus 1977) using a Shimadzu UV mini 1240 spectrophotometer.

*Alcian blue concentration*—The ratio of alcian blue to sample may affect the range of the assay. Reducing the alcian blue concentration at relatively low APS concentrations may increase accuracy and precision. Conversely, increasing the alcian blue concentration in samples containing high concentration of APS will extend the upper limit of the assay. Three curves were constructed in which either 0.5, 1, or 2 mL alcian blue solution was added to 5 mL standard over an extended range (0–100  $\text{mg L}^{-1}$ ) of gum xanthan made up in UHP water. Calibration curves were also constructed using a low concentration of alcian blue (0.15 or 0.3 mL added to 5 mL standard) and standards at concentrations in the range 0 to 5  $\text{mg L}^{-1}$ .

*Dye binding time*—The amount of alcian blue binding to APS, and therefore the amount of dye removed during the filtration step, may depend on the length of time that the dye is exposed to APS. Triplicates samples, containing 20  $\text{mg L}^{-1}$  gum xanthan, were analyzed using the method above after incubations with alcian blue of 7, 15, 30, 60, 120, 240, and 480 min at room temperature.

*Sample storage*—Formalin has been used as a preservative for water samples used in TEP assays, with negligible effects (Passow and Alldredge 1994; Corzo et al. 2000). To determine whether there was any effect of formalin on the APS assay, gum xanthan standards (0, 5, 10, 20, and 25  $\text{mg L}^{-1}$ ) were made up in 0%, 1%, 5%, and 10% formalin and analyzed in duplicate as described in the general procedure.

Access to a spectrophotometer may not be possible in the field. Therefore, it would be useful if the filtrates from samples analyzed in the field could be stored and measured on return to the laboratory. A calibration curve was constructed using duplicate gum xanthan standards (0, 5, 10, 20, and 25  $\text{mg L}^{-1}$ ) dissolved in UHP water. Absorbance of the filtrate was measured immediately and after 1, 7, and 11 d. The filtrate was stored in glass vials with Teflon-lined caps at room temperature in the dark between measurements.

*Filter effects*—Choice of filter will affect the color of the filtrate in two ways. Firstly, the pore size of the filter will determine how much of the precipitated APS is retained. Secondly, there is the possibility that the filter itself will bind alcian blue and remove it from the filtrate, an effect that may be influenced by the composition of the filter. Gum xanthan standards were made up in UHP water at concentrations of 0, 5, 10, 15, 20, and 25  $\text{mg L}^{-1}$ . Duplicate samples of each standard were filtered through 0.1 and 0.2  $\mu\text{m}$  pore size 25 mm diameter polycarbonate filters (Nuclepore, Whatman), 0.2  $\mu\text{m}$  pore size 25 mm diameter SFCA syringe filters (Nalgene), and 0.2  $\mu\text{m}$  pore size 25 mm diameter nylon syringe filters (Fisherbrand). A calibration curve was constructed for each filter type. Additional comparisons were made between 25 mm nylon syringe filters (Nalgene) and SFCA syringe filters (Nalgene) at standard concentrations in the range 0–25  $\text{mg L}^{-1}$  and 0–5  $\text{mg L}^{-1}$ . Gum xanthan and alginic acid were dissolved in UHP and used as standards.

To test how much of alcian blue is adsorbed by 0.2  $\mu\text{m}$  pore size 25 mm diameter SFCA syringe filters (Nalgene), 1 mL aliquots of alcian blue were diluted with 5 mL UHP water and the absorbance of 5 replicates was measured at 610 nm and compared with the absorbance of a further 5 replicates after filtration of the 6 mL through a 0.2  $\mu\text{m}$  pore size SFCA syringe filter (Nalgene). If a significant proportion of the alcian blue binds to the filter itself as it passes through, then the filtrate first coming out of the filter would be expected to be less blue than that coming out of the filter once the filter has become saturated with dye. To test for this effect, 5 replicate 1 mL aliquots of alcian blue were diluted with 5 mL UHP water. Each replicate was filtered through a 0.2  $\mu\text{m}$  pore size SFCA syringe filter (Nalgene), and the filtrate was collected directly into semi-micro cuvettes in aliquots of 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 mL. The absorbance of each aliquot was measured at 610 nm and compared with the absorbance of the unfiltered solution.

*Dialysis*—Most work on both EPS and TEP has been conducted in marine systems. However, inorganic salts affect the properties and efficiency of alcian blue staining (Hayat 2000;

Passow and Alldredge 1995). Therefore, marine water samples were de-salted by dialysis before the addition of alcian blue solution. Dialysis tubing with a relatively small MWCO of 1000 Da (Spectra/Por 7 regenerated cellulose, Spectrum Laboratories) or 100 Da (Spectra/Por cellulose ester, Spectrum Laboratories) was used to ensure minimum loss of APS. The dialysis tubing was soaked in UHP water for 30 min and rinsed to remove the sodium azide preservative. Eight to ten milliliter samples were loaded into the dialysis tubing, which was sealed with closures (Spectrum Laboratories). The tubing was dialysed in large, stirred bath of reverse osmosis purified water for approximately 24 h, during which time the water was changed at least once. A few drops of chloroform were added to the water bath to inhibit microbial growth (Underwood et al. 2004). When natural seawater samples were analyzed, or samples from cultures grown in artificial seawater salts (ESAW; Harrison et al. 1980), then the standards were made up in the appropriate media and the standards were analyzed after dialysis in the same way as the samples. Seawater used to make the standards was prepared as described above. The salinity of dialysed samples was checked with a hand-held refractometer to ensure that it was zero before analysis.

An experiment was conducted to assess if APS was lost from the dialysis tubing during dialysis. Gum xanthan was made up in UHP water, and 5 replicates of two concentrations (0 and 20 mg L<sup>-1</sup>) were analyzed before and after 24 h of dialysis (with 1000 Da MWCO; as described above). The standards were analyzed for APS (5 mL sample + 1 mL alcian blue) and carbohydrate using the phenol-sulfuric acid assay (Dubois et al. 1956). The carbohydrate assay was calibrated using D-glucose and carbohydrate concentrations were expressed as D-glucose equivalents.

A similar experiment was conducted using lower concentrations of gum xanthan (0 and 3 mg L<sup>-1</sup>) dissolved in UHP water. These samples were dialysed for 0, 24, and 48 h and compared with standards that were not placed in dialysis tubing. Approximately 10 mL gum xanthan solution was placed into 12 replicate dialysis bags (with 1000 Da MWCO; as described above) at each gum xanthan concentration and placed in a dialysis bath. Four replicates of each concentration were immediately sampled, with 1 mL frozen for carbohydrate analysis (-20°C) in a microcentrifuge tube and 7 mL placed in a 15 mL centrifuge tube and preserved with 70 mL formalin (i.e., approximately 1% v/v) and stored in the dark (4°C) until analysis. Four replicate dialysis bags at each concentration were sampled after a further 24 and 48 h of dialysis. At the end of the experiment, the frozen carbohydrate samples were analyzed using the phenol-sulfuric acid assay (Dubois et al. 1956). However, this time the assay was calibrated using gum xanthan and carbohydrate concentrations were expressed as gum xanthan equivalents. Standards and the samples were analyzed for APS (7 mL sample + 0.3 mL alcian blue).

*Analysis of samples from laboratory cultures of marine protists*—To test the applicability of the method to cultured organisms, experiments were carried out with cultures of the diatom *Tha-*

*lassiosira weissflogii* (CCMP 1051) and the heterotrophic protist *Cafeteria roenbergensis*. Measurements were made from these cultures with two objectives. The first objective was to show that a significant proportion of alcian blue staining material passes through a 0.4 µm pore filter (as used in TEP assays, Passow and Alldredge 1995). The second was to determine the effect of dialysis time on APS concentration in real samples.

*Cafeteria roenbergensis* was grown in 200 mL filtered autoclaved seawater to which 0.4 mL filter sterilized 10% (w/v) yeast extract was added, five grains of autoclaved rice and 2 mL trace metal solution (after Harrison et al. 1980, as modified by Thornton and Thake 1998). The flask was inoculated with 42 mL of an old culture. *C. roenbergensis* feeds on bacteria, which in turn grow on the yeast extract and rice added to the culture. *C. roenbergensis* was chosen as the cultures contain flocculent material resembling marine snow as they age. *Thalassiosira weissflogii* was grown in 245 mL autoclaved filtered seawater containing trace metals (after Harrison et al. 1980, as modified by Thornton and Thake 1998), vitamins (after Harrison et al. 1980), silicate (240 µM), phosphate (160 µM), and ammonium (240 µM). *T. weissflogii* was chosen as diatoms are often associated with TEP, EPS, and aggregates in the field (Thornton 2002; Passow 2002a).

The cultures were sampled in the stationary phase after 18 d growth. Samples (approximately 3 mL) were taken for cell counts from both cultures and preserved in Lugol's iodine (Parsons et al. 1984). Four samples from each culture were filtered under low vacuum onto 25 mm diameter 0.4 µm pore size polycarbonate filters and stained with alcian blue for TEP analysis using the method of Passow and Alldredge (1995). Triplicate 12 mL samples from each culture were placed in 15 mL centrifuge tubes with 120 mL formalin as a preservative (i.e., approximately 1% v/v). A further six 12 mL samples from each culture were gently filtered through a GF/C filter before being filtered through a 0.4 µm pore size polycarbonate filters. The filtrate was retained and preserved in formalin. Gum xanthan standards were made up in the same seawater used to grow the cultures and preserved with formalin. Preserved standards and culture samples were stored in the dark at 4°C until analysis. Samples for APS and standards were placed in dialysis tubing (1000 Da MWCO) and dialysed. After 24 h, the standards, whole culture samples, and 3 of 0.4 µm filtrate samples for each organism were analyzed for APS (5 mL sample + 1 mL alcian blue). Samples for cell counts were taken from the whole culture dialysis bags of *T. weissflogii*. The remaining 3 replicate 0.4 µm filtrate samples for each organism were dialysed for a further 24 h and analyzed for dissolved APS after a total of 48 h dialysis.

A second experiment was conducted with cultures of two marine diatoms (*Thalassiosira weissflogii* [CCMP 1051] and *Chaetoceros muelleri subsalsum* [CCMP 1316]) grown in filtered, autoclaved seawater supplemented with trace metals (after Harrison et al. 1980, as modified by Thornton and Thake 1998), vitamins (Harrison et al. 1980), and inorganic nutrients (500 µM nitrate, 500 µM silicate, 100 µM phosphate). One cul-

ture (300 mL) of each diatom was grown in a 1000 mL Pyrex flask. The cultures were sampled after 5 d of growth and samples were taken for cell counts (preserved in Lugol's iodine) and APS. APS was measured in samples processed using a cell disruptor (Disruptor Genie Scientific Industries) to break open the diatom cells using cold ( $-20^{\circ}\text{C}$ ) glass beads (425–600  $\mu\text{m}$  diameter) and 5 min disruption time. The disrupted culture was passed through a 0.2  $\mu\text{m}$  pore size SFCA syringe filter, and the filtrate was analyzed for APS. In addition, APS was measured in whole culture samples of *T. weissfloggi*. APS was measured in 5 mL samples to which 1 mL alcian blue solution was added. Five replicate sub-samples were taken from the culture for the measurement of each APS fraction. Results were expressed in gum xanthan equivalents and calibrated against standards made up in Gulf of Mexico blue water.

*Analysis of samples from freshwater ponds*—Two ponds were sampled to test the applicability of the method to natural freshwaters. The objective of these measurements was to determine whether there was any significant difference in APS concentrations in preserved samples compared with samples analyzed immediately after collection. Secondly, the hypothesis that significant APS passes through a 0.4  $\mu\text{m}$  pore filter was tested. Both ponds were located on the main campus of Texas A&M University and were sampled on 27 April 2006 when the water temperature was  $19^{\circ}\text{C}$ . Four replicate samples were taken in 250 mL polyethylene bottles from a pond located on the golf course ('golf course') fed by a small stream. Four replicate samples were also taken from a small pond containing macrophytes ('water garden'). On return to the laboratory, 5 mL from each bottle was filtered onto a 25 mm diameter 0.4  $\mu\text{m}$  pore size polycarbonate filter and stained with alcian blue for TEP analysis using the method of Passow and Alldredge (1995). Whole water samples and filtrates (0.4  $\mu\text{m}$  pore filter) from each bottle were analyzed for APS (5 mL sample + 1 mL alcian blue). A second set of APS samples were preserved in formalin to a final concentration of 1% v/v and stored in the dark ( $4^{\circ}\text{C}$ ) for 24 h before analysis for APS.

A second experiment was conducted with the golf course samples, which were stored in an incubator overnight at  $20^{\circ}\text{C}$ . Comparisons were made between the APS and TEP concentrations of whole water samples and samples processed using a cell disruptor (Disruptor Genie Scientific Industries) to break open cells in the water. Each bottle was sampled (5 mL) for APS and a second sample (3 mL) was filtered onto a 0.4  $\mu\text{m}$  pore size polycarbonate filter and stained with alcian blue for TEP analysis of whole water. A second set of samples were cell-disrupted using cold ( $-20^{\circ}\text{C}$ ) glass beads (425–600  $\mu\text{m}$  diameter) and 5 min of disruption time. The cell-disrupted samples were analyzed for APS and a further 3 mL was filtered down onto a 0.4  $\mu\text{m}$  pore size polycarbonate filter and stained with alcian blue for TEP analysis.

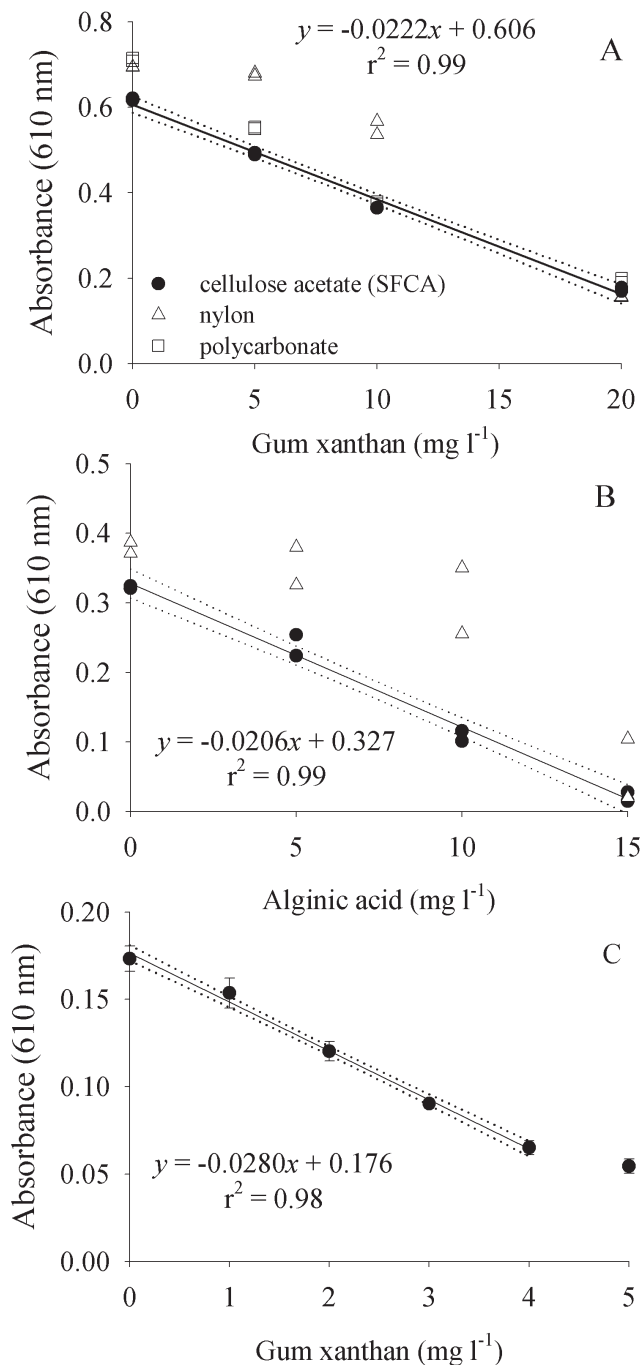
*Analysis of marine samples from the Gulf of Mexico*—Water samples were collected in the Gulf of Mexico from the *RV Gyré* on the 21–23 May 2005 at 3 stations over the Texas-Louisiana shelf. Samples were taken from the surface (0–0.3 m depth) at

each site using a bucket lowered over the side of the vessel. In addition, samples were taken from Niskin bottles closed at the chlorophyll maximum as determined by a chlorophyll fluorometer (Chelsea Aqua 3, Chelsea Technologies Group, UK) attached to a 12 bottle rosette with a CTD. Four 250 mL sub-samples were taken from the bucket or Niskin bottle. Each sample was preserved with 2.5 mL formalin (approximately 1% v/v) and stored in a 250 mL polycarbonate bottle (Nalgene) sealed with Parafilm around the bottle cap. Samples were stored upright in the dark at  $4^{\circ}\text{C}$  until analysis onshore. TEP were filtered (40–80 mL, depending on the site) from each bottle onto a 0.4  $\mu\text{m}$  pore size polycarbonate filter, which was stained with alcian blue. TEP concentrations were measured colorimetrically according to Passow and Alldredge (1995). APS was measured after 42 h of dialysis in tubing with a 1000 Da MWCO (Spectra/Por 7 regenerated cellulose, Spectrum Laboratories). APS was measured by adding 0.25 mL alcian blue to 8 mL sample at pH 2.5. The last 4 mL filtrate, after passing through a 0.2  $\mu\text{m}$  pore size syringe SFCA filter, was collected into 4 mL glass cuvettes and absorbance was measured at 610 nm. There is variation between polystyrene semi-micro cuvettes, which can significantly affect absorbance at low alcian blue concentrations, therefore matched glass cuvettes were used. Both the TEP and APS assays were calibrated against gum xanthan made up in APS-free blue water from the Gulf of Mexico prepared as described above.

*Statistics*—Statistical analyses were carried out using SigmaStat 3.1 (Systat Software) after checking that the data fitted the assumptions of the statistical test. Analysis of variance (ANOVA) was conducted on data which conformed to a normal distribution (Kolmogorov-Smirnov test) and equality of variance. If the data did not conform to these assumptions, then a  $\log(x + 1)$  transformation was applied to the data (Zar 1996). Nonparametric tests on ranks were performed when the data did not come from normally distributed populations after transformation.

## Assessment

*General procedure*—The alcian blue solution showed a linear relationship between concentration and absorbance (at 610 nm) up to a value of 1.3 in a 1 cm path cuvette, which is equivalent to a dilution of 1 in 3 of the alcian blue stock solution or 33.3% (v/v). Calibration curves were made using a range of alcian blue concentrations, from 2.9% to 28.6% (v/v) and a range of different standard concentrations made up in UHP water; three of these calibrations are shown in Fig. 1. The assay consistently produce linear calibration curves in which the coefficient of determination ( $r^2$ ) was  $> 0.95$ . The slopes of the regression were negative as color is removed by increasing the concentration of gum xanthan in the standard. The intercept on the y axis was the absorbance with no APS and was dependent on how much alcian blue was added to the assay. Therefore, adding 1 mL alcian blue solution to 5 mL UHP water (16.7% v/v) produced an absorbance of 0.61 compared



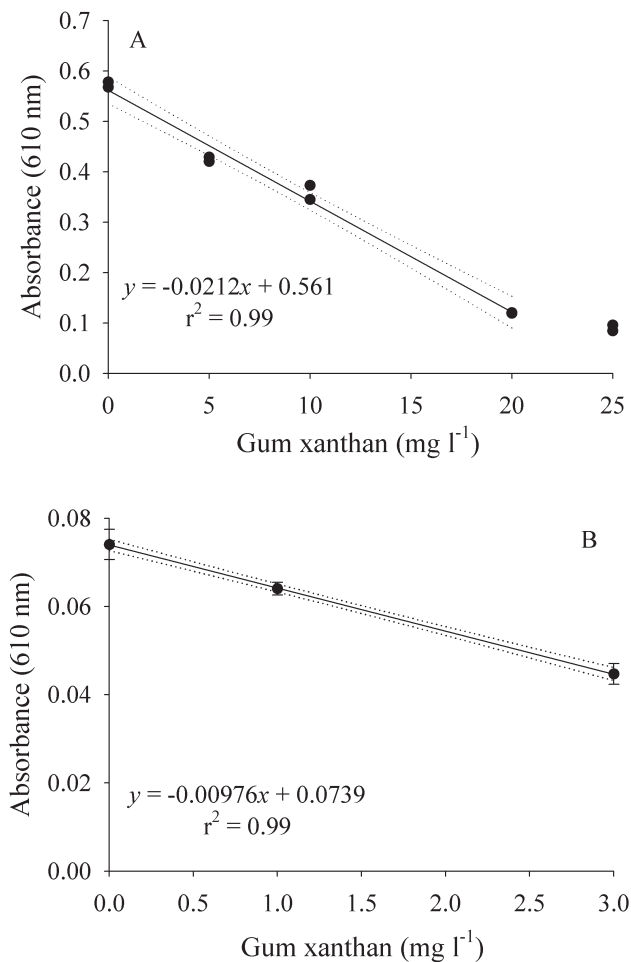
**Fig. 1.** Representative calibration curves for standard solutions made up in UHP water. (A) Calibration curves for gum xanthan solutions made with 3 different types filter and the addition of 1 mL alcian blue to 5 mL standard. Duplicates were analyzed at each concentration. (B) Calibration curves for alginate solutions made using cellulose acetate and nylon filters and the addition of 0.5 mL alcian blue to 5 mL standard. Duplicates were analyzed at each concentration. (C) Calibration for low concentrations of gum xanthan by the addition of 0.3 mL alcian blue to 5 mL standard using a cellulose acetate filter. Each data point shows the mean ( $\pm$  standard deviation,  $n = 5$ ). The solid line on each graph (A–C) is a linear regression through the data obtained using cellulose acetate filters and the dotted lines show 95% confidence limits.

with an absorbance of approximately half as much for an addition of 0.5 mL (9.1% v/v) alcian blue solution. The range of the assay depends on how much alcian blue is added to the sample; adding 0.5 mL, 1 mL, or 2 mL increases the upper limit of the assay to approximately 15, 20 or 30 mg L<sup>-1</sup> respectively, when added to 5 mL standard (data not presented). The time that the sample was exposed to alcian blue before it was filtered did not affect the results of the assay, indicating that the precipitation of APS with alcian blue was instantaneous once the dye was mixed with the sample. Furthermore, once filtered the filtrate could be stored in the dark at room temperature without any significant loss of color, even after 11 d (data not presented). Formalin did not significantly affect the calibration of gum xanthan standards, even at the high concentration of 10% (v/v) (data not presented).

Filters composed of different material produced calibration curves of different shapes. Cellulose acetate filters produced the calibrations with the most accuracy and precision, both with gum xanthan (Fig. 1a) and alginate (Fig. 1b) as standards. Nylon (Figs. 1a and 1b) and polycarbonate filters (Fig. 1a) produced calibration curves with a higher intercept than cellulose acetate. Carbohydrates that were not acid polysaccharides, such as D-glucose and D-glucuronic acid, did not produce a linear relationship between absorbance at 610 nm and concentration as they did not precipitate with alcian blue.

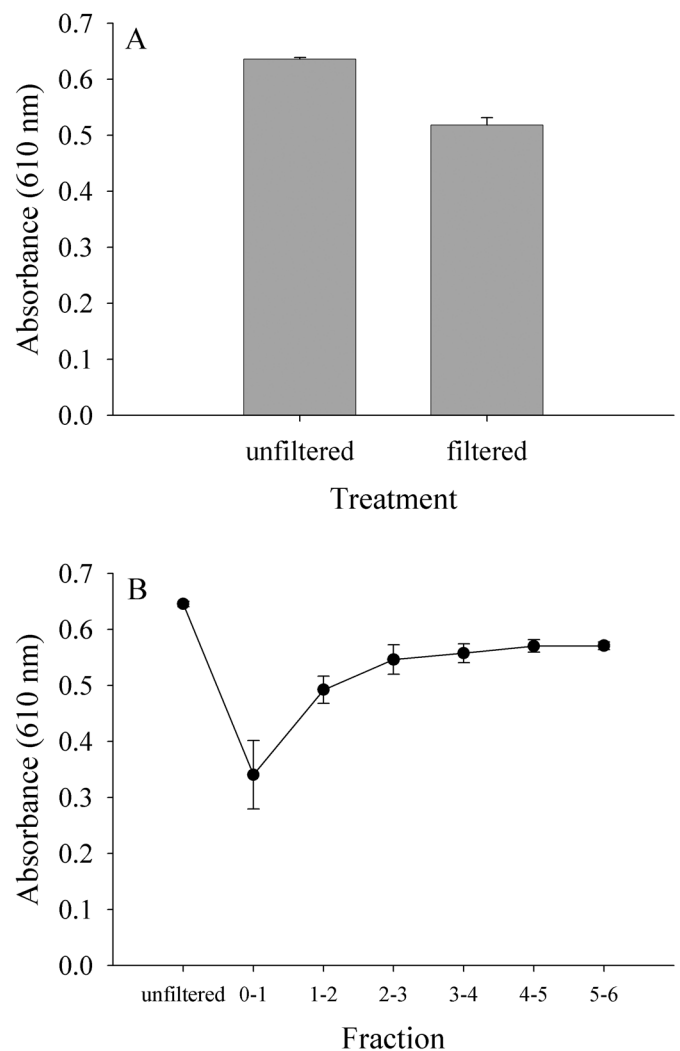
Salts interfere with the staining of APS by alcian blue, but introducing a dialysis step into the procedure to remove salts could affect accuracy and precision. Analysis of gum xanthan and alginate made up in seawater and dialysed prior to analysis, produced accurate and precise standard curves ( $r^2 > 0.95$ ). Fig. 2 shows two curves for gum xanthan standards made in seawater and dialysed for 24 h before analysis. The slope and intercept of the regression on Fig. 2a is similar to the equivalent curve on Fig. 1a, indicating that the dialysis process had little effect on the analysis other than removing salts.

The 0.2  $\mu$ m pore size SFCA filters did have an effect on the absorbance of alcian blue solutions passing through the filter (Fig. 3). Filtration of 1 mL alcian blue solution diluted with 5 mL UHP water significantly ( $P < 0.05$ , Mann-Whitney Rank Sum test) reduced absorbance from a mean of 0.636 to 0.518 (Fig. 3a), equivalent to a 19% loss of color. Fig. 3b shows that most of this color is lost as the first 1 mL passes through the filter. The mean absorbance of unfiltered solution was 0.645 compared with 0.341 for the first 1 mL. This indicates that the filter binds alcian blue and becomes saturated with the dye as more solution passes through it. Absorbance increased to a mean absorbance of 0.571 for the final two fractions, though it did not return to pre-filtration values. This was probably due to retention alcian blue, which had precipitated in the stock solution. As more 1 mL fractions passed through the filter, the variation of the measured absorbance decreased. These data indicate that the absorbance of the last 1 mL passing through the filter should be measured, as this is the least affected by adsorption to the filter.



**Fig. 2.** Representative calibration curves for gum xanthan solutions made up in natural seawater and dialysed (1000 Da MWCO) to remove the salts. (A) Duplicate standards analyzed by the addition of 1 mL alcian blue to 5 mL sample. (B) Calibration for low concentrations of gum xanthan by the addition of 0.25 mL alcian blue to 8 mL standard. Each date point shows the mean ( $\pm$  standard deviation,  $n = 4$ ). The solid line on each graph (A and B) is a linear regression through the data, and the dotted lines show 95% confidence limits. All standard alcian blue mixtures were filtered through cellulose acetate (SFCA) filters.

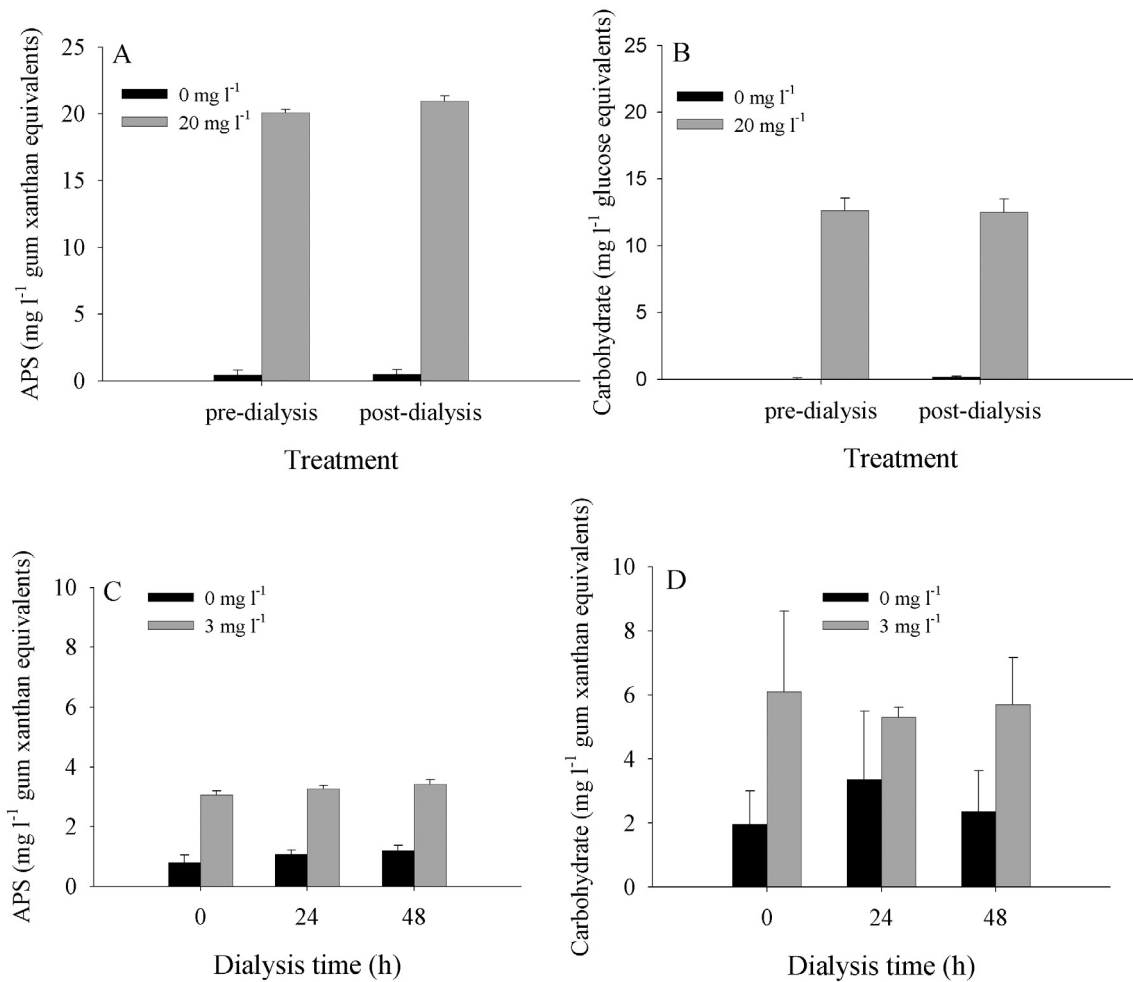
Two experiments were conducted to determine whether dialysis resulted in a loss of APS. In the first experiment (Figs. 4a & 4b), concentrations of APS and carbohydrate were measured before and after dialysis in gum xanthan standards. There was a slight increase in APS concentration in those standards which were dialysed for 24 h (Fig. 4a). The mean concentration in the 20 mg L<sup>-1</sup> standards increased from 20.1  $\pm$  0.3 to 21  $\pm$  0.4 mg X eq. L<sup>-1</sup> (mean  $\pm$  standard deviation). Two way ANOVA showed that there was a significant difference ( $P < 0.05$ ) between APS concentration before and after dialysis and that there was a significant interaction between dialysis and concentration. Post-hoc analysis showed that the source of this variation was between the 20 mg L<sup>-1</sup> standards pre- and post-dialysis, though there was no significant difference between



**Fig. 3.** Filtration effects on alcian blue absorbance using a 0.2  $\mu\text{m}$  SFCA syringe filter (Nalgene). (A) Reduction in absorbance in filtered compared with unfiltered reagent blanks. Bars show mean  $\pm$  standard deviation ( $n = 5$ ). (B) Absorbance of different volumetric fractions of reagent blanks passed through a 0.2  $\mu\text{m}$  SFCA syringe filter (Nalgene). Bars show mean  $\pm$  standard deviation ( $n = 5$ ).

the 0 mg L<sup>-1</sup> standards pre- and post-dialysis. Two way ANOVA (after a  $\log_{10}(x + 1)$  transformation of the carbohydrate data) showed that there was a significant difference ( $P < 0.05$ ) between carbohydrate concentration before and after dialysis and that there was a significant interaction between dialysis and concentration (Fig. 4b). Post-hoc analysis (Holm-Sidak method) showed that the source of this variation was between the 0 mg L<sup>-1</sup> standards pre- and post-dialysis. There was no significant difference between the 20 mg L<sup>-1</sup> standards pre- and post-dialysis in contrast to the APS analysis. A dialysis time of 24 h consistently reduced salinity to 0‰ as measured with a hand-held refractometer.

The second experiment examined the effect of dialysis time on the concentration of APS and carbohydrate concentration.

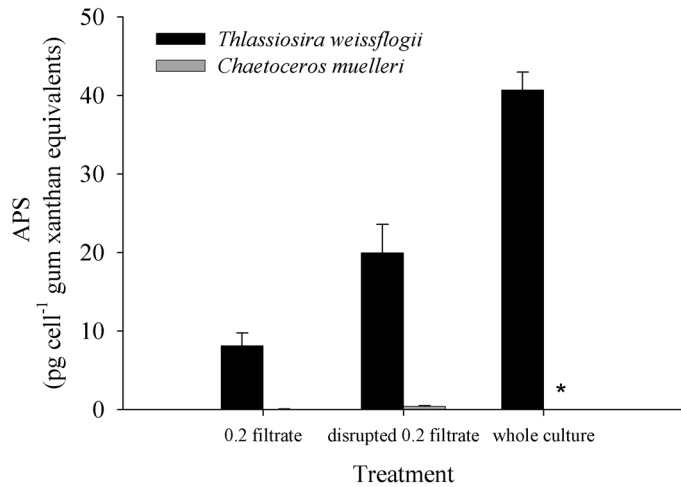


**Fig. 4.** Effect of dialysis on APS and carbohydrate concentrations of gum xanthan standards made up in UHP water. Concentrations of APS (A) and carbohydrate (B) pre- and post-24 h dialysis in 1000 Da MWCO tubing. Concentrations of APS (C) and carbohydrate (D) after placement in 1000 Da MWCO dialysis tubing and recovery after 0, 24, and 48 h dialysis. Bars show mean + standard deviation, where  $n = 5$  (A and B) and  $n = 4$  (C and D).

APS concentrations increased with dialysis time at both 0 and 3 mg L<sup>-1</sup> gum xanthan concentrations (Fig. 4c). Two-way analysis of variance showed that there was a significant ( $P < 0.05$ ) difference in concentration with time, however there was no significant interaction between concentration and time. Post-hoc analysis (Holm-Sidak method) showed that there was no significant difference in APS concentrations at 24 and 48 h, although there was a significant difference between APS concentrations at 0 and both 24 and 48 h. However, this analysis used the data at both 0 and 3 mg L<sup>-1</sup> together and compared these data at different times. A more relevant comparison is to determine whether there was a significant change in APS concentration between time 0 and 24 h at any of the standard concentrations, as 24 h is standard dialysis time used in the protocol;  $t$  tests showed that there was no significant change in concentration in APS at any given concentration between 0 and 24 h. There was no significant effect of dialysis time on carbohydrate concentration (Fig. 4d). Fig. 4d shows that sim-

ply placing the sample in the regenerated cellulose dialysis tubing increased carbohydrate concentrations, however a relatively small proportion of the added carbohydrate was APS as it did not stain with alcian blue (Fig. 4c).

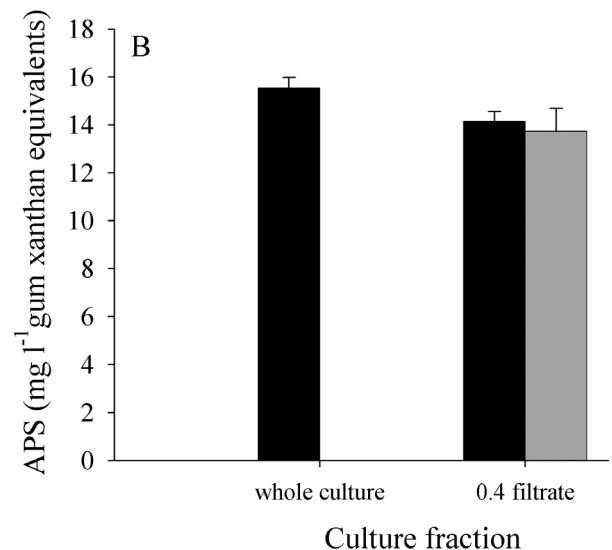
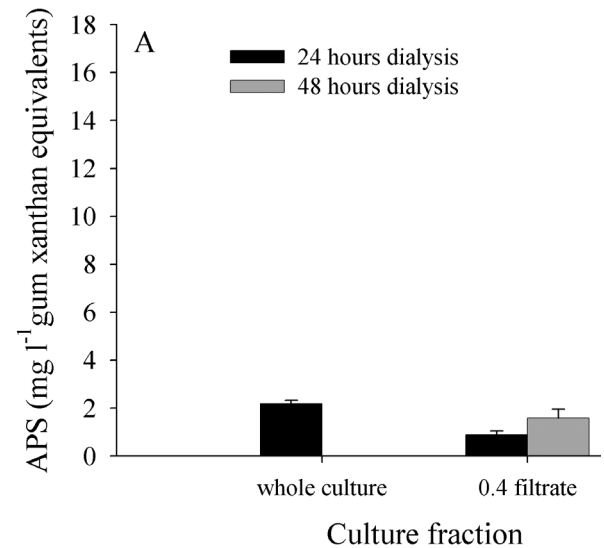
**Application to cultures of protists**—Fig. 5 shows APS concentrations in cultures of the diatoms *Thalassiosira weissflogii* and *Chaetoceros muelleri* greater than 1000 Da (the MWCO of the dialysis tubing used to de-salt the samples). There were negligible concentrations of extracellular APS that could pass through a 0.2  $\mu\text{m}$  pore size filter in cultures of *C. muelleri* compared with *T. weissflogii* in which there were  $3.03 \pm 0.62$  mg X eq. L<sup>-1</sup> (mean  $\pm$  standard deviation) or  $8.11 \pm 1.65$  pg X eq. cell<sup>-1</sup> (Fig. 5). Disrupting the cells showed that there was a significant internal pool of APS inside the diatom cells that would pass through a 0.2  $\mu\text{m}$  filter once released. Filter passing APS concentration in disrupted *C. muelleri* were  $1.73 \pm 0.38$  mg X eq. L<sup>-1</sup> compared with  $7.46 \pm 1.35$  mg X eq. L<sup>-1</sup> in *T. weissflogii*. Once normalized to cell density this was equivalent to  $19.96 \pm 3.62$  pg X eq. cell<sup>-1</sup>



**Fig. 5.** APS (> 1000 Da) concentrations in marine diatom cultures normalized to cell density. The asterisk (\*) indicates that no measurements were made from whole culture samples of *Chaetoceros muelleri*. Bars show mean + standard deviation ( $n = 5$ ).

in *T. weissflogii* and only  $0.40 \pm 0.09$  pg X eq. cell<sup>-1</sup> in *C. muelleri*. Measurements of APS in whole culture samples of *T. weissflogii* were  $15.21 \pm 0.85$  mg X eq. L<sup>-1</sup> ( $40.69 \pm 2.28$  pg X eq. cell<sup>-1</sup>). These data show that there are distinct internal and external pools of APS associated with microorganisms. Pre-treatment of the samples using filtration, cell disruption, and other techniques enables APS in different ecologically important pools to be measured relatively easily. For example, applying a simple mass balance approach to the data from the *T. weissflogii* culture indicates that of the 41 pg X eq. cell<sup>-1</sup> of APS, approximately 8 pg X eq. cell<sup>-1</sup> was released into the culture medium by the cells, 12 pg X eq. cell<sup>-1</sup> of dissolved APS was located inside the cells, and approximately 21 pg X eq. cell<sup>-1</sup> was associated with particulates retained on a 0.2  $\mu$ m filter (e.g., APS coatings on the frustule). Moreover, these data show that the APS assay can be used in comparative studies to investigate intra and extracellular pools of APS produced by different taxa of microorganism.

*Cafeteria roenbergensis* cultures contain visible white flocs after a few days. These flocs are probably generated by large numbers of bacteria growing within the culture. Despite the visible organic material in the culture, APS concentrations were relatively low (Fig. 6a) at  $2.19 \pm 0.88$  mg X eq. L<sup>-1</sup> (mean  $\pm$  standard deviation) and approximately half this material passed through a 0.4  $\mu$ m pore sized filter ( $0.88 \pm 0.16$  mg X eq. L<sup>-1</sup>). There was a significant difference ( $P < 0.05$ ) between APS concentrations in the whole culture compared with the 0.4  $\mu$ m filtrate. There was a significant increase ( $P < 0.05$ ) in APS concentrations in the filtrate that were dialysed for 48 h compared with 24. TEP concentrations in cultures of *C. roenbergensis* were  $2.59 \pm 0.89$  mg X eq. L<sup>-1</sup>, which was not significantly different from the APS concentrations for the whole culture.



**Fig. 6.** APS (> 1000 Da) concentrations in whole culture and filtrate (0.4  $\mu$ m pore size) from cultures of *Cafeteria roenbergensis* (A) and *Thalassiosira weissflogii* (B) grown in natural seawater. Bars show mean + standard deviation ( $n = 3$ ).

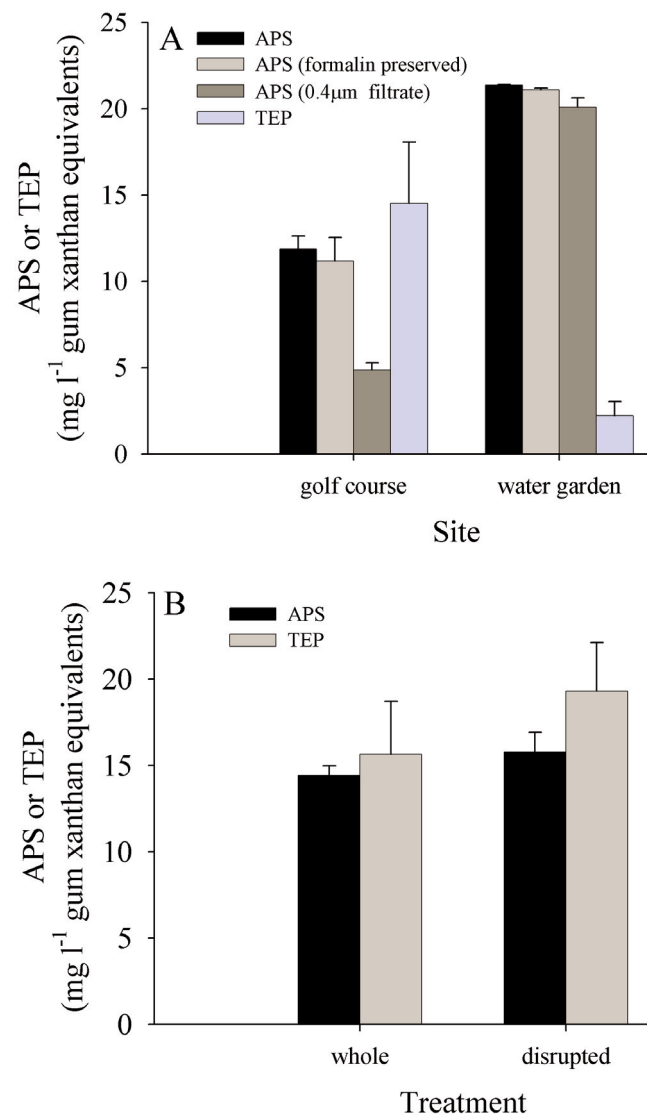
In the second experiment with *Thalassiosira weissflogii*, APS concentrations were  $15.54 \pm 0.44$  mg X eq. L<sup>-1</sup> in whole cultures and  $14.16 \pm 0.95$  mg X eq. L<sup>-1</sup> in the filtrate, indicating that the majority of APS in the culture was dissolved (or at least < 0.4  $\mu$ m; Fig. 6b). However, there was significantly ( $P < 0.05$ ) less APS in the filtrate compared with the whole cultures. There was no significant difference in APS concentrations in filtrate dialysed for 24 h compared with 48 h. Cell numbers in whole culture samples of *T. weissflogii* decreased from a mean of  $4.56 \times 10^5$  to  $4.19 \times 10^5$  cells mL<sup>-1</sup> during dialysis, probably due to cell lysis. This reduction in cell numbers was not significant ( $P > 0.05$ ,  $t$  test), however

it does not preclude leakage of intracellular APS from apparently intact cells.

There was a discrepancy between TEP concentrations ( $37.8 \pm 2.62$  mg X eq. L<sup>-1</sup>) and APS concentrations from the whole culture ( $15.54 \pm 0.44$  mg X eq. L<sup>-1</sup>) as TEP concentrations were significantly greater. Furthermore, as most of the whole culture APS passed through a 0.4  $\mu$ m filter, then it was predicted that TEP concentrations were approximately 1 mg X eq. L<sup>-1</sup>. There may have been a significant overestimation of TEP concentrations as a result of colored compounds, other than alcian blue, contributing to the absorbance at 787 nm. These compounds may have been extracted from *Thalassiosira weissflogii* cells during the extraction of alcian blue from the filters using 80% sulphuric acid. To test this hypothesis, samples of *T. weissflogii* cultures were filtered onto 0.4  $\mu$ m pore size polycarbonate filters, which were extracted with sulphuric acid without staining with alcian blue. It was found that the absorbance of the extract at 787 nm was proportional to the amount of culture filtered. This indicates that colored substances, other than alcian blue, may have led to an overestimation of TEP concentrations.

**Application to freshwater samples**—Two freshwater ponds on the main campus of Texas A&M University were sampled in April 2006. The ‘water garden’ pond was a small pond containing macrophytes. Microscopic examination of the water showed that it contained a low concentration of diatoms and some plant detritus. The ‘golf course’ pond water was golden-green in color and contained a dense bloom of a chlorophyte. Fig. 7a shows APS and TEP concentrations in various fractions from the two ponds. In the golf course pond, there was  $11.87 \pm 0.76$  mg X eq. L<sup>-1</sup> APS. Almost half the APS ( $4.87 \pm 0.42$  mg X eq. L<sup>-1</sup>) passed through a 0.4  $\mu$ m filter. There was no significant difference in APS concentrations in whole water samples that were analyzed immediately and those that were preserved in 1% (v/v) formalin and stored in the dark for 24 h at 4°C before analysis. Concentrations of APS in the water garden pond ( $21.34 \pm 0.05$  mg X eq. L<sup>-1</sup>) were twice those of the golf course pond and all of this material passed through a 0.4  $\mu$ m filter ( $21.10 \pm 0.09$  mg X eq. L<sup>-1</sup>). There was no significant difference in concentration between APS in whole water samples analyzed immediately the preserved sample. This indicates that formalin is a suitable preservative for APS in water samples.

TEP concentrations were very different in the two ponds. In the water garden pond, where most APS passed through a 0.4  $\mu$ m filter, TEP concentrations were  $2.22 \pm 0.81$  mg X eq. L<sup>-1</sup> and significantly lower ( $P < 0.05$ ) than the APS concentrations of all fractions. TEP concentrations ( $14.53 \pm 3.54$  mg X eq. L<sup>-1</sup>) were not significantly different from whole water APS in water samples taken from the golf course pond, where a lower proportion of the APS passed through a 0.4  $\mu$ m filter. A comparison was made between whole water samples from the golf course pond and water samples that were processed using a cell disruptor (Fig. 7b). Cell disruptor



**Fig. 7.** APS and TEP concentrations. Bars show mean + standard deviation ( $n = 4$ ). (A) Concentrations of TEP and APS fractions (whole water, formalin preserved whole water and 0.4  $\mu$ m filtrate) in two freshwater ponds (B). Concentrations of APS in whole water and disrupted whole water samples from the golf course pond.

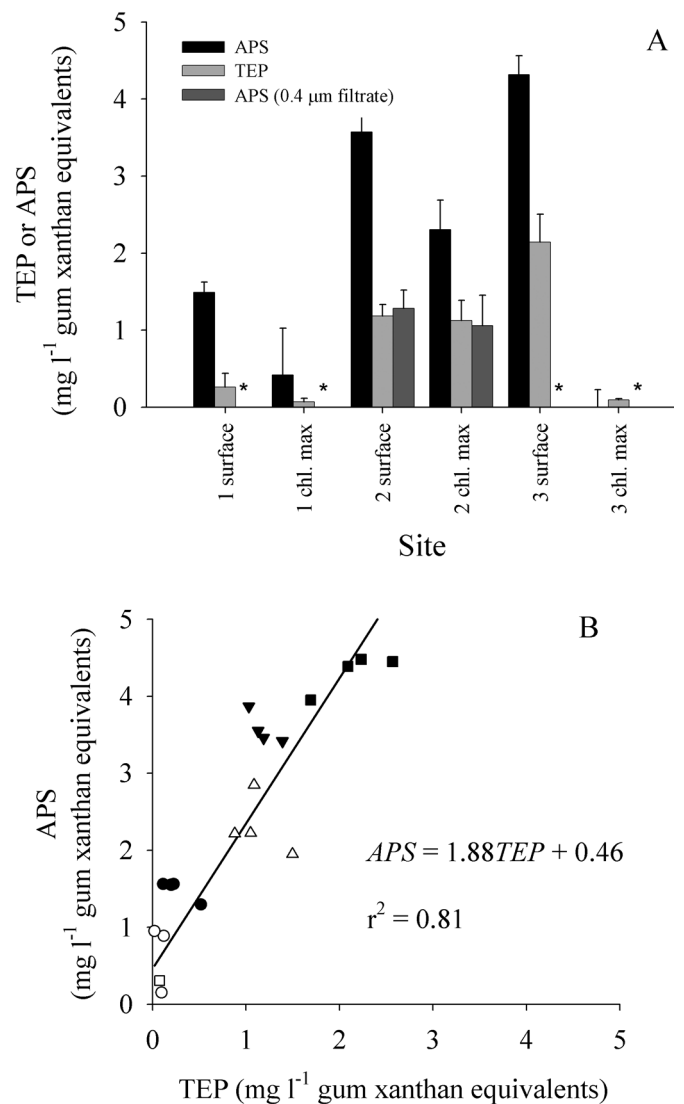
tion increased both the mean concentration of TEP and APS, the increase in concentration was not significant in either assay. This indicates that the applying the technique to whole water samples results in a substantial release of intracellular APS (probably to due the low pH of the assay). Such a release would be compounded in samples that are stored in formalin (Kiene and Linn 1999) before analysis and in marine samples, which are desalted by dialysis before analysis. Therefore, analysis of whole water samples is a measure of the total amount of APS in the water sample. To prevent ambiguity, cell disruption by sonication or mechanically should be used to ensure all the APS in the water sample is available for alcian blue staining.

**Table 1.** Locations and characteristics of 3 sites sampled on the Texas-Louisiana Shelf in the Gulf of Mexico (21–23 May 2005)

Site	Position	Depth (m)	Salinity	N ( $\mu\text{M}$ )	P ( $\mu\text{M}$ )	Particulates ( $\text{mg L}^{-1}$ )	Chlorophyll ( $\mu\text{g L}^{-1}$ )
1 (surface)	28.9529 N 91.9844 W	0	28.5	4.46	0.16		
1 (chl. max)	28.9529 N 91.9844 W	17	34.5	2.25	0.34	1.69	0.33
2 (surface)	28.8675 N 90.3719 W	0	26.5	0.77	0.21		
2 (chl. max)	28.8675 N 90.3719 W	5	30.9	0.95	0.25	2.02	1.87
3 (surface)	28.9860 N 89.5103 W	0	19.7	9.70	0.2		
3 (chl. max)	28.9860 N 89.5103 W	10	35.2	8.67	0.16	1.4	0.2

N is total inorganic nitrogen concentration ( $\text{NO}_3^- + \text{NO}_2^- + \text{NH}_4^+$ ), and P is phosphate concentration. Particulate concentrations were estimated from optical backscatter and chlorophyll concentrations from *in vivo* fluorescence.

**Application to seawater samples**—Table 1 shows the location and hydrographic data for the three sites sampled in the Gulf of Mexico. These were shallow water sites on the continental shelf with a total water depth of between 20.2 (site 2) and 21.8 m (site 1). Water temperature varied between 23.3°C and 26.3°C. The influence of outflow from the Mississippi river was apparent at site 3 (Table 1), where surface salinity was relatively low (19.7) and total inorganic nitrogen concentration was relatively high (9.36  $\mu\text{M}$ ). Fig. 8a shows that measurable concentrations of APS were detected in water from the Gulf of Mexico. Concentrations of APS (> 1000 Da) were significantly different between sites ( $P < 0.05$ ) and between depths ( $P < 0.05$ ). There were higher APS concentrations in surface waters at sites 2 ( $3.57 \pm 0.20 \text{ mg X eq. L}^{-1}$ ) and 3 ( $4.32 \pm 0.25 \text{ mg X eq. L}^{-1}$ ) compared with site 1 ( $1.49 \pm 0.13 \text{ mg X eq. L}^{-1}$ ). Concentrations of APS were greater in surface samples compared with those taken at the chlorophyll maximum, with negligible concentrations of APS measured at the chlorophyll maximum of sites 1 and 3. Filter passing APS (> 1000 Da) concentrations were measured in samples from site 2, where approximately a third to half the APS could pass through a 0.4  $\mu\text{m}$  pore sized filter. Unfortunately, this measurement was not made on samples from sites 1 and 3. Relatively high APS concentrations at the chlorophyll maximum at site 2 correspond to high relatively high chlorophyll fluorescence and particulate concentration at site 2 compared with sites 1 and 3 (Table 1). There was a positive correlation between TEP and APS concentration in the samples from the Gulf of Mexico ( $r = 0.90$ ) (Fig. 8b). Replicate samples from the same site and depth clustered together on the graph. A straight line was fitted to the data ( $r^2 = 0.81$ ) where APS concentration was 1.88 times greater than the TEP concentration with a background APS concentration of 0.46  $\text{mg X eq. L}^{-1}$ . This indicates that APS concentrations were consistently greater than TEP concentrations, an expected result as the APS pool of whole water samples includes both particulate and dissolved (> 1000 Da) APS. These data indicated that TEP formation did not occur until APS (> 1000 Da) concentration reaches approximately 0.5  $\text{mg X eq. L}^{-1}$  and illustrate how the two techniques can be used together to investigate dynamics of TEP and its precursors.



**Fig. 8.** APS (> 1000 Da) and TEP concentrations in the Gulf of Mexico. (A) APS and TEP concentrations at the surface and chlorophyll maximum at three sites. \* indicates no measurements were made. Bars show mean + standard deviation ( $n = 4$ ). (B) Relationship between APS and TEP concentrations. Points with the same symbol are replicates from the same site and depth.

The APS assay was conducted at the same pH and calibrated against the same standard as the TEP assay to facilitate comparison between the two techniques.

### Discussion

This technique enables the measurement of alcian blue staining APS in both the particulate and dissolved phase in water samples from marine and freshwater environments. The technique is simple and low cost, requiring only relatively cheap consumables and access to a spectrophotometer; therefore the technique can be used by most laboratories without any additional investment in equipment. Ramus (1977) used alcian blue (pH 2.5) to precipitate EPS produced by the soil alga *Porphyridium aeruginosum*, which was separated by centrifugation (27,000 *g*) and the absorbance of the supernatant was measured at 610 nm. Fatibello et al. (2004) used a similar technique to measure TEP in freshwater samples after concentration and colloid removal by tangential flow filtration. TEP was precipitated with alcian blue (pH 4.0) and separated by centrifugation (2,160 *g*) and absorbance of the supernatant was measured at 602 nm. Our technique is different from these methods for three reasons. Firstly, it does not require centrifugation. Secondly, by adding a dialysis step, measurements can be made of APS (> 1000 Da) in marine samples. Finally, by combining dialysis and filtration techniques, the APS pool can be fractionated by size before analysis. The method was successfully applied to cultures of protists in the laboratory and field samples from freshwater ponds and the northern Gulf of Mexico. Our samples were collected from relatively eutrophic environments; for example, TEP concentrations in the northern Gulf of Mexico (Fig. 8b) were relatively high compared with most previous measurements in the marine environment (Passow 2002a). Consequently, although the new technique may have a broad application in culture experiments, it will be limited to field samples containing relatively high concentrations of APS without an extra concentration step.

It is not necessary to complete the analysis at the time of sampling. Calibrations were not effected by formalin concentrations at 10% (v/v), an order of magnitude higher than the 1% concentration used to preserve samples in this work. Preservation of whole water samples from two ponds did not affect APS concentrations compared with unpreserved samples that were analyzed immediately. This indicates that the new technique can be used to measure APS in water samples preserved in formalin, a preservation technique which is commonly used for water samples for TEP assays with negligible effects (Passow and Alldredge 1994; Corzo et al. 2000; Thornton 2004). In samples that are dialysed, preservatives such as formalin would be removed during the dialysis process. However, the dialysis of formalin preserved samples has the potential to generate large volumes of formalin contaminated waste. The color in alcian blue containing filtrates was found to be stable for several days when stored at room temperature in the dark. This means that samples can be processed under rela-

tively primitive field conditions and measured on a spectrophotometer back in the main laboratory.

Filtration is a crucial step in the technique as it is used to separate precipitated alcian blue from the dissolved excess. Important considerations are pore size and filter composition. We have used a range of pore size of polycarbonate filters (Nuclepore, Whatman) and found that there was no difference in amount of material retained by 0.1 and 0.2  $\mu\text{m}$  pore size filters. Nuclepore filters (Whatman) produced good calibrations, however these are not as convenient to use as enclosed syringe mounted filters. SFCA syringe filters with a 0.2  $\mu\text{m}$  pore size proved to be the best choice of filter as these produced the most consistent calibration curves that were both accurate and precise. The one disadvantage of SFCA filters is that the filter retained significant alcian blue in the absence of APS, particularly at high stain concentrations. Background staining of filters also occurs with the polycarbonate filters used by Passow and Alldredge (1995) in the established colorimetric TEP assay. Secondly, the alcian blue precipitated with itself and therefore removal of the precipitates affected the color of the solution passing through the filter. Self-precipitation of alcian blue is well known (Passow and Alldredge 1995), and we filtered working stocks of alcian blue approximately once a week. For any one batch of alcian blue, these effects were constant, and although they affected the value of the intercept, they did not compromise the calibrations. Furthermore, these effects are reduced by not collecting the first few milliliters filtrate that comes through the filter. For example, in assays where 1 mL alcian blue is added to 5 mL sample, then only the last 1 mL should be collected in the cuvette for absorbance measurement.

Alcian blue precipitates even at relatively low salt concentrations. By removing salts, dialysis enables APS to be measured in seawater samples or samples from cultures grown in seawater media. A primary concern with dialysis is loss of the sample through the pores of the dialysis tubing, and it is the MWCO that will define the size of molecules that will be potentially lost. Tubing with a relatively small MWCO should be used to ensure that all the APS is retained within the dialysis tubing. We have used dialysis tubing with MWCO of 12,000–14,000 d (Visking, Medicell International), 1000 Da (Spectra/Por 7, Spectrum Laboratories), or 100 Da (Spectra/Por, Spectrum Laboratories) and found that 1000 Da tubing was the most effective. Although 100 Da tubing retained the smallest molecules, it proved unreliable as often the tubing would take up water by osmosis and the dialysis bags became turgid. Other researchers (Wustman et al. 1997; Underwood et al. 2004; Abdullahi et al. 2006; Apoya-Horton et al. 2006) have used dialysis to purify diatom EPS, and these studies do not consider loss of sample during the process as significant, even though the dialysis times were much longer than we used. However, Passow (2000) found that there was some loss of TEP precursors during dialysis using tubing with an 8000 Da MWCO. The calibrations of gum xanthan standards made up

in seawater and dialysed before analysis were similar to those of standards made up in UHP water (compare Figs. 1a and 2b), indicating no loss of gum xanthan during dialysis.

Our results show that placement into regenerated cellulose dialysis tubing may add alcian blue staining material to the sample. This occurs as soon as the sample is added to the dialysis tubing and did not significantly increase with dialysis time. This background APS is accounted for if the standards are treated in exactly the same way as the samples and dialysed. However, when APS concentrations are low, the addition of additional APS during dialysis may be a significant artefact. The source of the background APS was probably the dialysis tubing itself. As dialysis is a process that takes several hours, there is potential for microorganisms to grow and consume APS during dialysis, excrete additional APS or produce exoenzymes that degrade the walls of the dialysis tubing. To combat microbial activity, we added chloroform to the dialysis bath (Underwood et al. 2004). In addition, the dialysis tubing was stored in sodium azide before use and most of the samples placed in the dialysis tubing were preserved in formalin. Based on the extensive use of bactericidal chemicals and the instant addition of APS on addition of a sample to the dialysis tubing, it is unlikely that microbial activity contributed the additional APS. It may have been added abiotically by material sloughing or dissolving off the walls of the dialysis tubing on addition of the sample. This may be a disadvantage of using regenerated cellulose as the dialysis membrane and a possible solution to this problem would be to use a different type of material, such as polyethersulfone (PES). PES membranes are available for stirred ultrafiltration cells, although they are not available at MWCOs as low as regenerated cellulose.

Gum xanthan was chosen as the standard for the assay as this has become the standard of choice for calibrating TEP assays (Alldredge et al. 1993; Passow and Alldredge 1995; Passow 2002a; Corzo et al. 2000; Ramaiah et al. 2001; Thornton 2004) and TEP is expressed as gum xanthan equivalents. In addition to gum xanthan, the assay was also effective at measuring alginic acid, which could be used as an alternative acid polysaccharide for the calibration. The slope of calibrations with gum xanthan and alginic acid were remarkably similar. Hung et al. (2003) proposed that alginic acid is a more appropriate standard for APS analysis than gum xanthan as it is less sensitive to sample pretreatment and more representative of APS produced by microorganisms in marine environments than gum xanthan (Hung et al. 2003; Verdugo et al. 2004). Moreover, alginic acid has an anion density of almost 1 carboxyl group per monosaccharide, enabling the conversion of alginic acid equivalents to carbon concentrations (Hung et al. 2003; Verdugo et al. 2004). Choice of standard will depend on the question that the investigator is interested in answering. For example, gum xanthan is a good choice for studies on TEP as it behaves in a similar manner to TEP (Passow and Alldredge 1995) and has been used extensively to calibrate TEP assays (Passow 2002a). Alginic acid is a more suitable standard for general studies on APS.

D-Glucose did not react with alcian blue as is not an acid polysaccharide (data not presented). D-Glucuronic contains carboxyl groups and should stain with alcian blue, however it is highly soluble and non-polymeric and did not form a precipitate that was retained on the filter with alcian blue. The staining properties of alcian blue are affected by pH and salt concentration (Scott et al. 1964; Hayat 2000). A staining pH of 2.5 was used in the proposed technique as this was used to stain TEP (Passow and Alldredge 1995) and has previously been used to stain for APS from microalgae using alcian blue (Ramus 1977; Crayton 1982).

Alcian blue staining pools of APS in the environment are TEP, dissolved APS, APS associated with the external coating of organisms, and internal pools of APS within cells. To evaluate this technique, it is necessary to consider both what exactly the technique is measuring and how it relates to the established TEP assays. The colorimetric TEP assay (Passow and Alldredge 1995) may overestimate TEP concentrations if organisms have alcian blue stainable cell coatings, though Passow (2002a) maintains that this is not usually a significant problem. Passow and Alldredge (1995) did not believe that the assay included material that had leaked from the cells and was subsequently stained by alcian blue due to the low filtering pressure and brief exposure to alcian blue solution (2 seconds). However, both impact on the filter and exposure to low pH could result in the liberation of significant alcian blue staining material and subsequent overestimation of TEP. Secondly, colored compounds other than alcian blue may be extracted from filters and contribute to absorbance at 787 nm, leading to an overestimation of TEP concentrations. This interference was apparent when TEP was extracted from filter samples of *Thalassiosira weissflogii* cultures. In freshwater containing a dense bloom of a chlorophyte, there was no significant increase in either TEP or APS concentrations when the water samples were processed using a cell disruptor, indicating that both the APS assay and established TEP assay may lead to cell rupture and that the established method can include intracellular material. Our data show that the proposed APS assay on whole water samples includes TEP, dissolved APS which is precipitated on addition of alcian blue, cell coatings, and the internal pool of APS within the cells. It therefore represents the total amount of APS within the water sample. Cell integrity is likely to be compromised by the combination of formalin addition to preserve samples (Kiene and Linn 1999), the reduction of pH to 2.5, filtration, and dialysis of marine samples.

Ecologically, it is important to be able to distinguish between internal and external pools of APS. Analysis of whole water samples does not allow this, however, the main advantage of the proposed technique is that it can be used to measure filter passing APS and therefore complement established techniques for measuring TEP. By removing cells using gentle filtration before preserving the sample, dialysis or adding alcian blue, it is possible to get an accurate measure of APS in the dissolved pool (larger than the MWCO of any dialysis tubing used in pro-

cessing the sample). This is useful as TEP is generally believed to form abiotically from biologically produced precursors (Mopper et al. 1995; Mari 1999; Passow 2000) and there is probably a dynamic relationship between TEP and dissolved APS. Our data show that a significant proportion, and in some cases the majority, of APS passes through 0.4 and 0.2  $\mu\text{m}$  pore size filters.

### Comments and recommendations

This technique provides a simple, low-cost technique for measuring acid polysaccharides in both freshwater and marine environments, and in cultures. Analysis of samples pre-filtered through 0.4 or 0.2  $\mu\text{m}$  pore size filters is the most useful application of the technique. This provides a simple assay for dissolved APS in freshwater samples and  $> 1000$  Da APS in marine samples. Furthermore, it will enable the dynamics of TEP to be investigated in relation to its dissolved precursors. TEP and dissolved APS assays can be directly coupled by measuring APS concentrations in the filtrate produced during the TEP assay. This will enable researchers to test hypotheses and explore the relative dynamics of TEP and dissolved APS. Secondly, the technique will be useful to researchers investigating the relationship between POC fluxes, APS and  $^{234}\text{Th}$  binding in the ocean.

Samples analyzed with the technique can be fractionated using filtration or dialysis to isolate different size fractions of the APS. In our work, we used dialysis tubing with a relatively low MWCO to retain as much APS as possible. However, by selecting dialysis tubing with a range of MWCO, it would be possible to determine the concentration of APS in different molecular weight classes. In locations where APS concentrations are predicted to be relatively low, such as the open ocean, ultrafiltration could be used to concentrate the sample before analysis.

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