

# Measurement of acid polysaccharides (APS) associated with microphytobenthos in salt marsh sediments

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**ABSTRACT:** Microphytobenthos (MPB) are an important component of shallow-water habitats where there is sufficient light to support benthic photosynthesis. Production of extracellular polymeric substances (EPS) is characteristic of the MPB, and acid polysaccharides (APS) are a major component of these EPS. A simple technique was developed to extract and measure APS concentrations in sediments. Water-extractable APS (wAPS) were extracted with ultra high purity water from lyophilized sediments. After centrifuging to remove the sediment, wAPS in the supernatant was precipitated with alcian blue, a cationic dye. The APS-alcian blue precipitate was removed by filtration. The remaining alcian blue in solution was determined spectrophotometrically and was inversely proportional to the concentration of wAPS in the sediment. Chlorophyll (chl) *a*, carbohydrate fractions, and wAPS were measured in sediments from 5 contrasting sites in a microtidal salt marsh in the subtropics. wAPS concentrations, expressed as gum xanthan equivalents, ranged from 152 to 6268 mg m<sup>-2</sup> in the upper 5 mm of sediment. The biomass (determined as chl *a*) of MPB at all sites was highest in the winter compared to the summer. There was a significant correlation ( $r = 0.675$ ,  $p < 0.001$ ,  $n = 107$ ) between sediment wAPS and chl *a* concentrations. The ratio of wAPS to saline-extractable EPS (sEPS) was significantly higher in summer compared to winter, indicating a seasonal change in the composition of EPS. These data show that APS are a significant and variable component of the EPS pool in salt marsh sediments.

**KEY WORDS:** Exopolymers · EPS · Microphytobenthos · Colloidal carbohydrate · Alcian blue · Subtropics · Gulf of Mexico

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## INTRODUCTION

Microphytobenthos (MPB) are restricted to aquatic benthic environments where there is sufficient light to support net photosynthesis. In the ocean, the requirement for light limits MPB to intertidal environments and the inner continental shelf. Despite this restricted range, MPB are significant primary producers due to their high productivity (MacIntyre et al. 1996). Research has shown that MPB make a substantial contribution to the autotrophic biomass, primary productivity, and biogeochemistry of estuaries (reviewed by Underwood & Kromkamp 1999). MPB biomass on temperate intertidal mudflats is usually in the range of 10s to 100s mg m<sup>-2</sup> as chlorophyll (chl) *a* (De Jonge & Colijn 1994, Santos et al. 1997, Thornton et al. 2002). Annual pro-

ductivity on temperate intertidal mudflats is in the range of 10s to 100s g C m<sup>-2</sup> yr<sup>-1</sup> (Brotas & Caterino 1995, Thornton et al. 2002). Jahnke et al. (2000) estimated that primary productivity by subtidal MPB on the temperate continental shelf of the South Atlantic Bight accounted for 38% of daily primary productivity. While most research to date has focused on temperate systems, there is a growing body of work showing that MPB are significant primary producers in the tropics, such as the sands surrounding the Great Barrier Reef (Werner et al. 2008), and in the Arctic (Glud et al. 2002).

MPB carbon can be traced through grazers, suspension feeders, and carnivores using natural stable isotope abundance (Takai et al. 2004), indicating a significant contribution to higher trophic levels. MPB provide a food resource for deposit feeders and suspen-

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sion feeders, as a substantial proportion of the MPB is regularly re-suspended in many coastal systems (De Jonge & Van Beusekom 1992, Guarini et al. 2004). Middelburg et al. (2000) used a pulse-chase  $^{13}\text{C}$  tracer approach and found that carbon was rapidly transferred (within 1 h) from MPB to bacteria and nematodes within the sediment at a site that was dominated by diatoms. Middelburg et al. (2000) concluded that MPB have a 'central role' in 'moderating carbon flow in coastal sediments.'

A significant proportion of the carbon fixed by the MPB during photosynthesis is exuded into the surrounding environment, mainly as carbohydrates (Smith & Underwood 1998, De Brouwer et al. 2002, Bellinger et al. 2005). These carbohydrates are in the form of extracellular polymeric substances (EPS) and low molecular weight fractions. The terms 'extracellular polymeric substances,' 'exopolymeric substances,' and 'exopolymers' are used interchangeably in the literature and are all abbreviated to EPS. There is considerable interest in the biogeochemistry of EPS in marine ecosystems, both in the water column and in sediments. Verdugo et al. (2004) estimated that there are 70 Pg of carbon as EPS in the ocean, a carbon pool that is considerably larger than the biomass of living organisms (1 to 2 Pg C; Falkowski et al. 2000). Given the size of this carbon pool, there is a need to understand the production and fate of EPS. Epipellic diatoms and cyanobacteria dominate the MPB, and both groups produce copious amounts of EPS (Stal 2003, Underwood & Paterson 2003). EPS production rates by MPB in sediments range from 0 to  $18.2 \mu\text{g glucose equivalents } (\mu\text{g chl } a)^{-1} \text{ h}^{-1}$ , or up to  $1000 \mu\text{g C m}^{-2} \text{ h}^{-1}$  (reviewed by Underwood & Paterson 2003). The allocation of photosynthetically fixed carbon to the production of extracellular carbohydrates ranges from 1.7 to 73%, of which 0.05 to 20% is in the form of EPS (Underwood & Paterson 2003). EPS creates a complex microhabitat at the sediment surface (Fig. 1). The carbohydrates exuded by the MPB, including EPS, are utilized by other organisms in the ecosystem, such as bacteria and animals (Middelburg et al. 2000, Underwood & Paterson 2003). EPS also affects the biostabilization of sediments (Holland et al. 1974), although the relationship between sediment EPS and the erodibility of sediments is complex (Tolhurst et al. 2006).

Protocols to extract and measure EPS and other carbohydrate fractions from lyophilized sediments have become well established over the last decade (Underwood & Paterson 2003). These methods are simple, and therefore a large number of samples can be processed relatively quickly and at low cost, which is advantageous when working in patchy environments such as coastal sediments. However, there is debate on both the chemical structure and biological function of the different fractions extracted from natural sediments

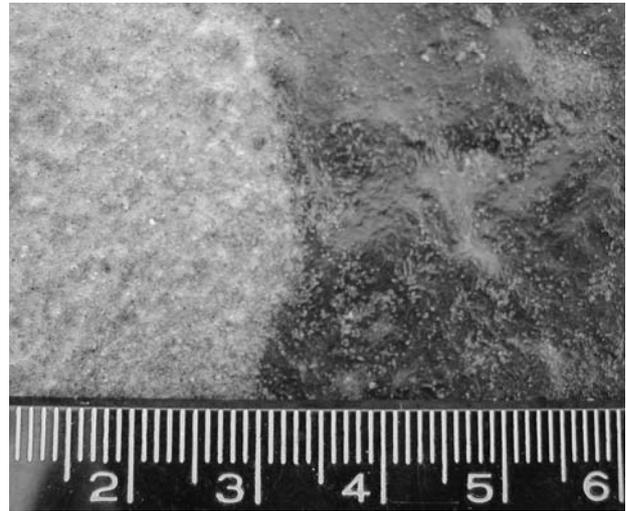


Fig. 1. A thick photosynthetic biofilm *in situ* on Galveston Island (14 March 2005). The biofilm is the dark area on the right of the image and was dominated by diatoms and cyanobacteria. Bare sand is on the left side of the image. The scale placed on the sediment surface shows centimeters

and laboratory-grown MPB cultures (Underwood & Paterson 2003, Chiovitti et al. 2004, De Brouwer & Stal 2004). Acid polysaccharides (APS) are a significant component of EPS (Hung et al. 2003, Santschi et al. 2003) and are the major component of transparent exopolymer particles (TEP) in the water column (Passow & Alldredge 1995, Passow 2002). APS are detected using alcian blue, a cationic dye that complexes with the carboxyl ( $-\text{COO}^-$ ) and half-ester sulfate ( $-\text{OSO}_3^-$ ) groups in APS, but not neutral sugars (Ramus 1977, Passow & Alldredge 1995). Consequently, alcian blue can be used to quantify APS in terms of these functional groups. Microalgae produce copious amounts of extracellular APS (Ramus 1977, Crayton 1982, Claquin et al. 2008). However, despite the high production rates of EPS by MPB, there have been no measurements of APS associated with natural MPB biofilms.

The objectives of this paper were 2-fold: firstly, to develop a simple technique that enabled the measurement of APS from sediments, and secondly, to apply the method to sediment samples from a subtropical salt marsh. This environment was chosen as it is representative of the coastal and intertidal systems that have been the focus of much of the recent work on sediment EPS. Secondly, there are limited data on EPS dynamics in subtropical coastal sediments compared to equivalent temperate environments. The development of an alcian blue method to quantify the APS fraction of sediment EPS would complement existing carbohydrate assays based on the phenol-sulfuric acid method of Dubois et al. (1956), provide information on the functional groups present in sediment EPS, and facilitate

the comparison of APS concentrations in the water column to those in the sediment. This would enable researchers to address questions on the potential coupling between sediment APS and APS pools in the water column, including TEP. Furthermore, unlike the carbohydrate method of Dubois et al. (1956), the staining of APS with alcian blue does not destroy the polymer (Ramus 1977).

## MATERIALS AND METHODS

**Field site and sample collection.** Samples were collected from a microtidal salt marsh located at East Beach (29° 20' N, 94° 44' W) on Galveston Island (Texas, USA). Sediment was collected during the winter (31 December 2006, 18 February 2007) and summer (11 June 2007, 5 September 2007) from 5 contrasting habitats within the salt marsh. Sites A (29° 20.052' N, 94° 44.295' W) and B (29° 20.042' N, 94° 44.292' W) were intertidal, unvegetated, and composed of muddy sand. Site C (29° 20.032' N, 94° 44.294' W) was supralittoral, unvegetated, and composed of sand. Site D (29° 20.021' N, 94° 44.195' W) was subtidal (water depth < 1 m), unvegetated, and composed of muddy sand. Site E (29° 20.010' N, 94° 44.324' W) was located in the vegetated zone of the high marsh and composed of sand. The sites were marked in December 2006 with a 55 cm long section of white PVC pipe (2.2 cm diameter), which was driven into the sediment so that a marker of 10 to 15 cm protruded above the sediment surface. Latitude and longitude were recorded using a GPS (Garmin Etrex Legend) with a maximum accuracy of 3 m.

Minicorers were made by cutting the end off 60 ml syringes (National Scientific). The minicorers were used to collect sediment cores of the surface 5 mm, with a surface area of 661 mm<sup>2</sup>. Six minicores were collected from Sites B to D on each sampling occasion. Minicores were taken from Site A during the winter, but not in the summer due to physical disturbance of the site by fishers in June and flooding in September. The sediment cores were collected within a 0.5 m radius of the site marker. Sediment surface temperature was measured using an electronic thermometer (VWR Scientific). Where overlying water was present, water temperature and salinity were measured. Salinity was measured using a handheld refractometer (VWR Scientific). Sediment from the core was placed in 20 ml scintillation vials on ice in a cooler and returned to the laboratory as quickly as possible, where they were frozen at -20°C.

**Laboratory preparation of the sediment.** The sediment cores were lyophilized for 24 h in the dark to remove water. The dried sediments were crushed and mixed with a mortar and pestle. Macroscopic plant

fragments were picked from the sediment samples with forceps. The lyophilized sediments were placed in scintillation vials and stored at -20°C in the dark in bags containing silica gel desiccant. All subsequent analyses were conducted with lyophilized sediments.

**Pigment analysis.** A sub-sample of sediment from each core (approximately 100 mg) was weighed into a 15 ml glass centrifuge tube. Pigments were extracted with 2 ml ice-cold methanol saturated with MgCO<sub>3</sub>. Extractions were carried out over 24 h, during which time the tubes were left in the dark at 4°C and shaken occasionally. The tubes were then centrifuged (Eppendorf centrifuge 5804R) for 15 min at 2880 × *g* and a temperature of 4°C. Chl *a* concentrations were estimated spectrophotometrically (Shimadzu 1240 UV-mini spectrophotometer) in 1 ml of extract, using a 1 cm path semi-micro cuvette, at wavelengths of 665 and 750 nm before and after acidification with 1 drop of 10% HCl. Chl *a* concentrations were calculated according to Stal et al. (1984).

**Carbohydrate analysis.** Carbohydrate fractions were extracted from the sediments and assayed according to widely used methods (Underwood et al. 1995, Smith & Underwood 1998, Hanlon et al. 2006). Saline extractions were made from 100 mg sub-samples of sediment, which were placed in 15 ml polyethylene centrifuge tube with 5 ml of 25‰ (w/v) NaCl solution. The centrifuge tubes were gently shaken on an orbital shaker for 15 min at room temperature. After centrifuging for 15 min at 2880 × *g* and a temperature of 4°C, 0.8 ml of the supernatant was removed and placed in a Pyrex boiling tube. The carbohydrates extracted by this method are generally termed 'colloidal carbohydrates' in the literature. However, this definition is inaccurate, as this fraction is composed of both low- and high-molecular weight dissolved carbohydrates, as well as colloidal material (Underwood & Paterson 2003, Haynes et al. 2007), and potentially gel particles (Verdugo et al. 2004). For accuracy, we used the term 'saline-extractable' to describe this pool. Carbohydrate concentrations in the extracts were measured using the phenol-sulfuric acid assay (Dubois et al. 1956) and were expressed as glucose equivalents using calibration curves generated with D-glucose as the standard. Phenol (0.4 ml of 5% w/v) was added to each sample followed by 2 ml of concentrated sulfuric acid. The acid was added rapidly using a dispenser bottle. The boiling tubes were agitated by hand and allowed to cool for 20 min. Absorbance was measured at 485 nm in a 1 cm path semi-micro cuvette zeroed against an ultra high purity (UHP) water blank.

EPS were extracted from approximately 300 mg sub-samples of sediment weighed into 15 centrifuge tubes. Extraction was carried out with 4 ml of 25‰ (w/v) NaCl solution for 30 min on an orbital shaker at room temperature. The samples were centrifuged for 20 min at 4500 × *g* and a temperature of 4°C. Saline-extractable

EPS (sEPS) was precipitated from the supernatant by placing 3 ml of supernatant into a 15 ml centrifuge tube with 7 ml of ice-cold reagent alcohol (i.e. 70% v/v reagent alcohol). The tubes were shaken, and the sEPS was allowed to precipitate overnight at 4°C in the dark. The next day, the sEPS was separated from the alcohol by centrifuging for 20 min at 4500 × *g* and a temperature of 4°C. The supernatant was removed from the tube with a pipette, and remaining alcohol was allowed to drain from the tube by standing the tubes upside down. Finally, the precipitate was dried in an oven for a few minutes at 85°C. The precipitate was re-suspended in 1 ml of UHP water, and 0.8 ml was assayed for carbohydrates, as described above. sEPS is the polymeric carbohydrate fraction of the saline-extractable carbohydrate. A proportion of the sEPS will be composed of APS.

Total carbohydrate was determined by weighing approximately 10 mg (summer) or 25 mg (winter) sediment into Pyrex boiling tubes. The sediment was re-suspended in 0.8 ml of UHP water and analyzed using the phenol-sulfuric acid assay described above. After completion of the reaction, the contents of the boiling tubes were placed in 15 ml polyethylene centrifuge tubes and centrifuged to remove suspended sediment prior to decanting the supernatant into cuvettes. Total carbohydrate includes both soluble saline-extractable carbohydrate and those particulate carbohydrates that are hydrolyzed by the addition of concentrated H<sub>2</sub>SO<sub>4</sub>.

**APS analysis: general protocol and method development.** The method for measuring APS in sediments is a modification of the method developed by Thornton et al. (2007), after Ramus (1977), to measure APS concentrations in marine water samples. The basis of the assay is that APS are both stained and precipitate with the cationic dye alcian blue at low pH. Alcian blue 8GX (Sigma) was made up to a concentration of 0.02% (w/v) in 0.06% acetic acid (Passow & 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 μm pore size, surfactant free cellulose acetate (SFCA) syringe filter (Nalgene; Passow & Alldredge 1995). Alcian blue solutions were stored refrigerated (4°C) in the dark.

Water-extractable APS (wAPS) were extracted from 200 mg samples of sediment. The exact weight of the sediment was recorded, the sediment was placed in 15 ml polyethylene centrifuge tubes and extracted with 10 ml of UHP water for 1 h at room temperature, while being gently mixed on a shaker. The tubes were centrifuged at 3645 × *g* at 20°C for 15 min. Supernatant (5 ml) was placed in a glass vial followed by the addition of 1 ml of alcian blue 8GX solution. The pH of the mixture was checked and adjusted to pH 2.5 (Mettler Toledo 7 easy pH meter) with concentrated acetic acid. The mixture was vigorously mixed and left at room tem-

perature for 5 min. The APS-alcian blue precipitate was removed from the sample by filtration through a 0.2 μm pore size SFCA syringe filter (Nalgene). The color of the filtrate was inversely proportional to the concentration of APS in the sample (Thornton et al. 2007). The final 1 ml of filtrate (6 ml total) was collected directly into a disposable 1 cm path semi-micro polystyrene cuvette, and absorbance was measured against UHP water using a spectrophotometer (Shimadzu UV-mini 1240) at 610 nm (Thornton et al. 2007). The absorbance at 610 nm was also measured for 1 ml of supernatant from the extract to check that there was no significant interference due to suspended particles or the extraction of colored substances from the sediment.

The assay was calibrated against gum xanthan (Sigma) in UHP water, and the data are expressed as gum xanthan equivalents (Thornton et al. 2007). Gum xanthan (0.1 g) was mixed with 100 ml of UHP water to produce a stock of 1 g l<sup>-1</sup>. Gum xanthan does not fully dissolve in water; it forms gel particles analogous to TEP (Passow & Alldredge 1995). After mixing on a stirrer for 2 h, the gum xanthan suspension was homogenized using a manual tissue homogenizer (Kontes Glass Company). Standards were made by dilution with UHP water. A standard curve over the range 0 to 20 mg l<sup>-1</sup> gum xanthan was made for each analysis.

Spike experiments were conducted to test 2 hypotheses. Firstly, spiking the sediment with APS should result in an increase in the amount of wAPS recovered from the sediment that corresponds to the amount of APS added. Secondly, there should be a linear relationship between the amount of sediment in the extraction and the amount of wAPS extracted. Spike experiments were conducted using sediment collected from Site A on 18 February. Four replicate series of sediment weights (0, 50, 100, 150, 200, and 250 mg) were weighed out into 15 ml centrifuge tubes. No additions were made to the first series of sediment weights. APS was added to the second and third series of sediment weights in the form of 50 and 100 μl of gum xanthan (1 g l<sup>-1</sup>), resulting in a spike of 50 and 100 μg of APS, respectively. Glucose (100 μl of 1 g l<sup>-1</sup>) was added to the fourth set of sediment weights, resulting in a spike of 100 μg. The volume of liquid in all extractions was made up to a total volume of 10 ml with UHP, and the wAPS was extracted from the sediments as described above. Samples were analyzed immediately for APS concentrations. A 1 ml sample of supernatant was placed in microcentrifuge tubes and frozen at -20°C for analysis of water-extractable carbohydrates.

Serial extractions from the same sediment were conducted to determine whether the majority of wAPS were recovered from the sediment on the first extraction. The general wAPS analysis protocol described above was carried out for 5 replicate samples collected

at Site D on 18 February 2007. At the end of the wAPS extraction, 5 ml of supernatant were placed in a glass vial and immediately analyzed for wAPS, and a 1 ml sample was placed in a cuvette to check for interference at 610 nm. A 1 ml sample of supernatant was frozen for analysis of water-extractable carbohydrates. The remainder of the supernatant was removed from the centrifuge tube, and the sediment was re-suspended in 10 ml of UHP water for a second wAPS extraction. Samples were once again analyzed for wAPS and collected for water-extractable carbohydrate analysis. This process was repeated to produce a total of 4 wAPS extractions from the same sediment. The water-extractable carbohydrate samples were thawed and analyzed in 1 batch according to the protocol described above (Dubois et al. 1956).

Potentially, wAPS will be composed of dissolved APS (dAPS), colloids, and gel particles. Five samples collected from 3 contrasting sites (A, C, and D) in February 2007 were re-analyzed to determine whether the wAPS can be fractionated into a dissolved pool and a pool that is retained on a filter. A second purpose of this test was to verify that dissolved APS precipitates with alcian blue (Thornton et al. 2007). APS was extracted as described above. The extract was centrifuged to remove sediments and the supernatant was passed through a 0.2  $\mu\text{m}$  pore size SFCA syringe filter (Nalgene) prior to the addition of alcian blue. The filtrate was analyzed using the standard procedure.

**APS analysis: field samples.** wAPS was extracted from approximately 200 mg of sediment using the protocol described above. All 6 replicate samples from each site on each sampling occasion were analyzed.

**Data analysis.** Data were analyzed using Sigmasat 3.1. (Systat Software). Analysis of variance (ANOVA) was conducted on data that met the assumptions of normality and equality of variance. Data that did not meet these assumptions were log ( $n+1$ ) transformed before analysis or a non-parametric ANOVA was carried out on ranks (Kruskal-Wallis ANOVA). Pairwise comparisons were made using post hoc tests. Dunn's pairwise comparison was used for datasets in which there were missing data, and the Holm-Sidak method was used to make pairwise comparisons in data where group sizes were equal. Correlation analysis was carried out using the Pearson product moment correlation.

## RESULTS

### APS method development

There was a linear relationship between sediment weight in the extraction and the amount of wAPS recovered (Fig. 2). Fig. 2A shows wAPS concentrations

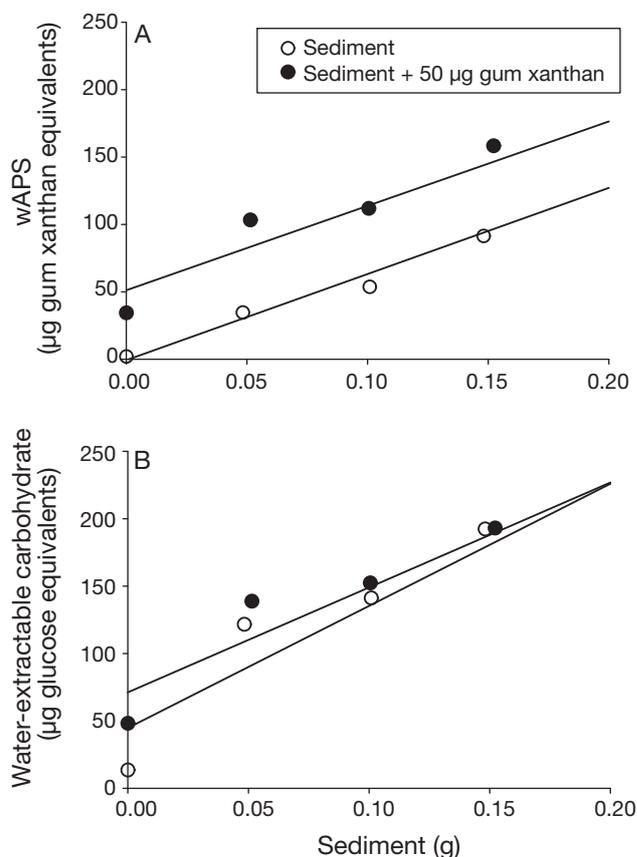


Fig. 2. Relationship between (A) water-extractable acid polysaccharide (wAPS) content and (B) water-extractable carbohydrate content and sediment weight in control sediment and sediment spiked with 50  $\mu\text{g}$  gum xanthan. Coefficients of the linear regressions fitted through these data are shown in Table 1

for sediment alone and sediment spiked with 50  $\mu\text{g}$  of gum xanthan. The slopes of both curves are very similar. The y-axis intercept of the sediment to which 50  $\mu\text{g}$  of gum xanthan was added was 51  $\mu\text{g}$  gum xanthan equivalents, compared to  $-1$   $\mu\text{g}$  gum xanthan equivalents for sediment to which no gum xanthan was added (Table 1). Fig. 2B shows water-extractable carbohydrates in the same samples. The y-intercept of the regression line for sediment samples spiked with 50  $\mu\text{g}$  gum xanthan was higher (71  $\mu\text{g}$  glucose equivalents) than that of the sediment to which no gum xanthan was added (45  $\mu\text{g}$  glucose equivalents). There was also a difference in the slope of the 2 fits (Fig. 2B). Data for wAPS extracted from 0.25 g of sediment are not shown in Fig. 2 and were not used to calculate the regressions in Table 1. These samples, when spiked with gum xanthan, were at the upper detection limit of the assay (see 'Discussion'). Moreover, samples to which a 100  $\mu\text{g}$  spike of gum xanthan was added were above the detection limits of the wAPS assay beyond a sediment weight of 0.0 g. Consequently, there was no significant

Table 1. Results of the spike experiment. Coefficients calculated from linear regressions between sediment weight and concentrations of water-extractable acid polysaccharides (wAPS) or concentrations of water-extractable carbohydrate. NS: the regression was not a significant ( $p > 0.05$ ) linear fit to the data. Coefficients are rounded to the nearest integer. Each regression was fitted to 5 data points over the range of 0 to 0.2 g sediment

Treatment	wAPS		Water-extractable carbohydrate	
	Intercept	Slope	Intercept	Slope
Control	-1 <sup>a</sup>	638	45 <sup>a</sup>	906
+50 $\mu\text{g}$ gum xanthan	51	626	71	779
+100 $\mu\text{g}$ gum xanthan	NS	NS	130	926
+100 $\mu\text{g}$ glucose	-21 <sup>a</sup>	848	111	1269

<sup>a</sup>The intercept of the regression was not significantly different from 0

relationship between sediment weight and wAPS in samples spiked with 100  $\mu\text{g}$  gum xanthan (Table 1). Table 1 shows that the addition of 100  $\mu\text{g}$  glucose to the sediment produced a negative intercept of -21  $\mu\text{g}$  gum xanthan; however, the  $y$ -intercept of the regression was not significantly different from 0 ( $p = 0.145$ ).

Fig. 3 shows the effect of multiple extractions from the sediment sample. The extraction of wAPS and

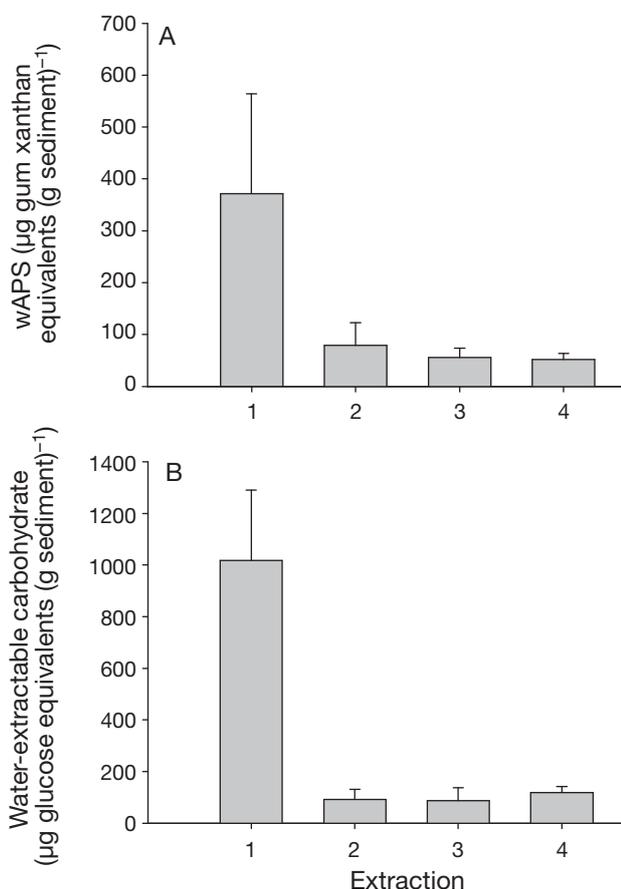


Fig. 3. Effect of repeated extractions from the same sediment samples. (A) Water-extractable acid polysaccharide (wAPS) concentrations. (B) Water-extractable carbohydrate concentrations. Bars show mean + SD ( $n = 5$ )

water-extractable carbohydrates showed a similar pattern, with the first extraction resulting in the greatest yield of both wAPS and water-extractable carbohydrates. There was a significant difference ( $F_{3,16} = 13.4$ ,  $p < 0.001$ ) between the concentrations of wAPS recovered during repeat extractions. Post hoc analysis showed that extraction 1 was significantly different from extractions 2, 3, and 4 ( $p < 0.05$ ). There was no significant difference in wAPS concentrations among extractions 2, 3, and 4 (Fig. 3A). There was a significant difference

in water-extractable carbohydrate concentrations ( $F_{3,16} = 26.4$ ,  $p < 0.001$ ), and post hoc analysis showed the same pattern as observed for wAPS. wAPS concentration was  $372 \pm 192$  (mean  $\pm$  SD) for extraction 1, compared to a combined mean of  $62 \pm 29$  for extractions 2 to 4. Water-extractable carbohydrate concentration for extraction 1 was  $1017 \pm 273$ , compared to a combined mean of  $100 \pm 38$  for extractions 2 to 4.

Fig. 4 shows that the wAPS extracted from sediments was composed of dAPS that passed through a 0.2  $\mu\text{m}$  pore sized filter and material that was retained on a filter prior to precipitation with alcian blue. There was a consistent pattern at each site, with higher wAPS than dAPS concentrations. Two-way ANOVA indicated a significant difference between sites ( $F_{2,24} = 29.0$ ,  $p < 0.001$ ) and between filtered and unfiltered samples ( $F_{1,24} = 15.0$ ,  $p < 0.001$ ). However, there was no significant interaction between site and whether the sample was filtered ( $F_{2,24} = 1.3$ ,  $p = 0.290$ ).

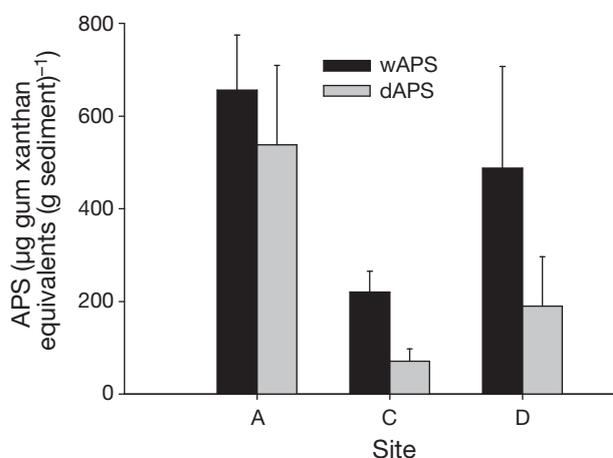


Fig. 4. Effect of filtering the acid polysaccharide (APS) extract through a 0.2  $\mu\text{m}$  pore size filter before analysis. Samples were collected in February 2007. Extracts for water-extractable APS (wAPS) analysis were not filtered prior to analysis. Extracts for dissolved APS (dAPS) were filtered prior to analysis. Bars show mean + SD ( $n = 5$ )

### Field samples

Winter sediment surface temperatures were  $16.1 \pm 1.2$  and  $15.1 \pm 2.2^\circ\text{C}$  (mean  $\pm$  SD) in December 2006 and February 2007, respectively. Summer sediment surface temperatures were  $36 \pm 1.2$  and  $31.7 \pm 0.4^\circ\text{C}$  in June 2007 and September 2007, respectively. ANOVA on ranks showed a significant difference ( $p = 0.003$ ) between summer and winter temperatures at the sites,

but not within each season. Salinity of the water overlying Site D varied between 20 (September 2007) and 26 (December 2006).

Concentrations of chl *a*, wAPS, and carbohydrate fractions are shown on an areal basis in Fig. 5. Total sediment carbohydrate (Fig. 5A) concentrations were approximately 5 times those of saline-extractable carbohydrates (Fig. 5B) and an order of magnitude greater than sEPS (Fig. 5C). ANOVA on ranks showed a signif-

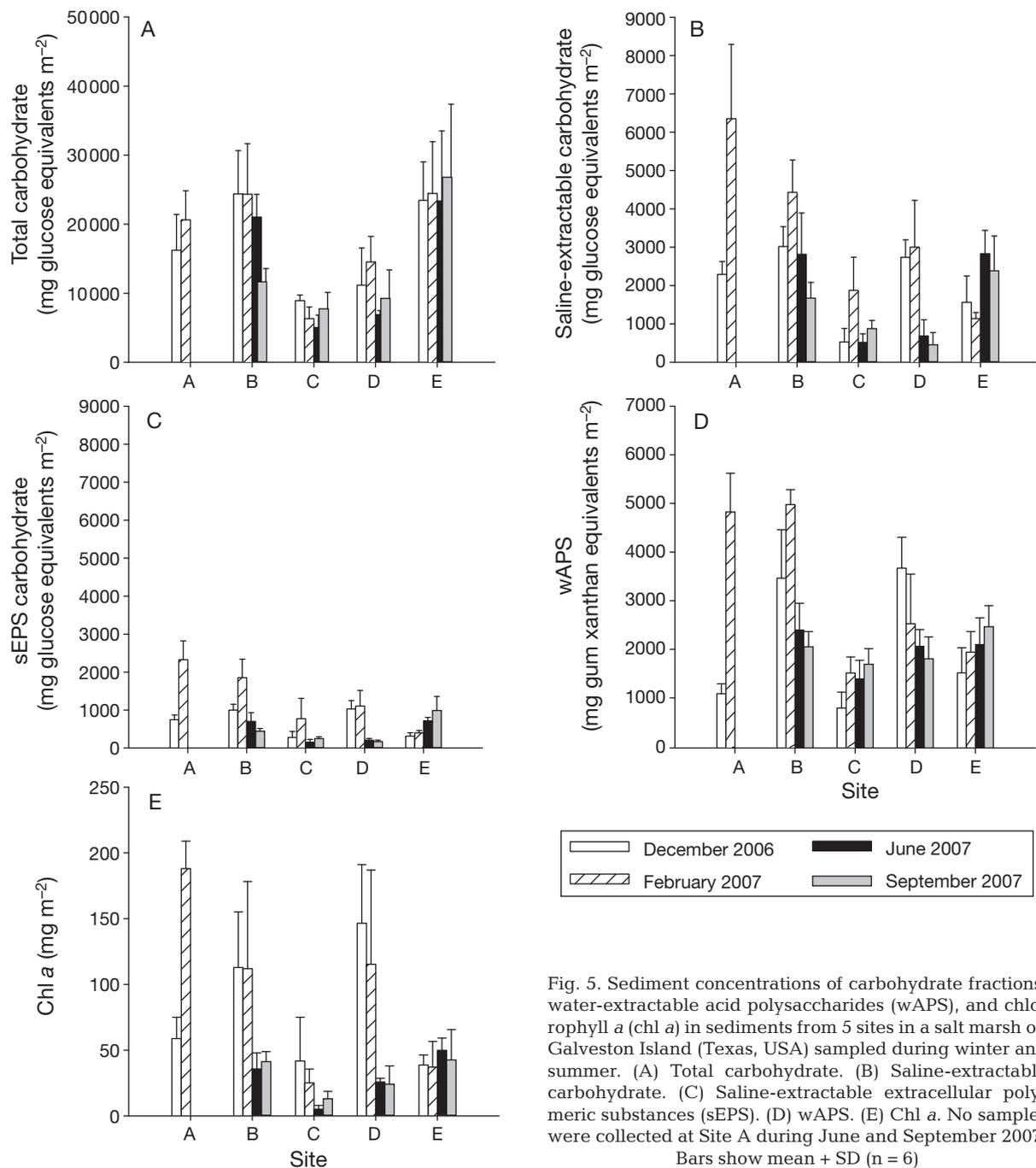


Fig. 5. Sediment concentrations of carbohydrate fractions, water-extractable acid polysaccharides (wAPS), and chlorophyll *a* (chl *a*) in sediments from 5 sites in a salt marsh on Galveston Island (Texas, USA) sampled during winter and summer. (A) Total carbohydrate. (B) Saline-extractable carbohydrate. (C) Saline-extractable extracellular polymeric substances (sEPS). (D) wAPS. (E) Chl *a*. No samples were collected at Site A during June and September 2007. Bars show mean  $\pm$  SD (n = 6)

ificant ( $p < 0.05$ ) difference in each carbohydrate fraction with both time and between sites. Total carbohydrate concentrations in the upper 5 mm of sediment varied from 1011 to 38 170  $\text{mg m}^{-2}$  as glucose equivalents. Total carbohydrate concentrations were generally low at Site C and high at Site E in the vegetated marsh (Fig. 5A). Saline-extractable carbohydrates were higher during the winter than in the summer at Sites B, C, and D. The pattern was reversed for Site E (Fig. 5B). This pattern was also observed in sEPS concentrations (Fig. 5C). wAPS concentrations varied from 152 to 6268  $\text{mg m}^{-2}$  (Fig. 5D). Patterns of wAPS concentrations were similar to those of the saline-extractable carbohydrate and sEPS fractions, with generally higher concentrations observed in the winter at Sites B and D compared to summer, and low concentrations throughout the year at Site C (Fig. 5D). Chl *a* concentrations ranged from 0 to 228  $\text{mg m}^{-2}$ . Mean chl *a* concentrations at Site E were relatively constant throughout the year, from  $37 \pm 19$  to  $50 \pm 9$   $\text{mg m}^{-2}$  (mean  $\pm$  SD) in February and June, respectively. The highest mean concentration of chl *a* ( $188 \pm 21$   $\text{mg m}^{-2}$ ) was observed in February 2007 at Site A. A distinct seasonality was observed at Sites B, C, and D; winter chl *a* concentrations were generally more than twice those measured during the summer.

The data from each site and each sampling occasion were pooled to produce a large dataset in which relationships between the different variables were determined. Table 2 shows significant positive correlations between wAPS concentrations, chl *a*, and the carbohydrate fractions measured. wAPS was strongly correlated with chl *a* (0.675), sEPS (0.699), and saline-extractable carbohydrates (0.683). Correlations between total carbohydrate and the other variables were relatively weak; the correlation between wAPS and total carbohydrate was 0.333, and only 0.192 between total carbohydrate and chl *a*. Scatterplots show the relationship between MPB biomass (as chl *a*) and

Table 2. Pearson product moment correlation coefficients between concentrations of sediment water-extractable acid polysaccharides (wAPS), chl *a*, total carbohydrates, saline-extractable carbohydrates, and saline-extractable extracellular polymeric substances (sEPS) for all sites on all sampling occasions. All correlation coefficients were significant ( $p < 0.05$ ;  $n = 107$  for wAPS;  $n = 108$  for the carbohydrate fractions and chl *a*)

	wAPS	sEPS	Saline-extractable	Total
Chl <i>a</i>	0.675	0.713	0.692	0.192
wAPS		0.699	0.683	0.333
sEPS			0.875	0.350
Saline-extractable				0.425

wAPS or the various carbohydrate fractions extracted from the sediment (Fig. 6). There were significant linear regressions between chl *a* and all other variables, including wAPS. Generally, when data from a single site were considered in isolation, correlations between total carbohydrates and the other carbohydrate fractions, chl *a*, or wAPS were not significant (Tables 3, 4). At Site D, there was a strong positive correlation between chl *a*, wAPS, and carbohydrate fractions other than total (Table 3). At Site C, where the biomass of MPB was consistently low, correlations between variables were generally not significant (Table 4). There was a significant negative correlation ( $-0.465$ ) between chl *a* and wAPS at Site C.

Fig. 7 shows the relationship between MPB biomass as chl *a* and wAPS concentrations for each site on each sampling occasion and enables the comparison of changes in the relative concentrations of wAPS and MPB biomass between sites and over time. The variability of both wAPS and chl *a* concentrations between sites was much greater during the winter (Fig. 7A, B) compared to summer (Fig. 7C, D). Furthermore, error bars show that the variation between samples taken from the same site was much greater in the winter months compared to the summer. This was particularly the case for Sites B and D and indicates that the biofilms at these sites were relatively patchy. Site C was consistently a low MPB biomass and low wAPS

Table 3. Pearson product moment correlation coefficients between sediment concentrations of water-extractable acid polysaccharides (wAPS), chl *a*, total carbohydrates, saline-extractable carbohydrates, and saline-extractable extracellular polymeric substances (sEPS) for Site D. NS: non-significant correlation ( $p > 0.05$ );  $n = 24$

	wAPS	sEPS	Saline-extractable	Total
Chl <i>a</i>	0.762	0.871	0.866	NS
wAPS		0.761	0.779	NS
sEPS			0.970	0.415
Saline-extractable				NS

Table 4. Pearson product moment correlation coefficients between sediment concentrations of water-extractable acid polysaccharides (wAPS), chl *a*, total carbohydrates, saline-extractable carbohydrates, and saline-extractable extracellular polymeric substances (sEPS) for Site C. NS: non-significant correlation ( $p > 0.05$ );  $n = 24$

	wAPS	sEPS	Saline-extractable	Total
Chl <i>a</i>	$-0.465$	NS	NS	NS
wAPS		NS	NS	NS
sEPS			0.488	NS
Saline-extractable				NS

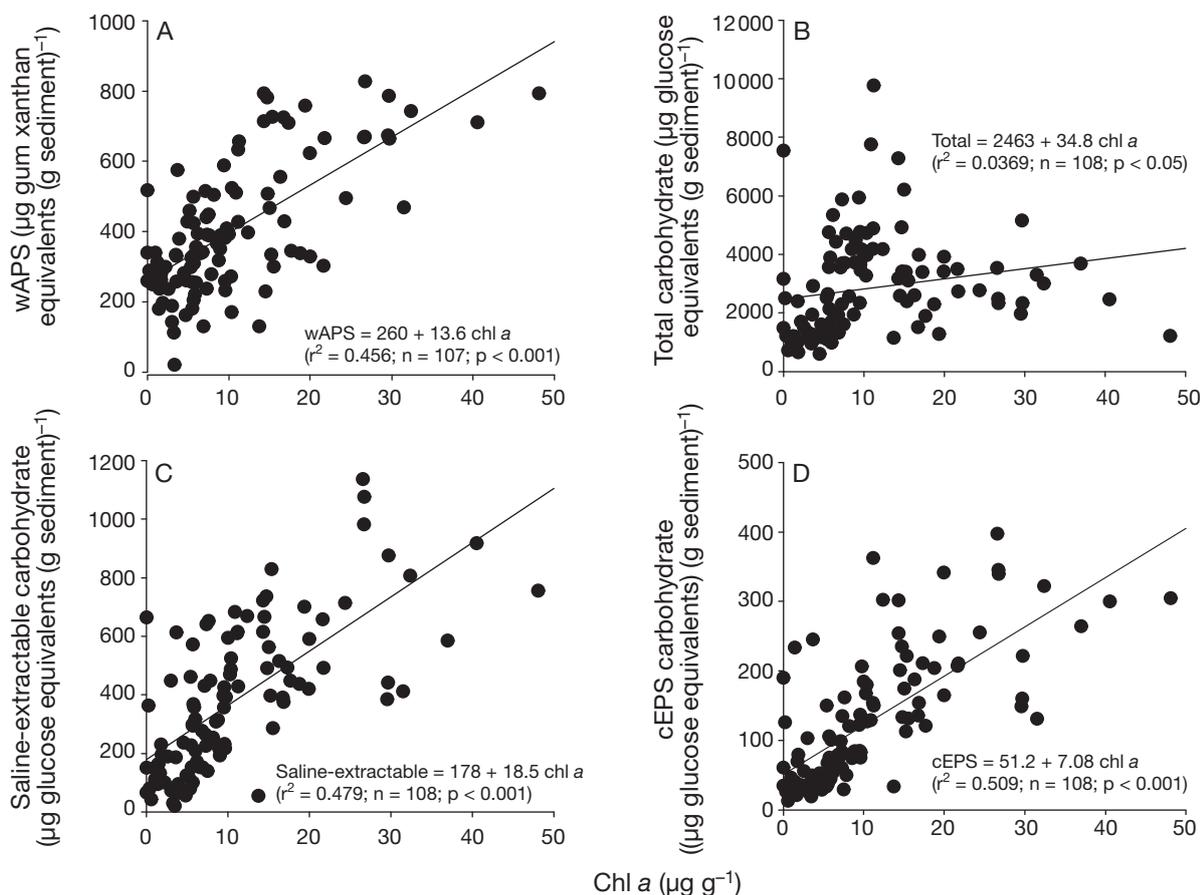


Fig. 6. Relationship between concentrations of chlorophyll *a* (chl *a*) and carbohydrates or water-extractable acid polysaccharides (wAPS) in salt marsh sediments. The data were combined for all sites on all sampling occasions. (A) wAPS ( $n = 107$ ). (B) Total carbohydrate ( $n = 108$ ). (C) Saline-extractable carbohydrate ( $n = 108$ ). (D) Saline-extractable extracellular polymeric substances (sEPS) ( $n = 108$ ). Weights are for dry sediment

site. Site E had relatively low concentrations of wAPS and chl *a* during the winter, although it was a high wAPS site in September.

The wAPS:sEPS ratio was not constant (Table 5). Two-way ANOVA showed that there was a significant difference in this ratio between sites ( $F_{4,99} = 6.47$ ,  $p < 0.001$ ) and between months ( $F_{3,99} = 10.29$ ,  $p < 0.001$ ). The interaction between site and season was not tested due to missing data. Post hoc multiple comparisons showed a seasonal difference in the wAPS:sEPS ratio. There was no significant difference ( $p > 0.05$ ) in the ratio between samples taken in either the 2 winter months (December 2006 and February 2007) or between the 2 summer months (June and September 2007). However, there was a significant difference ( $p < 0.05$ ) in the ratio between samples taken in different seasons. Site D was significantly different ( $p < 0.05$ ) from all sites, except C. Site A was significantly different from Site C; however, all other sites were not significantly different from one another.

## DISCUSSION

### Method development

The precipitation of EPS with alcian blue was first used by Ramus (1977) as a method to measure APS concentrations in cultures of a soil microalga in laboratory culture. Thornton et al. (2007) modified the method in 2 ways to enable the measurement of APS in seawater samples. Firstly, the need for high-speed centrifugation (Ramus 1977) was removed from the protocol, as the APS-alcian blue precipitate was recovered from the sample by filtration. In the method presented here, centrifuging at a relatively low speed enabled separation of the sediment from the APS, which remained in solution or suspension. A sample of supernatant was then removed and stained with alcian blue in the absence of sediment, which could potentially adsorb alcian blue. Secondly, Thornton et al. (2007) added a dialysis step to de-salt seawater samples, as

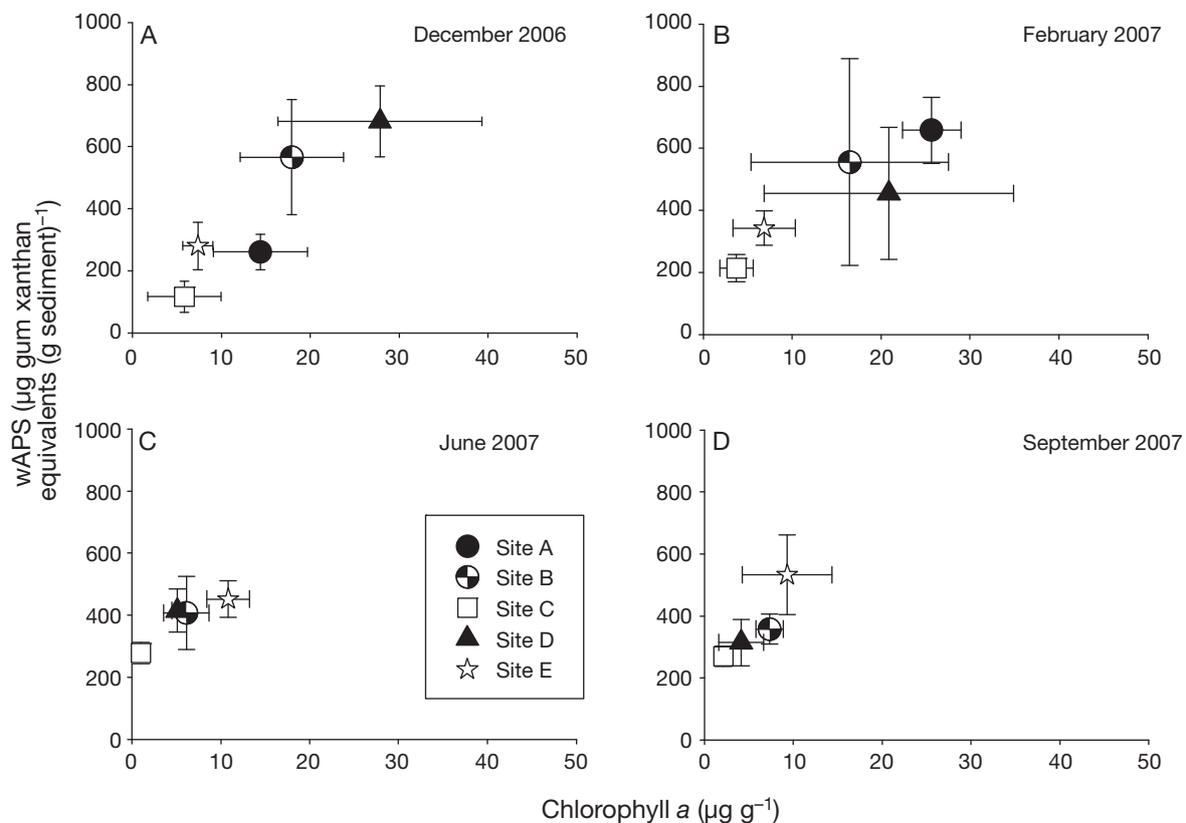


Fig. 7. Relationship between sediment concentrations of chl *a* and water-extractable acid polysaccharides (wAPS) in salt marsh sediments at 5 sites. (A) December 2006. (B) February 2007. (C) June 2007. (D) September 2007. Each point shows the mean  $\pm$  SD ( $n = 6$ ). Weights are for dry sediment

Table 5. Ratio between concentrations of water-extractable acid polysaccharides (wAPS,  $\mu\text{g}$   $\text{g}^{-1}$ ) and saline-extractable extracellular polymeric substances (sEPS,  $\mu\text{g}$   $\text{g}^{-1}$ ) in sediments from 5 sites in a salt marsh on Galveston Island (Texas, USA) sampled during winter and summer. wAPS was measured as gum xanthan equivalents, and sEPS was measured as glucose equivalents. Values are means  $\pm$  SD ( $n = 6$ )

Site	December 2006	February 2007	June 2007	September 2007
A	$1.51 \pm 0.41$	$2.14 \pm 0.49$	no data	no data
B	$3.46 \pm 0.80$	$2.77 \pm 0.70$	$3.74 \pm 1.19$	$4.61 \pm 0.71$
C	$3.44 \pm 1.87$	$2.61 \pm 1.53$	$10.81 \pm 4.52$	$6.99 \pm 2.12$
D	$3.71 \pm 1.03$	$2.29 \pm 0.38$	$10.54 \pm 2.25$	$11.15 \pm 4.25$
E	$4.97 \pm 1.41$	$4.86 \pm 0.55$	$3.01 \pm 0.83$	$2.82 \pm 1.22$

alcian blue does not stain APS in salt solutions at the concentration of seawater. A potential interfering factor with the method presented could be salts within porewater, which will remain in the sediment sample after lyophilization. However, a large volume (10 ml) of UHP water was added to a relatively small sample of sediment (0.2 g), which diluted the small amount of salts in the sample. This was verified by measurement

of the salinity of the extractions with a handheld refractometer, which showed they were not significantly different from 0. The salinity of the overlying water at East Beach was generally  $<25$ . Interference from salts in the lyophilized sediments could potentially affect the staining of APS with alcian blue at salinities higher than observed in this work. Under those circumstances, dialysis could be used to desalt the extract before the addition of alcian blue.

It was expected that the amount of wAPS extracted would increase with increasing sediment weight. There was a linear relationship between wAPS recovered and sediment weight. At high sediment weights, the amount of wAPS extracted from the sediment was sufficient to precipitate all the alcian blue added to the sample. Therefore, wAPS concentrations calculated from near 0 absorbance values should be viewed with caution as these are likely to be underestimates of the true amount of wAPS. This was clearly observed in samples spiked with 100  $\mu\text{g}$  of gum xanthan, in which 172  $\mu\text{g}$  of wAPS (gum xanthan equivalents) were consistently recovered over a range of sediment weights from 0.15 to 0.25 g. This limitation may be overcome by extracting

from less sediment or by adding more alcian blue. It was hypothesized that the addition of a consistent APS spike to the sediment would increase the intercept of the relationship between sediment weight and wAPS, but not the slope. This was the case with the addition of a 50 µg gum xanthan spike (Table 1), which increased the intercept from -1 to 51. These data show that there was a complete recovery of the spike from the sediment, indicating that wAPS (or at least gum xanthan) does not become significantly adsorbed to the surface of the sediment grains and that it can be separated from the sediment using UHP water. The addition of a 100 µg glucose spike did not affect the intercept of the relationship between sediment weight and wAPS, as it was not significantly different from 0. This was expected, as glucose should not be detected by the wAPS assay because it does not contain the carboxyl and half-ester sulfate groups that bind to alcian blue at the staining pH of 2.5. The glucose spike was detected using the phenol-sulfuric acid assay for carbohydrates, indicating that it was recovered from the sediment during the extractions. Serial extraction from the same sediment sample indicated that the majority of wAPS was extracted in the first hour and that the pattern of wAPS extraction was the same as that for water-extractable carbohydrates. Therefore, a single extraction of wAPS was equivalent to a single extraction of water-extractable carbohydrates.

Dialysis membranes are available with molecular weight cut-offs over a wide range of molecule sizes. Potentially, dialysis could be used as a tool to size-fractionate the APS (Thornton et al. 2007) extracted from the sediment. In this work, filtration was used to separate the dAPS from the bulk extract. There was consistently less APS in the dAPS fraction, indicating that a fraction of the APS were extracted as gel particles, or at least formed particles during the extraction process. This is consistent with the observation that EPS make a major structural contribution to photosynthetic biofilms, as observed in low temperature scanning electron micrographs (Paterson 1989). As gel particles have a similar density to water, they are not removed from the supernatant by centrifuging. An alternative explanation is that the filtration step removed cells that were not removed from the supernatant by centrifuging, although it is unlikely that diatoms and chain-forming cyanobacteria were left in suspension. Cells were not observed in the supernatants of extracts made by Underwood et al. (1995), who centrifuged at a lower force. Passing the sample through a 0.2 µm filter before the addition of alcian blue verified that APS was being precipitated by the addition of alcian blue.

Carbohydrates may be extracted from both fresh and lyophilized sediments (Underwood & Paterson 2003). Factors affecting the amount and composition of the

carbohydrate extracted include temperature, extraction time, and extraction medium (Underwood & Paterson 2003). The sediments for sEPS and wAPS used in this study were lyophilized before extraction of these polymeric fractions. This has been the standard approach for over a decade, as lyophilization enables greater gravimetric accuracy, which is important in ecological studies where carbohydrate concentrations are expressed per unit sediment (Underwood & Paterson 2003). However, lyophilization may change carbohydrate yield and polymer properties (Underwood et al. 1995, Underwood & Paterson 2003). One possible contributing factor to these differences may be the degree to which intracellular carbohydrates contaminate EPS extracted from sediment prepared in different ways. Consequently, it should be noted that the wAPS may contain some intra- as well as extracellular APS.

Saline (25%) is commonly used to extract sEPS from sediments (Underwood et al. 1995, Bellinger et al. 2005, Hanlon et al. 2006). However, saline cannot be used to extract APS (unless a dialysis step is added), due to salt interference. This operational difference means that the extractions for carbohydrates and APS may not contain exactly the same concentrations and composition of carbohydrates. Underwood et al. (1995) found that increasing the salinity of the extraction solution had no effect on the yield of carbohydrate extracted, but proposed using saline rather than pure water as the salts caused fine sediment particles to flocculate, thus making them easier to remove from the extract by centrifugation. This suggests that the wAPS extractions are comparable to saline extractions for sEPS and saline-extractable carbohydrates. Extractions made from sediments with water or saline at room temperature contain EPS (including APS) that are generally considered to be unbound or loosely bound to MPB cells (Underwood & Paterson 2003). However, a significant proportion of the total polysaccharides may be in the form of EPS that is bound to the surface of MPB cells or located within the cells as storage products (Underwood & Paterson 2003). Serial extraction procedures have been developed to extract these fractions from sediments. The initial extraction at room temperature is followed by a hot water (HW) extraction at 95°C and then a hot bicarbonate (HB) extraction with 0.5 M NaHCO<sub>3</sub> at 95°C. The HW extract is composed largely of glucan, an intracellular cell storage product and polymer of glucose (Bellinger et al. 2005, Abdullahi et al. 2006). The HB is predominantly associated with polymers tightly bound to the cell wall (Abdullahi et al. 2006, Hanlon et al. 2006). Analyzing these extracts with both the carbohydrate assay of Dubois et al. (1956) and for APS would give additional information on the structure of these differ-

ent fractions. For example, if the HW extract is predominantly glucan, then APS concentrations in this extract should be relatively low, even if carbohydrate concentrations are high, as glucan is not an APS.

The wAPS method gives wAPS concentrations as an equivalent of the standard, rather than an absolute amount of carbon. This limitation of the wAPS method is also true for the commonly used phenol-sulfuric acid assay, which is usually calibrated with glucose and carbohydrate concentrations are expressed as glucose equivalents. In this work, measurements of wAPS were calibrated against gum xanthan, which is the default standard for measuring TEP (Passow & Alldredge 1995, Passow 2002). However, other APS, such as alginic acid, can be used as a standard (Thornton et al. 2007). Further research on the composition of wAPS in sediments will enable researchers to determine a standard that is both similar in composition and behavior to wAPS. Hung et al. (2003) suggested that alginic acid is a more suitable standard for measuring APS as it is more representative of APS produced by microorganisms in the marine environment than gum xanthan. Moreover, alginic acid has an anion density of 1 carboxyl group per monosaccharide, and therefore the amount of alcian blue that binds to alginic acid is directly proportional to its carbon content, enabling the conversion of alginic acid equivalents to carbon (Hung et al. 2003, Verdugo et al. 2004). Similarly, if the anion density of natural APS was known, then it would be relatively straightforward to convert equivalents to carbon. However, the ratio of wAPS to sEPS was not constant *in situ* (Table 5). Changes in the wAPS to sEPS ratio will be affected by changes in the proportion of EPS in the form of APS or changes in the anion density of individual polymers within the APS pool. Variability in the anion density of polymers within the APS pool would add uncertainty to conversions of alcian blue staining to carbon based on alginic acid equivalents. The ratio of wAPS to sEPS was affected by site and time, with the highest ratios observed in the summer months, indicating that the relative importance of APS as a component of the EPS pool was greatest during summer. This may have been due to the dominance of different microorganisms at different times of the year or the production of different types of EPS in response to environmental factors such as temperature.

#### APS dynamics *in situ*

The positive correlation between MPB biomass (as chl *a*) and wAPS concentrations in the upper 5 mm of sediment suggests that the APS were produced directly by the MPB, or produced by other organisms (e.g. bacteria) utilizing photosynthates produced by

the MPB. The relationships between biomass and wAPS, saline-extractable carbohydrates, or sEPS were explained by simple linear regressions between the data (Fig. 6), indicating that all 3 carbohydrate pools were directly affected by MPB biomass. The relationship between MPB biomass and total carbohydrate was weaker than that for the other carbohydrate fractions and wAPS. Chl *a* is relatively labile and turns over relatively rapidly (hours to days) as cells grow and are eaten (Pinckney et al. 2003) or are resuspended (De Jonge & Van Beusekom 1992, Guarini et al. 2004). Much of the saline-extractable carbohydrate pool is composed of low molecular weight carbohydrates, which are also labile. A significant proportion of saline-extractable carbohydrates may be lost from the sediment by wash-away (Hanlon et al. 2006) and exoenzyme activity (Hanlon et al. 2006, Haynes et al. 2007). However, the total carbohydrate pool includes refractory carbohydrate that is not of MPB origin, such as cellulose and lignin from plants. Although macroscopic pieces of plant material were removed from the samples, they still would have contained many small fragments. Total carbohydrates were generally highest at Site E, the only site with a covering of vegetation. The linear relationship between MPB biomass and wAPS suggests that APS, like saline-extractable carbohydrates and sEPS, are relatively labile and that wAPS does not accumulate in the sediments.

When all data from all 5 sites were pooled, wAPS had the strongest positive correlation (0.699) with sEPS (Table 2). The strongest positive correlation in Table 2 was between saline-extractable carbohydrate and sEPS, which suggests that sEPS was the most significant component of the saline-extractable carbohydrate pool or that there was a relatively fixed ratio between the production of sEPS and the low molecular weight fraction of the saline-extractable carbohydrate pool. While Table 2 illustrates overall patterns in the salt marsh sediment, there were differences between sites within the salt marsh. This is illustrated by the correlations between variables at Sites D (Table 3) and C (Table 4). Generally, the relationships between different carbohydrate pools and chl *a* were not significant at Site C because MPB biomass was relatively low, and it is likely that a greater proportion of the carbohydrates were either imported into the site or produced by organisms other than MPB. The conditions at Site C were not conducive to the formation of a thick MPB biofilm as it was a supralittoral site composed of well-drained sand. In contrast, a visible MPB biofilm formed at Site D, which was subtidal and composed of muddy sand. At Site D, there were strong correlations between chl *a* and the different carbohydrate fractions, indicating that the MPB were the primary source of carbohydrates at this site.

MPB biomass was generally higher in the winter compared to summer months. Winter blooms of MPB on the sediments of the East Beach salt marsh have been observed previously (D. Thornton unpubl. data), and this may be the general seasonal pattern at this location. It is likely that high temperature, high irradiance, and dehydration (high on the shore) play a role in affecting the lower summer biomass. Sediment chl *a* concentrations were 0 to 228 mg m<sup>-2</sup>, which is similar to the range reported for intertidal sediments in many other systems (Underwood & Kromkamp 1999). As most previous research has focused on temperate systems, our data make a valuable contribution to our limited knowledge of MPB dynamics in the intertidal of subtropical systems.

MPB biomass was recently measured in Galveston Bay by Pinckney & Lee (2008), who found a maximum chl *a* concentration of 17.5 mg m<sup>-2</sup>, with a mean of 4.2 ± 2.9 (± SD) mg m<sup>-2</sup>. We measured chl *a* concentrations generally 1 or 2 orders of magnitude greater than those of Pinckney & Lee (2008). The major difference between our work and that of Pinckney & Lee (2008) is that they sampled the subtidal central axis of the estuary, with a depth range of 2.2 to 3.6 m. Light attenuation was great in Galveston Bay; on average the 1% irradiance level was at 2 m depth, indicating that light limited the accumulation of MPB biomass at the sites surveyed by Pinckney & Lee (2008). Pinckney & Lee (2008) hypothesized that MPB biomass would be substantially higher at sites <2 m deep, and this is confirmed by our data from Site D (subtidal; <1 m deep). A secondary factor contributing to differences may be the techniques used in these 2 studies to extract and measure photopigments. While it is acknowledged that high performance liquid chromatography (HPLC; used by Pinckney & Lee 2008) is more reliable, there is generally a good agreement between HPLC and spectrophotometric methods for chl *a* extractions from sediments (Brotas et al. 2007).

These data indicate that APS are a significant component of photosynthetic biofilms at this subtropical location and in a variety of contrasting microhabitats. wAPS were found in significant concentrations in the high vegetated marsh where *Salicornia* was the dominant plant (Site E), in bare supralittoral sediment that was regularly re-worked by crabs (Site C), on intertidal sediments (Sites A and B), and at a shallow water subtidal site (Site D). The extraction of wAPS was comparable to methods used to extract the carbohydrate fractions. Both carbohydrate fractions and wAPS were extracted from the same lyophilized sediment sample. The measurement of wAPS was simple, and the total analysis time was less than that for sEPS, as the precipitation step took minutes rather than hours.

The measurement of sEPS from sediments is simple and enables researchers to determine the concentrations of sEPS in time and space. However, the technique does not reveal any information about the structure of sEPS except that it contains polysaccharide. The wAPS technique provides additional compositional information, as alcian blue is specific for acid (i.e. carboxylated and half-ester sulfated) polysaccharides at the staining pH of 2.5. When used in conjunction with other carbohydrate assays, the measurement of wAPS provides insight into the relative importance of APS in the total EPS pool. Chemical structural information such as this is required to relate chemical composition to the function of EPS associated with photosynthetic and other microbial biofilms. APS are an important component of the organic carbon pool in the water column in the form of TEP and its precursors (Passow 2002, Thornton et al. 2007). The measurement of APS in sediments using alcian blue will enable comparable measurements of APS concentrations in both the water column and sediments and therefore enable researchers to explore the coupling between these APS pools and the role of APS originating in sediments in the formation of TEP in the water column.

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