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Highlights

- Salinity and surrogate type greatly impact the accuracy of TEP quantification.
- Total carbohydrate more accurately quantifies TEP surrogates than Alcian blue.
- Report surrogate, salinity, recovery, and calibration factor for TEP studies.
- Xanthum gum is a better surrogate for precursor TEP than for particulate TEP.

Comparison of Alcian blue and total carbohydrate assays for quantitation of transparent exopolymer particles (TEP) in biofouling studies.

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Abstract

Transparent exopolymer particles (TEP) and their precursors are gel-like acidic polysaccharide particles. Both TEP precursors and TEP have been identified as causal factors in fouling of desalination and water treatment systems. For comparison between studies, it is important to accurately measure the amount and fouling capacity of both components. However, the accuracy and recovery of the currently used Alcian blue based TEP measurement of different surrogates and different size fractions are not well understood. In this study, we compared Alcian blue based TEP measurements with a total carbohydrate assay method. Three surrogates; xanthan gum, pectin and alginic acid; were evaluated at different salinities. Total carbohydrate concentrations of particulates (>0.4 μm) and their precursors (<0.4 μm, >10 kDa) varied depending on water salinity and method of recovery. As xanthan gum is the most frequently used surrogate in fouling studies, TEP concentration is expressed as xanthan gum equivalents (mg XG_{eq}/L) in this study. At a salinity of 35 mg/L sea salt, total carbohydrate assays showed a much higher particulate TEP fraction for alginic

acid (38%) compared to xanthan gum (9%) and pectin (12%). The concentrations of particulate TEP therefore may only represent ~10% of the total mass; while precursor TEP represents ~80% of the total TEP. This highlights the importance of reporting both particulate and precursor TEP for membrane biofouling studies. The calculated concentrations of TEP and their precursors in seawater samples are also highly dependent on type of surrogate and resulting calibration factor. A linear correlation between TEP recovery and calibration factor was demonstrated in this study for all three surrogates. The relative importance and accuracy of measurement method, particulate size, surrogate type, and recovery are described in detail in this study.

Keywords: Transparent exopolymer particles (TEP), precursor TEP, total carbohydrate assay, membrane fouling, biofouling, Alcian blue

1. Introduction

Interest has steadily risen over the past decade in the role played by transparent exopolymer particles (TEP) in the biofouling of membranes (Bar-Zeev et al. 2009, Berman 2005, Berman et al. 2011, de la Torre et al. 2008). This interest quickly expanded from a focus only on particulate TEP to include smaller size fractions of these extracellular polymeric substances (EPS), now termed colloidal and precursor TEP (Bar-Zeev et al. 2015, Discart et al. 2015, Li et al. 2016a, Villacorte et al. 2015, Villacorte et al. 2009a, b). TEP was itself identified as an important player in marine ecosystems and biogeochemical processes about a decade prior to that when a tractable means of quantitatively identifying this gel-like, natural material was developed by Passow and Alldredge (1995). The likelihood that these biogenic particles were implicated in ocean desalination plant reverse osmosis membrane fouling seemed

mechanistically plausible due to their ubiquitous presence, particulate nature, documented tendency to aggregate and adhere to surfaces, and identification as a food source for microbial growth (Chin et al. 1998, Passow 2000, Rochelle-Newall et al. 2010, Uthicke et al. 2009, Verdugo 2012). TEP are a subset of EPS, which are released from microbial aquatic organisms and so, despite their microbial origin, contribute to biofouling of membranes even when no viable microorganisms are present (e.g., after disinfection).

TEP is an operationally defined entity. It is the mass of material retained on a 0.4 μm filter which stains with Alcian blue (denoted herein as $TEP_{0.4\mu m}$, 'particulate' TEP, or pTEP) (Bar-Zeev et al. 2015, Passow and Alldredge 1995). Subsequently, extensions of this concept have been utilized. Colloidal TEP (cTEP) is defined as Alcian blue (AB) stainable material smaller than 0.4 μm but larger than 0.05 μm (Bar-Zeev et al. 2015, Villacorte et al. 2009a, b), although filtration bounds of 0.2 μm and 0.1 μm have also been studied (Passow 2000, Villacorte et al. 2009a, b). In addition, TEP and their precursors (denoted here as TEP_{10kDa}) have been defined and studied as the mass of material which is retained by a 10 kDa filter and stains with Alcian blue (Villacorte et al. 2015). By this definition, precursor TEP includes cTEP plus any additional AB stainable material retained by a 10 kDa filter, which are smaller than 0.4 μm. Consequently, precursor TEP is a measure of any "non-dissolved" (i.e., filterable) constituent passing a 0.4 μm filter which stains with Alcian blue.

It is worth noting that Alcian blue stain is not specific to acid polysaccharides, which are typically referenced as the components of TEP (Discart et al. 2015, Villacorte et al. 2015). Alcian blue only stains the carboxylated ($-COO^-$) and sulfated ($-OSO_3^-$) polyanionic (at pH 2.5) functional groups on a complex, typically high molecular weight material. Other compounds, such as glycopolymers and proteins with these functional components (i.e. bacterial cell walls) stain as well. TEP's quantification is therefore only a quantification of

the concentration of stainable (and membrane retained) functional groups, not concentration of total EPS mass, nor biofouling potential.

The difficulties and variabilities in collecting natural TEP samples motivate the use of more easily prepared TEP surrogates. Even more critically, controlled experiments such as quantitative and mechanistic biofouling studies require use of a reproducible, quantifiable material rather than an operationally defined material to which no standard value or mass balance can be applied. Thus, for controlled behaviour trials, TEP surrogates have been routinely used (Fatibello et al. 2004, Passow and Alldredge 1995, Thornton et al. 2007, Villacorte et al. 2015, Villacorte et al. 2009a, b). Relatively easily quantified surrogates allow normalization of measurements of natural samples to some known and reproducible standard (e.g., TEP expressed as mg equivalents xanthum gum/L (Passow and Alldredge 1995)). In addition, and particularly relevant to controlled parametric trials on membrane fouling, it allows known quantities of a foulant (albeit a surrogate) to be utilized and the response observed under a variety of conditions (Le Lan et al. 2015, Li et al. 2016a, Villacorte et al. 2009a, b). This approach gives rise to several questions, both in evaluating the 'real' world impact of study results, as well as when comparing the results between different studies. These include how well do the surrogates mimic TEP including size variants, how well do different surrogates' behaviours compare one to another, to what interferences or conditions are the measurements sensitive, and how well are the surrogates (and thus by implication the TEP) recovered and size fractionated in the various conditions and methods commonly utilized by researchers? However, few studies have attempted to answer these questions and none have done so comparatively among surrogates and over the range of salinities and TEP size fractions utilized in membrane biofouling trials.

In this study, we investigate TEP method recovery of different fractions of TEP using three surrogates (xanthan gum (XG), pectin (PN) and alginic acid (AA)); at three selected salinities

representative for fresh, brackish and seawater conditions; and for the size fractions of TEP implicated in biofouling studies. To understand the influence of recovery on TEP quantification, the relationship between TEP recovery and calibration factor was simulated. The surrogates are then compared to a natural seawater TEP material to evaluate the correlation between surrogate behaviour and behaviour of a real world TEP. The seawater TEP was also analysed to compare with previous TEP studies using different calibration factors.

2. Materials and Methods

2.1 TEP Quantification

To be most consistent with the previous TEP definitions, in this study $TEP_{0.4\mu m}$ is defined as particulate TEP and is the AB stainable mass retained by a 0.4 μm filter. $TEP_{0.1\mu m}$ is defined as colloidal TEP and is the AB stainable mass passing 0.4 μm filtration and retained by 0.1 μm filtration. TEP and their precursors (TEP_{10kDa}), on the other hand, are defined as all AB stainable mass retained by a 10 kDa filter, which includes particulate plus colloidal TEP plus any AB stainable material retained by a 10 kDa filter.

The procedure for measuring the 0.4 μm fraction of TEP was based on Passow and Alldredge (Passow and Alldredge 1995). The procedure for measuring the 0.1 μm fraction of TEP was based on Villacorte (Villacorte et al. 2009a, b). Fifty milliliters of sample was filtered through a 0.4 μm polycarbonate filter (Nuclepore Track-Etch membrane, Whatman, 47 mm diameter) and the filtrate was filtered through a 0.1 μm polycarbonate filter (Nuclepore Track-Etch membrane, Whatman, 47 mm diameter) by applying a constant vacuum of 0.2 bar (Bettervac Pty Ltd, Australia). To remove residual salt from the filter-retained material, 2.0 mL of distilled MilliQ water was subsequently added to each filter and filtered. 1.00 mL of pre-filtered 0.02% AB solution in 0.06% acetic acid (pH 2.5) was added to each filter so that the filter was soaked in the stain. After 10 s, the excess dye was rinsed off using 2.0 mL of

distilled MilliQ water and vacuum filtration. The filter was then transferred to a 50 mL glass beaker face down and 6.0 mL of 80% sulphuric acid added. The beaker was covered with parafilm and mixed on a shaker table for 2 hrs. Samples were taken from the beaker and absorbance measured at 787 nm wavelength (UVmini-1240, Shimadzu). Concentrations of $TEP_{0.4\mu m}$ and $TEP_{0.1\mu m}$ were calculated as follows and expressed as surrogate equivalents ($\mu g/L$ $surrogate_{eq}$):

$$TEP_{0.4\mu m} = \frac{1}{m_{787}^{0.4\mu m} V}\left(A_{sample} - A_{filter\ blank} - A_{sample\ blank}\right)$$

$$TEP_{0.1\mu m} = \frac{1}{m_{787}^{0.1\mu m} V}\left(A_{sample} - A_{filter\ blank} - A_{sample\ blank}\right)$$

$$f_{787} = \frac{1}{m_{787}}$$

Where $TEP_{0.4\mu m}$ and $TEP_{0.1\mu m}$ are defined previously; $m_{787}^{0.4\mu m}$ and $m_{787}^{0.1\mu m}$ are the slopes of the calibration curves for 0.4 μm and 0.1 μm fractions [(abs/cm)/μg $surrogate_{eq}$]; V is the volume of filtered sample (L); A_{sample} is the absorbance of Alcian blue stained sample (abs/cm); $A_{filter\ blank}$ is the absorbance of Alcian blue from a negative control filter (abs/cm); $A_{sample\ blank}$ is the absorbance from an unstained sample filter (abs/cm); and $1/m_{787}$ is the calibration factor (f_{787}) [μg/(abs/cm)].

The procedure for measuring seawater TEP and their precursors was based on Villacorte (Villacorte et al. 2015). 50.0 mL of sample was filtered through regenerated cellulose filters (Ultracel Ultrafiltration Discs, 24 mm diameter, 10 kDa, Millipore) using a disposable syringe. Injection of ten milliliter of air followed to ensure the excess sample liquid was filtered. 2.0 mL of distilled MilliQ water was then filtered to remove residual salt. Finally, 10 mL of air was injected to remove excess MilliQ water. The filter was then soaked in 10.0 mL

of distilled deionized water with TEP side facing down in a 40 mL plastic container, vortexed for 10 s and sonicated for 2 hrs. 4.00 mL of the sonicated solution was transferred to a 7 mL glass vial, and acetic acid added to adjust the pH to 2.5. Then 0.50 mL of pre-filtered AB was added, vortexed for 10 s, and 4.0 mL withdrawn and filtered through a 0.1 μm polycarbonate membrane filter (Nuclepore Track-Etch membrane, Whatman, 24 mm diameter,) manually using a syringe. The absorbance of the filtrate was measured at 610 nm wavelength. Concentration of TEP_{10kDa} was calculated from the following and expressed as surrogate equivalents (mg $surrogate_{eq}$/L):

$$TEP_{10kDa} = \frac{1}{m_{610}} \frac{V_F}{V} \left(A_{sample} - A_{filter\ blank} - A_{sample\ blank}\right)$$

$$f_{610} = \frac{1}{m_{610}}$$

Where TEP_{10kDa} is defined previously; m_{610} is the slope of the calibration curve [(abs/cm)/(mg $surrogate_{eq}$/L)]; V is the volume of filtered sample (L); V_F is the final re-suspended sample volume, which is 10 mL for this method (L); A_{sample} is the absorbance of Alcian blue stained sample (abs/cm); $A_{filter\ blank}$ is the absorbance of Alcian blue on a negative control filter (abs/cm); and $A_{sample\ blank}$ is the absorbance of unstained sample filter (abs/cm). Also, $1/m_{610}$ is defined as the calibration factor (f_{610}) in [(mg/L)/(abs/cm)].

2.2 Total carbohydrate analysis

To quantify recovery and comparatively evaluate the TEP assay, the phenol-sulfuric acid method was used. The phenol-sulfuric acid method is a widely used colorimetric method to determine total carbohydrate concentration (Dubois et al. 1956). The predominant monomers found in biofilms and exocellular polysaccharides are glucose, mannose, galactose, glucuronic acid and galacturonic acid based on the composition analysis of both pure strain (Uhlinger and White 1983) and activated sludge (Horan and Eccles 1986). Therefore, these

five sugars were selected for phenol-sulfuric acid (total carbohydrate) method validation. In addition, three TEP surrogates were investigated: XG, PN and AA. Each surrogate contains one or more of the corresponding sugar monomers: XG consists of glucose, mannose and glucuronic acid units; PN consists of galacturonic acid units; and AA consists of glucuronic acid. Among the three surrogates, XG is the most widely used standard in TEP measurement due to its reported better replicability (Passow and Alldredge 1995, Villacorte et al. 2015, Villacorte et al. 2009b). AA is less frequently used (Hung et al. 2003), while to our knowledge no studies have used PN as a surrogate for TEP. However, PN contains the AB stainable galacturonic acid groups absent from either XG or AA, and was therefore included in this study.

Stock sugar solutions were made by dissolving 20 mg of D-glucose (Sigma-Aldrich), D-Mannose (Sigma-Aldrich), D-Galactose (Sigma-Aldrich), D-Glucuronic acid (Sigma-Aldrich) or D-Galacturonic acid (Fluka) to 500 mg of distilled milliQ water. A mixed sugar solution was made from D-glucose, D-Mannose and D-Glucuronic acid with a molar ratio of 2.8: 2.0: 2.0 which mimics the molar polysaccharide composition of XG.

For calibration curves, 0.25 - 1.75 mL of above stock solutions containing 10-70 μg sugar was pipetted to a 10 mL glass tube to make calibration standards between 1.52 and 10.65 mg/L. Distilled milliQ water was added to reach a volume of 2.00 mL, followed with 80% phenol solution (VWR) and 5.0 mL of sulfuric acid (95%, VWR). To obtain a good reaction, the sulfuric acid needs to be added rapidly and directly to the liquid surface. The vial was capped, vortexed (Crown scientific) for 10 s, and set aside for 20 min. Full scans of each standard were examined before reading the absorbance (UVmini-1240, Shimadzu). The maximum wavelength for each standard is listed in Table 1. All samples were measured in triplicate.

2.3 Surrogates and Alcian blue

Stock surrogate solutions were freshly made by adding 15.00 mg of XG (Sigma-Aldrich), AA (Sigma-Aldrich) or PN (Sigma-Aldrich) to 200.0 mL of distilled deionized water. The solution was mixed constantly with a magnetic stir bar, and then ground with a tissue grinder 3 times before use.

Stock Alcian blue solution was prepared by dissolving 40.00 mg of Alcian blue (Sigma-Aldrich) to 200.0 mL of distilled deionized water. Acetic acid (ACS reagent, Sigma-Aldrich) was added dropwise to adjust pH to 2.5. The solution was stirred for 12-18 hr and stored in the dark at 4^{o}C. Stock AB solution was filtered drop by drop through a 0.05 μm polycarbonate membrane (Nuclepore Track-Etch membrane, 24 mm diameter, Whatman) using a syringe prior to staining. A new stock solution was prepared every 4 weeks or more frequently. New calibration curves were generated for each batch of total carbohydrate analysis, and TEP or surrogate measurement.

2.3 TEP recovery method

Recoveries of the TEP surrogates; XG, PN and AA; were tested under three different salinities: representative of fresh water (prepared in distilled deionized water), brackish water (4,000 mg/L sea salt (Sigma-Aldrich)) and seawater (35,000 mg/L sea salt). Surrogate processing followed the TEP methods discussed above including filtration, rinsing, staining with Alcian blue, and re-rinsing. Total carbohydrate concentrations of the initial surrogate solution and the filtrate were measured following the total carbohydrate analysis method. Finally, total carbohydrate concentration of the re-suspended filter solution was measured. Recoveries were calculated as:

$$R\ (\%) = \frac{C_{filter}V_{filter} + C_{filtrate}V_{filtrate}}{C_{initial}V_{initial}} \times 100$$

where C_{filter}, $C_{filtrate}$ and $C_{initial}$ are total carbohydrate concentrations of the re-suspended filter, filtrate, and initial surrogate solutions, respectively (μg/mL); and V_{filter}, $V_{filtrate}$ and $V_{initial}$ are volume of the re-suspended filter, filtrate and initial surrogate solutions, respectively.

The percentage of recovered carbohydrates in the surrogate retained on the membrane filters (F_{filter}) and the percentage in the filtrate ($F_{filtrate}$) were calculated as:

$$F_{filter}(\%) = \frac{C_{filter}V_{filter}}{C_{filter}V_{filter} + C_{filtrate}V_{filtrate}} \times 100$$

$$F_{filtrate}(\%) = \frac{C_{filtrate}V_{filtrate}}{C_{filter}V_{filter} + C_{filtrate}V_{filtrate}} \times 100$$

2.4 Seawater samples

Raw seawater samples were collected from the Trigg Beach in Perth, WA, Australia (Water Corporation) in February 2017. TEP and total carbohydrate analyses were carried out within 24 hours after sample collection.

3. Results and discussion

3.1 Monomer and surrogate quantification

To evaluate phenol-sulfuric acid method performance, three parameters were considered: method linearity, linearity range and method precision. Calibration results showed that the coefficient of determination for linear regression was higher than 0.98 for sugar standards within the range of 10 μg (1.52 mg/L) to 70 μg (10.65 mg/L) and higher than 0.99 for surrogates within the range of 7.5 μg (1.14 mg/L) to 75 μg (11.42 mg/L) (Table 1). The relative standard deviations (RSD %) of all sugars and surrogates were less than 9% and 7%, respectively. The standard deviation of 10 independent blank measurements was 0.02 abs/cm.

237 The lower limits of detection (LOD) of the phenol-sulfuric acid method were 1.39, 1.67, 1.67
238 mg/L for XG, PN, and AA, respectively.

239

240 **3.2 Alcian blue effect on quantification by phenol-sulfuric acid**

241 In this study, the phenol-sulfuric acid assay was used to quantify the total carbohydrate
242 concentration of both surrogate and seawater samples processed using the TEP filtration
243 method. TEP analytic processing is a two-step process; filtration and Alcian blue (AB)
244 staining. Typically filtration occurs before staining, although the reverse order has also been
245 utilized (Passow and Alldredge 1995, Thornton et al. 2007). Thus, for this study effects of the
246 filtration process were separated from those of AB staining. AB is a blue-purple dye while
247 phenol-sulfuric acid presents a yellow-orange color. Thus, the effect of AB on phenol-
248 sulfuric acid analysis was subtracted in the measure of total carbohydrate of any TEP (filter
249 or filtrate) sample.

250 Spectral scans of AB in XG, PN and AA were performed using the total carbohydrate
251 analysis. The peak wavelength for the three surrogates remained unchanged with the presence
252 of AB: 485 nm for XG and 480nm for both PN and AA. However, AB did increase the
253 absorbance for all three surrogates. In subsequent analyses this was corrected by subtracting
254 the AB control in the phenol-sulfuric acid assay. To minimize AB batch variations, a new
255 correction was done for each batch of AB solution.

256 **3.3 TEP method recovery**

257 Although method recovery (mass balance closure) is an important parameter in method
258 validation, it has not been done often in TEP analysis (Table 2). Recovery in a filtration
259 method is expressed as the sum of the mass retained on the filter and the mass in the filtrate
260 divided by the initial mass subjected to filtration. The Passow and Alldredge, 1995 method
261 (Passow and Alldredge 1995) measured the 0.4 μm retained fraction of surrogate XG on the

filter by dry weight measurement, but didn't measure the amount in the filtrate nor in the initial solution. This follows the logic used in measuring TEP in natural samples in which there is no independent means of measuring the "total TEP" in the sample subjected to filtration, since TEP is itself operationally defined by filtration. In contrast, Villacorte et al, 2009 method (Villacorte et al. 2009a) measured the total organic carbon (TOC) of the XG surrogate in the initial solution and in the filtrate, but didn't analyse the TOC of the filter, deducing that quantity by difference (and *de facto* assuming 100% recovery). Neither of these two methods directly tested method recovery. To determine TEP method recovery, phenol-sulfuric acid method was used in this study to analyse the initial surrogate solution, filter, and filtrate.

In this study, $TEP_{0.4\mu m}$ and $TEP_{0.1\mu m}$ and TEP_{10kDa} represent the AB stainable fraction larger than 0.4 μm; fraction between 0.1 and 0.4 μm; and fraction larger than 10 kDa, respectively. Recoveries using four versions of the TEP method were investigated, namely: $TEP_{0.4\mu m}$ by Passow and Alldredge (Passow and Alldredge 1995), $TEP_{0.1\mu m}$ by Villacorte et al (Villacorte et al. 2009a), TEP_{10kDa} by Villacorte et al (Villacorte et al. 2015), and $TEP_{pre\text{-}stain}$ by Thornton et al (Thornton et al. 2007) using AB staining prior to filtration by 0.4 μm PC filter. The first three methods were selected because the Passow and Alldredge, 1995 method (Passow and Alldredge 1995) is the most widely used method for TEP (> 0.4 μm) monitoring, Villacorte et al, 2009 method (Villacorte et al. 2009a) is developed based on the Passow and Alldredge's method by using a series of filtration targeted at colloidal TEP (0.05 μm < TEP size < 0.4 μm) measurement, and Villacorte et al, 2015 method (Villacorte et al. 2015) is a recent developed method for TEP/TEP precursor (>10 kDa) analysis. $TEP_{pre\text{-}stain}$ of Thornton (Thornton et al. 2007) is also of interest since the method offers some operational advantages over the other methods (such as ability to measure total stainable mass of the initial sample)

and since the fouling mechanisms as a function of particle size and solution composition for any kind of TEP are not fully understood.

Mass balances quantified using the phenol-sulfuric acid method show recoveries for the three surrogates at three different salinities (DDI: distilled deionized water, 4000 mg/L sea salt and 35000 mg/L sea salt) were in the range of 80 - 120% (Table 3). Across the variables of both salinity and size fraction, no surrogate showed noticeably better or worse recovery than the others. Likewise, the relative standard deviation among replicates was not noticeably different for one surrogate or set of conditions than another.

3.4 Recovered carbohydrate of $TEP_{0.4\mu m}$

In DDI water, the XG $TEP_{0.4\mu m}$ fraction was only 13% of the total XG mass and similarly the PN $TEP_{0.4\mu m}$ fraction was only 12% of the total mass (Fig.1). For XG, this was consistent with recent TOC results for seawater in which $TEP_{0.4\mu m}$ was only 16.5% of the TOC of the initial feed seawater (Li et al. 2016a). In contrast, the particulate fraction for AA was 68%. However, the higher $TEP_{0.4\mu m}$ fraction of AA is not consistent with its relative molecular weight (32000 - 400000 g/mol (Lee and Mooney 2012)) compared to XG (2 MDa - 20 MDa g/mol (Garcia-Ochoa et al. 2000)) and PN (60 - 130000 g/mol (Muzzarelli et al. 2012)). One possible explanation is that AA has a higher particulate fraction than XG and PN, due to a greater propensity for aggregation. However, the decreasing filter retained mass with increasing salinity and Ca^{++} concentration is not expected if this is the case (Verdugo, 2012). However, this explanation was not further tested. Since TEP is operationally defined as the mass of material retained on a 0.4 μm filter and stained by AB, then use of XG or PN as surrogates is problematic in that only a small fraction of surrogate mass in a sample would meet the TEP criteria of filterability in fresh water. Choosing AA as a surrogate for $TEP_{0.4\mu m}$, will capture much more of the AB stainable material than using XG or PN as $TEP_{0.4\mu m}$ surrogates.

Two salinities, 4000 mg/L and 35000 mg/L of sea salt, were selected to simulate brackish water and seawater, respectively (Fig.1). As the sample salinity increases the mass retained by the 0.4 μm filter is unchanged for PN (12%, 11% and 12%, respectively) and only modestly diminished for XG (13%, 13% and 9%, respectively). The greatest observed change caused by salinity is for AA; the retained $TEP_{0.4\mu m}$ fraction gradually decreased from 68% to 38% as salinity increased. Therefore, the drawback of selecting AA as TEP surrogate is its sensitivity to salinity change, even though in fresh water it behaves much more like a true $TEP_{0.4\mu m}$ surrogate (than XG or PN) in which the AB stainable material would be completely retained on a 0.4 μm filter. The issue of poor retention of $TEP_{0.4\mu m}$ for the three surrogates is unmitigated or worsened in seawater salinity concentration samples compared to fresh or brackish water salinity concentration samples.

The $TEP_{0.4\mu m}$ surrogate results also expose the reproducibility problem with using $TEP_{0.4\mu m}$ by itself as a quantification method. The $TEP_{0.4\mu m}$ measure of different compounds (in this case represented by the different surrogates) varied substantially within the same salinity matrix. For instance, $TEP_{0.4\mu m}$ quantification of seawater salinity samples containing 75mg/L of XG, PN, or AA resulted in $TEP_{0.4\mu m}$ measured concentrations of 6.8mg/L, 9.0mg/L, and 28.5mg/L, respectively for the three compounds. These compound-specific differences in $TEP_{0.4\mu m}$ measurement are caused by differences in molecular weight as well as likely differences in other factors such as relative self-assembly and hence filterability (see for instance, Chin et al. 1998, Rochelle-Newall et al. 2010, Verdugo 2012). Thus, $TEP_{0.4\mu m}$ quantification of EPS made up of different proportions of different compounds would result in very different values even though the total EPS concentration present might be the same.

3.5 Recovered carbohydrate of $TEP_{0.1\mu m}$

In terms of filter retention, $TEP_{0.1\mu m}$ of XG, PN and AA in DDI water contrasted with $TEP_{0.4\mu m}$ in that the retention for AA was much less than XG and PN (Fig.2). However, the retention difference of $TEP_{0.1\mu m}$ between AA, XG and PN shrunk.

The same as with $TEP_{0.4\mu m}$ results, the retention of $TEP_{0.1\mu m}$ for PN was not affected by sample salinity (within the range of one standard deviation between the means), while that for AA declined with increased salinity (Fig.2). However, $TEP_{0.1\mu m}$ for XG reduced from 43% to 26% at salinity of 4000 mg/L and to 10% at salinity of 35000 mg/L. This declining retention trend indicates the salt interference effect is more pronounced on the colloidal size particles (less than 0.4 μm) than on the larger particles for XG. Possible explanations are that higher salinity causes some disaggregation of particles and stabilization of colloids or that conformational change of the XG polymeric material is toward a less filterable (perhaps flexible) geometry due to increased salinity and compression of diffuse and fixed layer charge proximate to charged functional groups (Verdugo et al. 2004). However, this decrease in filterability with increasing size is seemingly at odds with the enhancement of dissolved organic carbon (DOC) self-assembly and annealing as salinity increases reported by Verdugo (2012). Passow and Alldredge (Passow and Alldredge 1995) did compare post sample rinse and no rinse of the 0.4μm filtered material, but their results showed no measurable difference for $TEP_{0.4\mu m}$. This is consistent with this study's results, as a rinse of the material retained on the filter would remove only salts and increase the material's filterability, whereas the additional fraction that would have been retained in less saline water has already been passed through to the filtrate prior to rinsing.

3.6 Recovered carbohydrate of TEP_{10kDa}

Unlike $TEP_{0.4\mu m}$, the retention of TEP_{10kDa} for XG (93%) in DDI water is much better than PN (77%) and AA (73%) (Fig.3). Also, the XG and PN mass retained by the 10 kDa filter were minimally affected by salinity change. This suggests that XG may be a reasonable

surrogate for precursor TEP across a range of salinities using the Villacorte et al., 2015 method (Villacorte et al. 2015). This is in stark contrast to its poor performance as a surrogate for p-TEP and c-TEP compared particularly to AA, but also PN (Fig. 4). While the TEP_{10kDa} retention of AA declined with increasing salinity, this trend is similar to the effect of salinity on $TEP_{0.4\mu m}$ and $TEP_{0.1\mu m}$ retention of AA.

3.7 Recovered carbohydrate of different TEP size fractions

The surrogates XG and PN exhibit a broad (filterable) size distribution across the spectrum from 0.4 μm to 10 kDa retention with $TEP_{10kDa} > TEP_{0.1\mu m} > TEP_{0.4\mu m}$ (Fig.4). This is expected when a sample contains a broad range of particle sizes or in terms of gels, a wide range of tendencies toward self-assembly. In general, the $TEP_{0.1\mu m}$ fractions of XG were higher than $TEP_{0.4\mu m}$, but their mass ratio varied depending on salinity. The $TEP_{0.1\mu m}$ fractions of PN were about three times the $TEP_{0.4\mu m}$ fraction, and TEP_{10kDa} fractions were about seven times the $TEP_{0.4\mu m}$ fraction. However, the $TEP_{0.1\mu m}$ fractions of AA were far less than $TEP_{0.4\mu m}$, and even less than the 10 kDa filterable fraction (Fig.4). This suggests that most of the filterable AA is in particles of effective diameter greater than 400 nm with the balance in a dissolved state of <10 kDa. Despite its molecular weight range being much less than that of XG, its polymeric units appear to much more readily assembled or aggregated to large microgels, than those of XG. Interestingly, as the salinity of the AA sample decreases, the fraction of the total AA mass in the particulate state increases without a significant fraction appearing in the intermediate size fractions of 0.1 μm and 10 kDa.

It is recognized and important to keep in mind that all of the surrogates studied are deformable chain organic molecules, so particle size is more of an operational concept than a hard-solid characterization. This is particularly noticeable in the trials at the various salinities described above. Xanthan gum is the most widely used TEP surrogate (Fatibello et al. 2004, Passow and Alldredge 1995, Thornton et al. 2007, Villacorte et al. 2009a). However, the

reported XG $TEP_{0.4\mu m}$ concentration is only a small portion of the total XG TEP (particulate, colloidal and dissolved). On the other hand, a much greater fraction of XG is observed in the 0.1 μm to 10 kDa size range than for either AA or PN. Thus to the degree that the colloidal and precursor TEP fractions are implicated in membrane biofouling, as has been suggested by several studies (Bar-Zeev et al. 2015, Li et al. 2016a, Li et al. 2016b), XG may be a better surrogate than PN or AA. Consequently, in studies on the mechanisms of biofouling, which use XG as a surrogate, the possible effects of these smaller size fractions shouldn't be ignored.

3.8 Recovered carbohydrate of $TEP_{pre\text{-}stain}$

The TEP method of Thornton (Thornton et al. 2007) adds Alcian blue to the sample prior to filtration. In marked contrast to the results of $TEP_{0.4\mu m}$ fractional retention, $TEP_{pre\text{-}stain}$ of XG in DDI water retains over 90% of the sample mass and much more than compared to PN and AA (Fig.5). Particulate XG (> 0.4 μm) retained in the $TEP_{pre\text{-}stain}$ method showed a much greater fraction (93%) of total carbohydrate compared to the $TEP_{0.4\mu m}$ fraction (Fig.1) in DDI water. The likely explanation is that the AB dye forms insoluble substances and promotes stable XG assembly into microgels from otherwise sub 0.1μm fractions (Horobin and Flemming 1990).

For XG, the retained $TEP_{pre\text{-}stain}$ at salinity of 4000 mg/L (91%) was very close to that in DDI water (93%), but dropped significantly to 13% at seawater salinity (35000 mg/L) (Fig. 5). Presumably the seawater stabilized the small size XG fractions and largely prevented their aggregation into particulate size TEP. AA showed the same trend as XG, albeit with a lesser fraction no longer retained in seawater. This illustrates that the effect of AB on the size distribution of XG and AA was essentially nullified at seawater salinity as the fractions retained were nearly the same for the pre-stain and post-stain cases. Like XG and AA, pre-staining of PN increased the 0.4μm retained fraction, but the effect was more evenly

409 distributed across the three size fractions investigated. From the perspective of biofouling
410 potential and based on the surrogates' results, pre-staining of samples with brackish or
411 freshwater salinities significantly increases the apparent particle size and therefore overstates
412 the predicted fouling potential. This effect is not pronounced for seawater samples.

413 **3.9 Seawater analysis**

414 When using XG as a TEP surrogate, seawater $TEP_{0.4\mu m}$ and $TEP_{0.1\mu m}$ concentrations were
415 0.49 mg XG_{eq} per liter and 0.44 mg XG_{eq} per liter, respectively (Fig.6). The calibration factor
416 was 213 [μg XG_{eq}/(abs/cm)] for seawater $TEP_{0.4\mu m}$ and $TEP_{0.1\mu m}$, and -42 [(mg
417 XG_{eq}/L)/(abs/cm)] for TEP_{10kDa}. Total carbohydrate concentrations of seawater 0.4 μm and
418 0.1μm fractions were lower than TEP concentrations. The calibration factor used for seawater
419 total carbohydrate concentration was 164 [μg XG_{eq}/(abs/cm)]. In order to compare the
420 seawater TEP results to other studies, we also took calibration factors from two widely used
421 TEP method studies to calculate our seawater TEP concentrations. Two of the calibration
422 factors were from Passow and Alldredge (Passow and Alldredge 1995): 88 [μg
423 XG_{eq}/(abs/cm)] and 139 [μg XG_{eq}/(abs/cm)]; another two were from Villacorte: 476 [μg
424 XG_{eq}/(abs/cm)] (Villacorte et al. 2009b) and -30 [(mg XG_{eq}/L)/(abs/cm)] (Villacorte et al.
425 2015). As shown in Fig. 6, TEP concentrations were proportional to calibration factors. That
426 is to say, the higher the calibration factor, the higher the TEP concentration results, and a
427 greater difference in calibration factor results in a greater difference in calculated TEP
428 concentration. Villacorte attributed their big calibration factor difference compared to Passow
429 and Alldredge's to a lower concentration of Alcian blue stain. In this study, we noticed the
430 apparent calibration factor difference could be due to projected TEP recovery ($R_{projected}$)
431 difference, which is shown in Fig.7. By manipulating the projected TEP recovery, it changes
432 the mass of surrogates on the filter, and the slope of calibration curve (the reciprocal of
433 calibration factor) changes thereafter. Its impact is especially important when using TOC or

total carbohydrate method to calculate the mass of surrogates on the filter. Unlike direct mass measurement including method recovery, indirect methods have a default assumption that TEP method recovery is 100%. This is problematic, since TEP method recovery changes between different batches of experiments, and varies between different surrogates at different salinities (Table.3). Linear regression between calibration factor and projected TEP recovery showed a coefficient of determination (R^2) of 0.9992 for XG, 0.9981 for PN and 0.9486 for AA. Therefore, a higher calibration factor could be due to a higher TEP recovery.

Since the calibration factor also changed when using different surrogates for TEP analysis, Figure 8 illustrates how the calculated seawater TEP concentrations changes when expressed as different surrogate equivalents. The calibration factor for surrogate XG, PN and AA were 213, 909 and 833 [μg XG_{eq}/(abs/cm)], respectively. The TEP recoveries of the surrogates for the same batch seawater analysis were 84%, 85% and 85%, respectively.

4. Summary and conclusions

- TEP measurement by definition means quantification using the Alcian blue method. However, because of the variability in results demonstrated here as a function of salinity and calibration factor employed, it is at best semi-quantitative and cannot be used as a comparative metric between studies even within a single generic matrix such as "seawater". The use of surrogates to simulate TEP ($TEP_{0.4\mu m}$) and its smaller size fraction variants ($TEP_{0.1\mu m}$ and TEP_{10kDa}) is commonly practiced in membrane biofouling research in an effort to undertake more controlled and quantifiable parametric studies than is possible using natural water TEP. However, there is a high degree of variability in quantification as TEP of a single surrogate with variation of the water's salinity and the filter size applied, as well as between surrogates for the same water salinity and filter size applied. This makes comparison between studies using different surrogates and/or different salinities and size fractions problematic. Furthermore, to the degree that surrogate

behaviour is indicative of the behaviour of TEP in general, there is little basis on which to confidently compare results from different studies whose results are based on measuring natural TEP and between which either the water salinity or size fraction considered vary.

- $TEP_{0.4\mu m}$ only represents a small fraction (~10%) of the total mass for the xanthan gum surrogate. The major component of TEP is in the form of precursor TEP. Therefore, measuring the concentration of $TEP_{0.4\mu m}$ alone will likely underestimate the fouling potential for a membrane system. Thus, it is recommended to report both concentrations of particulate TEP and precursor TEP in biofouling studies.
- Measured TEP concentrations were dependent on surrogate, salinity, method of recovery, and calibration factor. Thus, it is recommended to determine and report each of these for each study. It is especially useful when a comparison needs to be done between studies by different research groups or in different conditions.
    - Alcian blue based TEP staining method is also limited by batch variability of the standard. It is also laborious. Although semi-quantification of TEP levels in feed water is possible, the variability of standards makes evaluation and comparison of biofouling potential between studies suspect. Another drawback of the Alcian blue method is that it also stains other organic polyanions than just the acid polysaccharides (i.e. bacterial cell walls) that are classified as TEP. The colorimetric total carbohydrate method was more reproducible and simple than the AB stain method. If one correlates total carbohydrate concentration to TEP concentration, then the total carbohydrate analysis can be used as a more comparatively accurate measurement tool for TEP.

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569 **Table 1** - Parameters for phenol-sulfuric acid method validation.

570 **Table 2** - Comparison of surrogate calibration methods used in transparent exopolymer particles

571 (TEP) assays.

572 **Table 3** - Transparent exopolymer particles (TEP) recovery (R (%)) by phenol-sulfuric acid assay.

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592 **Fig.1** - Percentage of recovered carbohydrate in xanthan gum, pectin and alginic acid retained by 0.4
593 μm membrane filter versus in the filtrate at different salinities. Error bars show one standard deviation
594 measured from triplicate samples.

595 **Fig.2** - Percentage of recovered carbohydrate in xanthan gum, pectin and alginic acid passing a 0.4
596 μm membrane filter, but retained by a 0.1 μm filter versus that in the 0.1 μm filtrate at different
597 salinities.

598 **Fig.3** - Percentage of recovered carbohydrate in xanthan gum, pectin and alginic acid retained by 10
599 kDa membrane filter versus in the filtrate at different salinities.

600 **Fig.4** - Percentage of recovered $TEP_{0.4\mu m}$, $TEP_{0.1\mu m}$ and TEP_{10kDa} fractions of carbohydrate in xanthan
601 gum, pectin and alginic acid.

602 **Fig.5** - Percentage of recovered carbohydrate in pre-stained xanthan gum, pectin and alginic acid
603 retained by 0.4 μm membrane filter versus in the filtrate at different salinities.

604 **Fig.6** - Comparison of seawater $TEP_{0.4\mu m}$, $TEP_{0.1\mu m}$ and TEP_{10kDa} concentrations by using different
605 calibration factors from references versus this study. (expressed as xanthan gum equivalent)

606 **Fig. 7 - Projected effect of transparent exopolymer particles (TEP) method recovery ($R_{projected}$**
607 **(%)) on calibration factor of surrogate xanthan gum, pectin and alginic acid**

608 $$\boldsymbol{R_{projected}}(\%) \times \boldsymbol{C_{initial}V_{initial}} - \boldsymbol{C_{filtrate}V_{filtrate}} = \boldsymbol{m_{filter}}$$

609 where $R_{projected}$ is calculated TEP recovery in percentage; $C_{filtrate}$ and $C_{initial}$ are total
610 carbohydrate concentrations of the filtrate and initial surrogate solution (μg/mL),
611 respectively; $V_{filtrate}$ and $V_{initial}$ are volume of filtrate and initial surrogate solution (mL),
612 respectively; and m_{filter} is the mass of retained surrogates on the filter (μg).

613 **Fig. 8** - Seawater $TEP_{0.4\mu m}$, $TEP_{0.1\mu m}$ and TEP_{10kDa} concentrations expressed as different surrogate
614 equivalents.

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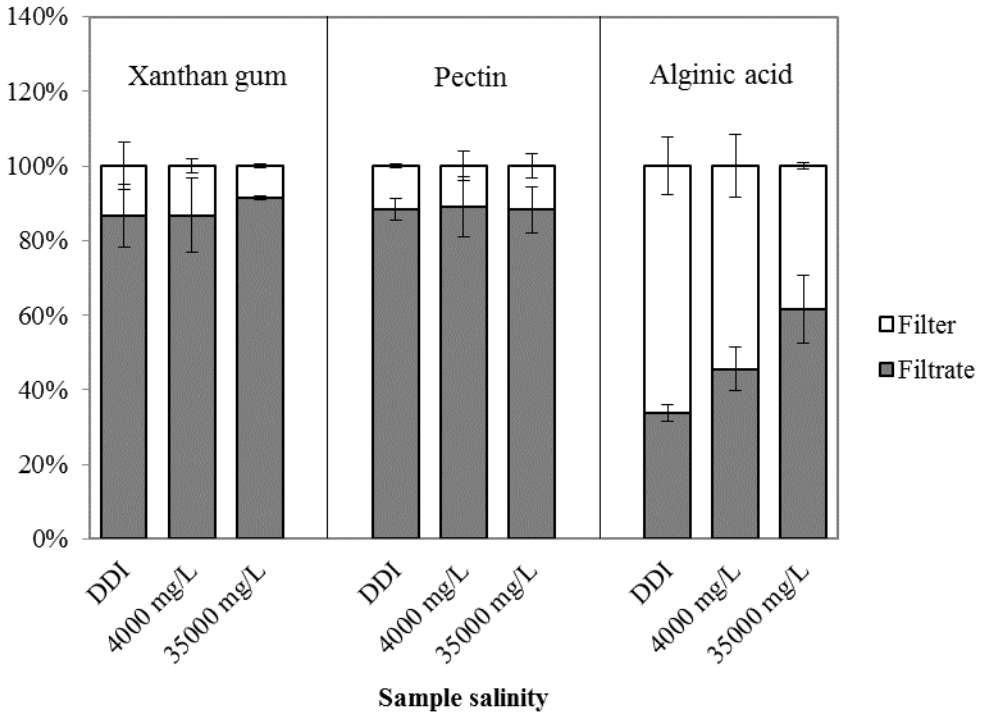

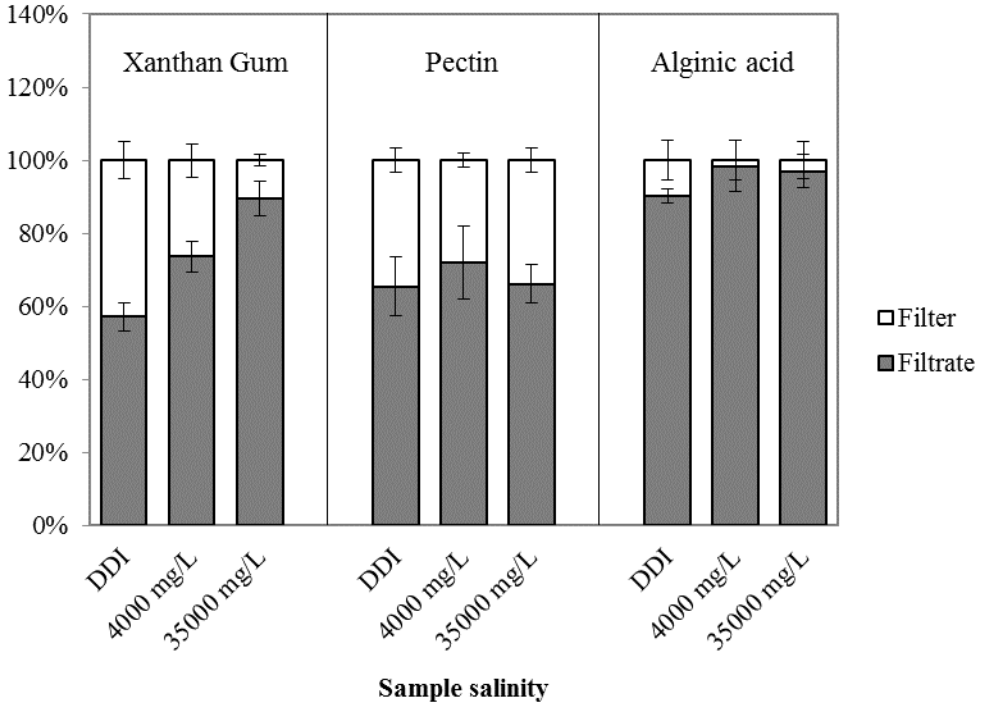

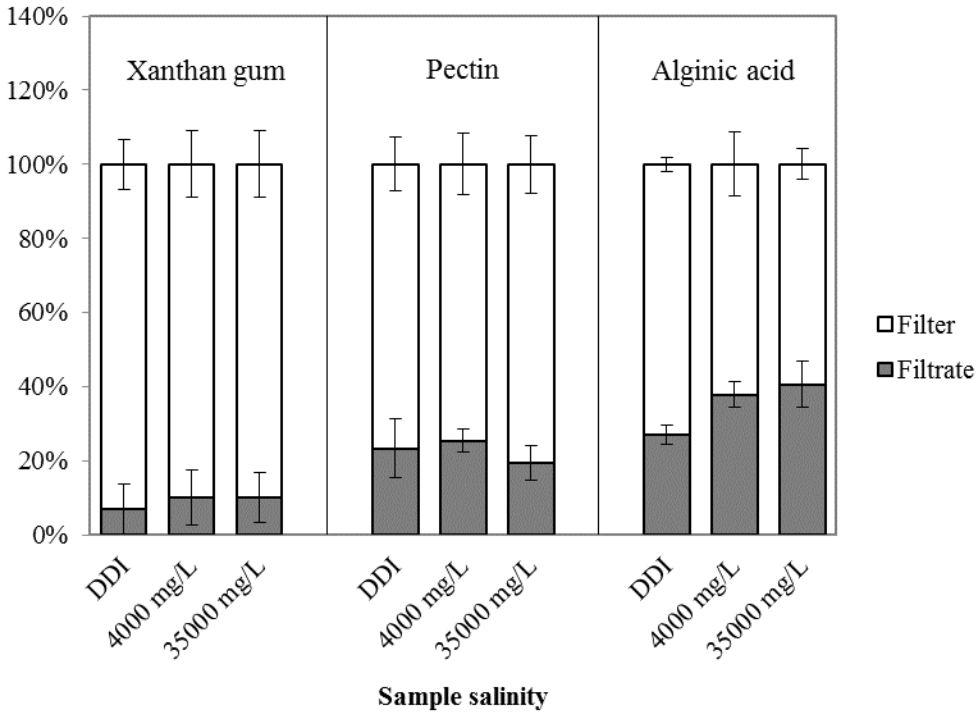

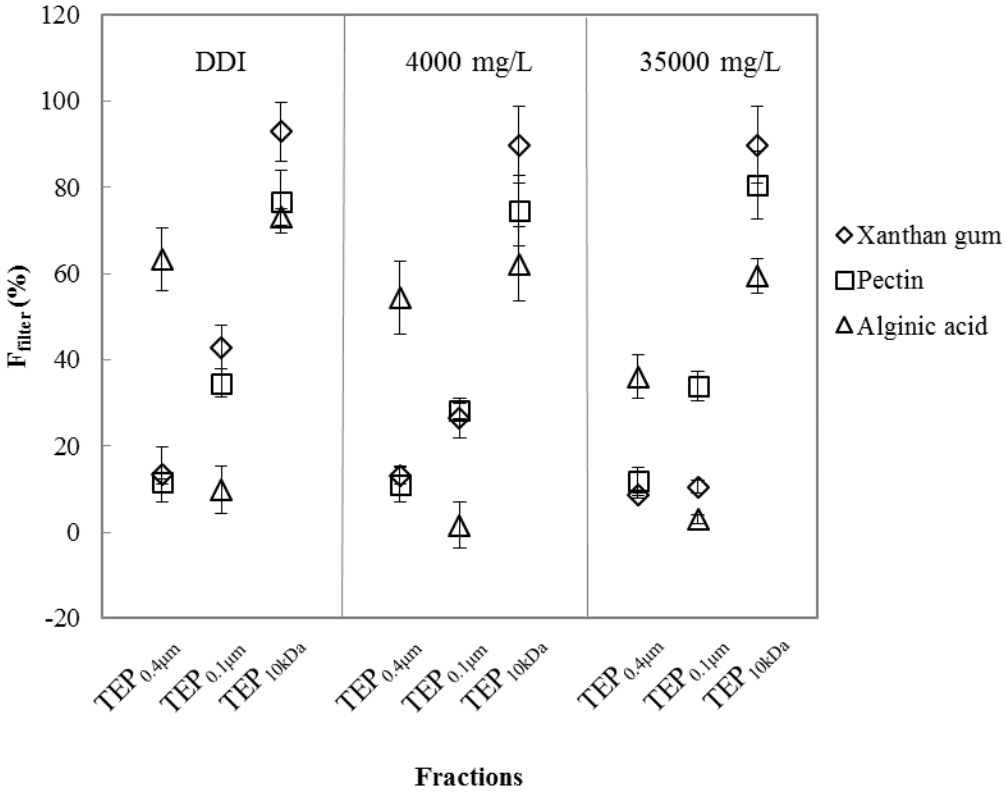

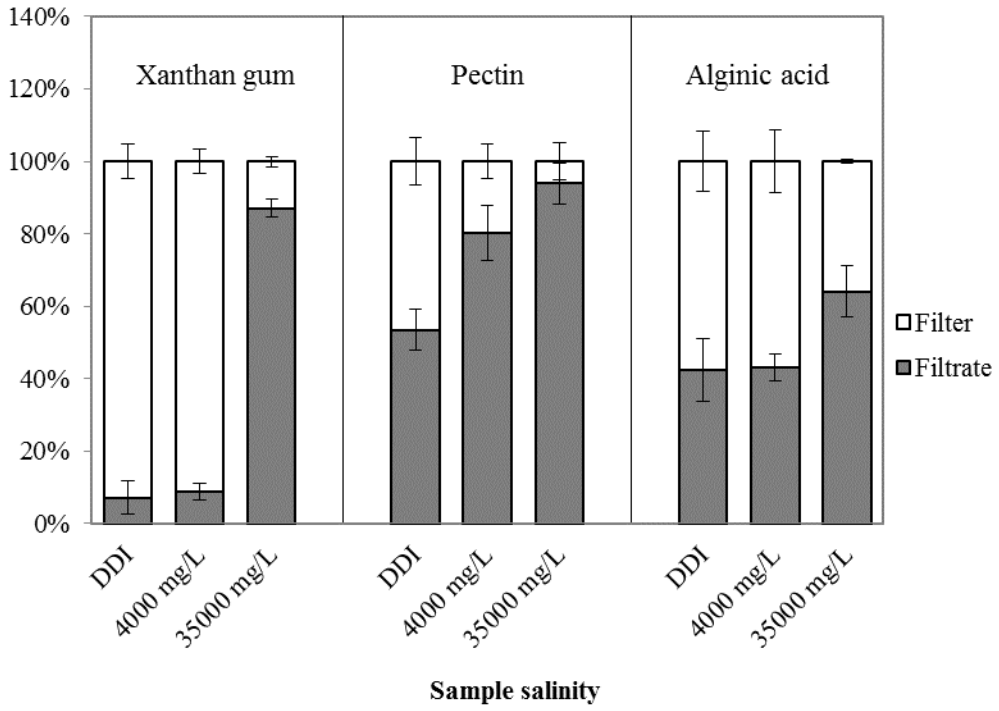

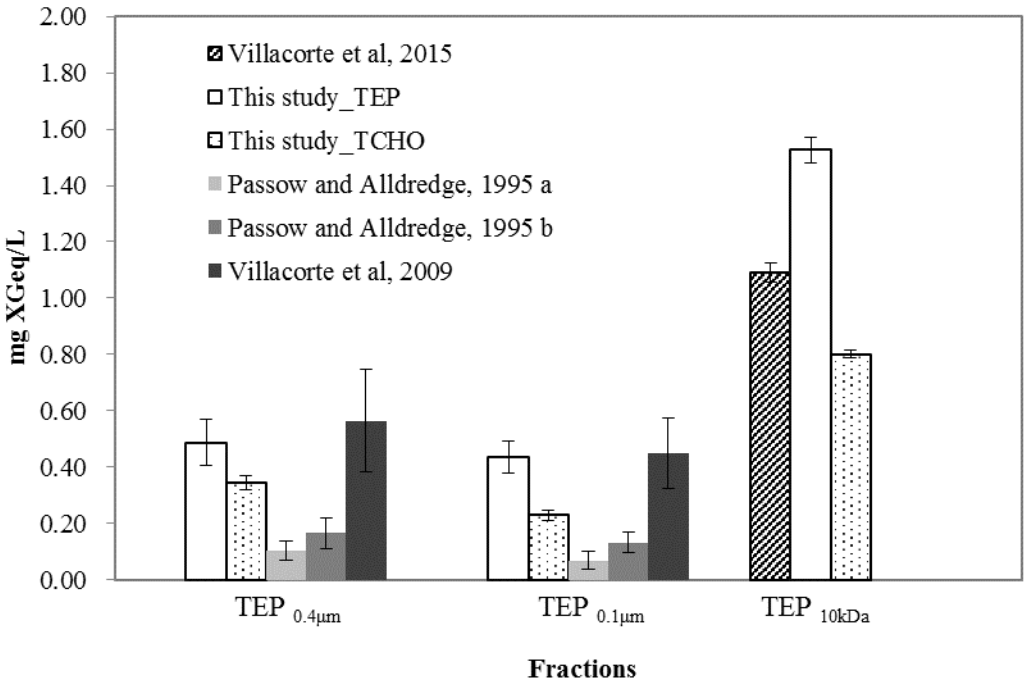

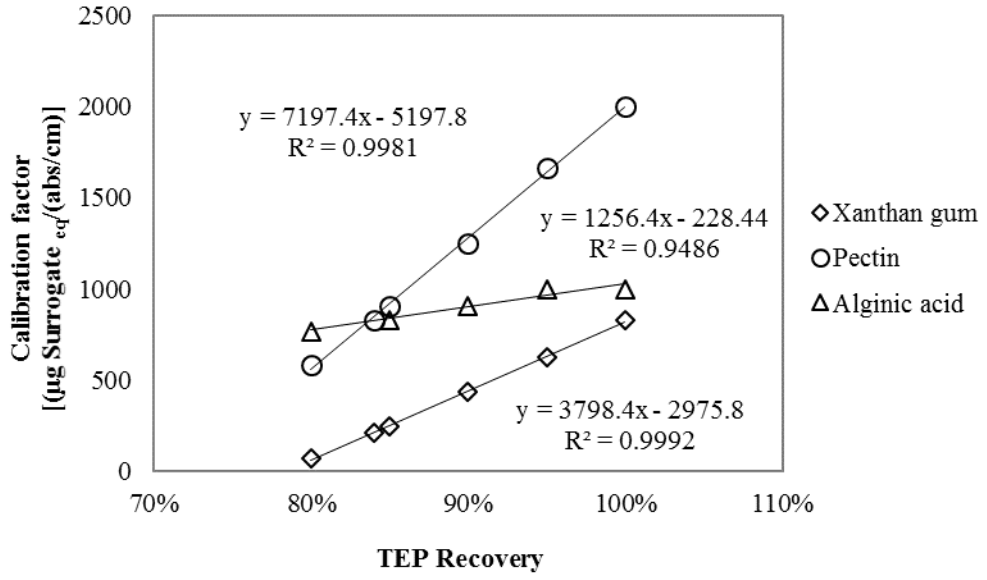

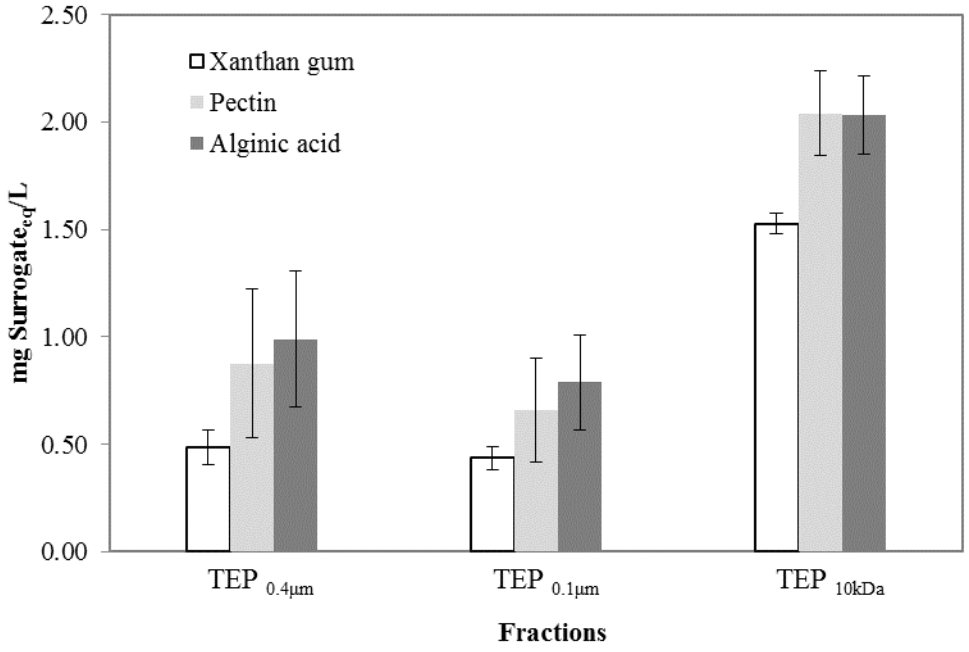

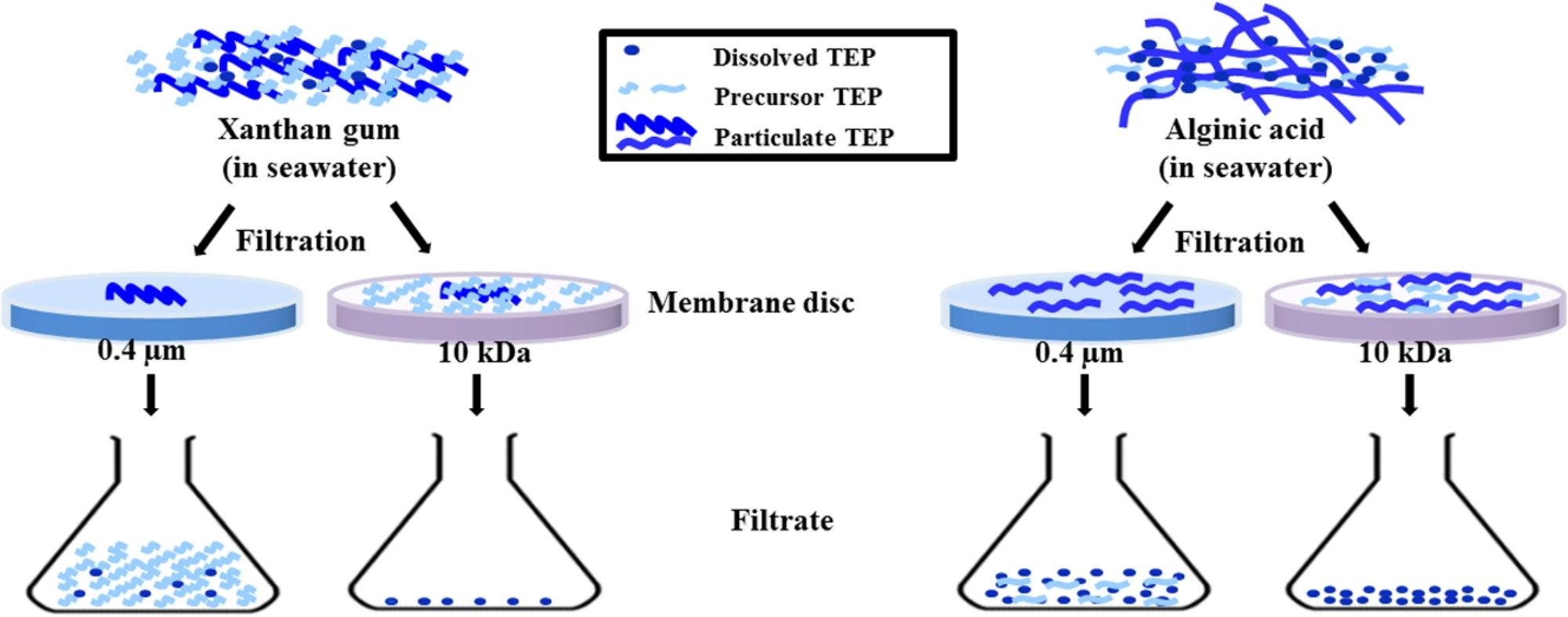
Xanthan gum
(in seawater)
Dissolved TEP
Precursor TEP
Particulate TEP
Alginic acid
(in seawater)
Filtration
Filtration
Membrane disc
0.4 µm
10 kDa
0.4 µm
10 kDa
Filtrate

Sample	Wavelength (nm)	Concentration range (mg/L)	RSD[a] (%)	R^2
D-Glucose	485	1.52-10.65	1.19-8.12	0.9921-0.9965
D-Mannose	487	1.52-10.65	0.95-3.84	0.9967-0.9982
D-Galactose	487	1.52-10.65	0.36-6.63	0.9913-0.9953
D-Glucuronic acid	480	1.52-10.65	2.09-7.25	0.9898-0.9943
D-Galacturonic acid	480	1.52-10.65	1.05-8.31	0.9909-0.9942
Mixed sugar standards[b]	485	1.52-10.65	1.04-4.94	0.9901-0.9941
Xanthan Gum	485	1.14-11.42	7.03-15.20[c]	0.9985-0.9999
Pectin	480	1.14-11.42	1.05-6.14	0.9985-0.9996
Alginic acid	480	1.14-11.42	1.32-6.37	0.9987-0.9992
Seawater	485	N/A[d]	N/A	N/A

a. Relative standard deviation of three samples using total carbohydrate analysis.

b. Mixed sugar solution was made from D-glucose, D-Mannose and D-Glucuronic acid with a molar ratio of 2.8: 2.0: 2.0 to mimic the composition of xanthan gum.

c. RSD value was 1.1% for repeated measurements (n=10) of a 5.71 mg/L of xanthan gum sample.

d. Not available.

	Passow and Alldredge, 1995	Villacorte et al. 2009b	This study
	Dry weight method[a]	TOC[b] method	Phenol-sulfuric acid method
Initial feed	×[c]	√[d]	√
Filter	√	×	√
Filtrate	×	√	√
Mass balance	×	×	√

a. Dry weight method of measuring surrogates on pre-weighed filter (Sharp 1991).

b. Total organic carbon.

c. not measured.

d. measured by surrogate calibration method.

Salinity	$TEP_{pre\text{-}stain}$			TEP_{10kDa}			$TEP_{0.1\mu m}$			$TEP_{0.4\mu m}$		
	XG[a]	Pectin	AA[b]	XG	Pectin	AA	XG	Pectin	AA	XG	Pectin	AA
DDI[c]	89.39	87.33	84.83	85.44	82.31	113.17	90.15	111.56	92.98	84.20	112.67	85.26
	(5.78)[d]	(2.94)	(9.79)	(7.64)	(6.14)	(5.75)	(4.43)	(1.35)	(2.97)	(6.02)	(2.13)	(5.25)
4000 mg/L SS[e]	95.02	97.65	106.30	96.39	108.70	102.20	95.9	102.06	93.31	87.38	98.05	100.62
	(4.46)	(5.61)	(10.42)	(12.45)	(4.57)	(4.72)	(7.10)	(7.53)	(10.80)	(7.24)	(8.75)	(3.98)
35000 mg/L SS	98.19	93.86	103.39	87.03	97.29	84.25	97.36	95.16	101.06	95.31	101.17	94.09
	(1.19)	(7.09)	(5.98)	(8.01)	(7.90)	(2.61)	(5.15)	(2.14)	(1.02)	(0.36)	(4.25)	(7.48)

a. xanthan gum

b. alginic acid

c. distilled deionized water.

d. standard deviation of triplicate filtration sample measurements.

e. sea salt