An updated method for the calibration of transparent exopolymer particle measurements

Thais B. Bittar,1* Uta Passow,2 Liti Hamaraty,3 Kay D. Bidle,3 Elizabeth L. Harvey1

1Department of Marine Sciences, Skidaway Institute of Oceanography, University of Georgia, Savannah, Georgia, USA
2Marine Science Institute, University of California Santa Barbara, Santa Barbara, California, USA
3Department of Marine and Coastal Sciences, Rutgers University, New Brunswick, New Jersey, USA

Abstract

Transparent exopolymer particles (TEP) are biologically derived, polysaccharide-rich, gel-like particles that play important roles in ocean carbon cycling. The original spectrophotometric method for TEP quantification published in 1995 by Passow and Alldredge describes staining TEP with the dye Alcian Blue (AB), and calibration of the AB staining solution using a commercially available polysaccharide xanthan gum (XG) powder as the reference material. In the original method, a XG solution is prepared to create gel-like particles that resemble TEP. The solution is filtered to collect the XG gels onto a filter, and the filters are stained with AB solution. However, recently, it has been found that the XG powder commercially available today has higher solubility and forms negligible amounts of gel particles in solution, which precludes its use as described in the original method. Here, we present an updated calibration method to generate reproducible XG calibration curves using the currently available XG powder, whereby a XG dilution series is stained with AB solution prior to filtration resulting in AB-stained XG gels that are retained on a filter. Subsequent extraction and spectrophotometric analysis are performed as described in the original method. The updated calibration method described here yielded calibration curves consistent with those reported in the original method. The updated method should only be used in the preparation of a calibration curve when the new soluble XG is available, whereas samples for TEP quantification should be processed as described in the original method.

Transparent exopolymer particles (TEP) are polysaccharide-rich, gel-like particles that comprise an ecologically and biogeochemically relevant fraction of the organic carbon in the ocean (Alldredge et al. 1993). TEP display adhesive properties, can trap living and nonliving particles, and adsorb dissolved and colloidal organic matter and mineral elements, producing large aggregates known as marine snow (Passow 2002). Marine snow is a nutrient-rich micro-environment for particle-attached bacteria and can serve as food to zooplankton and benthic fauna (Caron et al. 1982; Simon et al. 2002). Given TEP is sticky and attaches to detrital and/or mineral particles, the size and sinking velocities of TEP-rich aggregates increase through accumulated incorporation. The vertical transfer of particulate organic carbon by sinking TEP-rich marine snow aggregates represents an important mechanism of carbon and energy export from the surface to the mesopelagic and deep ocean (Passow et al. 2001). Owing to the ecological and biogeochemical relevance of these particles, TEP quantification has important implications for our understanding of food web and mesopelagic interactions, and carbon cycling in the ocean.

TEP is operationally defined as particles (>0.22 μm) stainable by the cationic dye Alcian Blue (AB; Alldredge et al. 1993; Passow 2002). AB selectively stains acidic polysaccharides (Ramus 1977) by ionically binding with \(-\text{COOH}\) and \(\text{O}^\text{SO}_3^-\) groups, making TEP visible and enabling quantification. However, there are two main procedural challenges in TEP quantification via AB staining. First, the staining efficiency of TEP is dependent upon the presence and the number of acidic groups \(-\text{COOH}\) and \(\text{O}^\text{SO}_3^-\) “available” to bind with AB. The chemical composition of in situ TEP is expected to be diverse; thus the AB staining efficiency of TEP is likely variable. As AB staining is a proxy of an inherent TEP property (i.e., its acidity), rather than a direct measure of TEP mass, the technique provides only a semiquantitative assessment of TEP concentration. Second, AB precipitates in the presence of salts and binds...
with acidic dissolved polysaccharides (<0.22 μm), resulting in precipitation of these compounds out of solution and into gel-like particles. Thus, if the staining solution is added directly to seawater, TEP is overestimated. To accurately measure TEP concentrations, the dissolved fraction of a water sample (operationally defined as anything in seawater that passes through a 0.22 μm filter) must be removed (Passow and Allerdridge 1995). Vacuum filtration is the most convenient way to remove salts and dissolved polysaccharides in terms of effort, time, and cost efficiency and allows for easy adjustments in sample volume (from milliliters to liters) needed to estimate TEP depending on TEP concentration in a given sample.

The original method described by Passow and Allerdridge (1995) remains the most widely applied methodology for TEP quantification in oceanographic research. It has been used to quantify TEP in a myriad of environments, revealing patterns of TEP distribution in the ocean and broadening our understanding of the dynamics of these particulate organic carbon constituents (Simon et al. 2002). According to the original method, water samples are filtered onto 0.45 μm polycarbonate membrane filters and TEP retained on filters are stained with AB solution. Stained TEP is extracted from the filters in 80% sulfuric acid solution, and AB absorbance is measured spectrophotometrically at 787 nm.

The spectrophotometric quantification of TEP described above relies on calibration against a reference material for two reasons: (1) given the unknown nature of TEP chemical composition, a “model” polysaccharide with constant AB staining efficiency over a specific mass range provides a standardized unit of equivalence for AB-staining efficiency of natural TEP and (2) the amount of dye per weight of AB powder is variable, so a reference material is needed to standardize the AB staining solution. Xanthan gum (XG), a polysaccharide excreted by the bacterium Xanthomonas campestris, is the most commonly used reference material. According to the original method (Passow and Allerdridge 1995), a XG stock solution in distilled water is prepared from commercially available XG powder. Grinding the stock solution with a tissue grinder leads to the formation of gel-like XG particles that resemble TEP. Known volumes of this unstained XG stock solution are filtered under gentle vacuum, and XG gels retained on the filters are stained with AB solution. Blank filters are also stained as a measure of inherent AB staining of polycarbonate membrane filters in the absence of XG. Extraction and absorbance measurements are performed as detailed earlier. The mass of XG on the filters is determined as dry weight from a second set of unstained filters. The resulting calibration curves are fit using a linear regression. In the original calibration method, the f-value, defined as 1/slope of the regression, is used to calculate TEP concentrations in a sample, in units of XG equivalents per unit volume of sample filtered, from AB absorbance values. Using this approach, the vast majority of TEP data in the literature are reported as μg-XG equivalents L⁻¹.

Our recent attempts to reproduce the XG calibration curves according to the original method have been unsuccessful. To some extent, these difficulties are also reflected in the literature (Discart et al. 2015), where AB staining solutions (and resulting TEP measurements) are not calibrated and data are reported as unit-less absorbance values or as relative changes in absorbance, such as percentage (Kuznetsova et al. 2005; Harlay et al., 2007; Kahl et al. 2008; Vardi et al. 2012). We designed a cross-laboratory calibration experiment, in a joint institutional effort, to determine the factors hindering, and often precluding, the successful reproduction of the original calibration method. This comprehensive exercise discovered that the currently available XG powder no longer exhibits the same solubility properties as the XG powder that was utilized in the original method. Most notably, the solubility of the currently available XG is much higher, and negligible amounts of gel-like particles are formed in solution, regardless of whether a tissue grinder is used in the solution preparation. These differences between the old and new XG, both as a powder and in solution, are fundamental and visible to the naked eye. Once in solution, the old XG forms visible, insoluble gels that give the solution a “cloudy” appearance, while the new XG in solution dissolves completely, appearing clear and with no gels (Fig. 1).

Given these significant differences in solubility properties, the original calibration method, which relies on capturing unstained XG gels on filters, cannot be used to produce calibration curves when the new XG is used. In this study, we describe a protocol to generate calibration curves using the new, commercially available XG powder. A XG stock solution is prepared in ultrapure (or deionized) water at a known concentration, without grinding. From the stock solution, a XG dilution series is prepared, AB solution is added, and the mixture is filtered to collect AB-stained XG gels. Filters are extracted and absorbance is measured as detailed in the original method. XG mass is determined by XG concentration in the dilution series, with the assumption that all XG reacted with AB. This updated protocol was tested in three independent laboratories across the United States, with three different batches of XG and AB powders, and solutions. The procedure is simple, time efficient, and provides calibration curves consistent with to those reported in the original method. Ultimately, the updated calibration protocol detailed here helps to streamline TEP calibration, allowing for inter-laboratory comparability, and enhancing the quantitative understanding of the role of TEP in the ocean.

Materials and procedures

Precleaning procedures

All plastic and glassware were acid cleaned by overnight soaking in freshly made 2–5% HCl solution (pH 2) and rinsed thoroughly with ultrapure (or deionized) water. Glassware (except volumetric flasks) was also combusted at 450 °C.
overnight. Appropriate cleaning procedures are critical to avoid contamination of materials with built up salts and organic matter that could react with the dye and/or compromise filtration efficiency.

Solutions

All solutions were made in ultrapure (or deionized) water. Standard solutions used the new XG powder purchased in 2010 (Lab A, Harvey), 2003 (Lab B, Bidle), and 2017 (Lab C, Passow) (cat no. G-1253, Sigma) and were prepared at 75 mg L\(^{-1}\) dissolved in ultrapure (or deionized) water. The solutions were made in acid washed glass volumetric flasks and the exact concentration of the stock solution (exact amount of XG weighed) was noted. When added to ultrapure water, the new XG dissolves completely after a few minutes of mixing by inverting the flask several times until clumps of XG are no longer visible.

The AB (8GX, cat. no. A5268, Sigma) staining solution (400 mg L\(^{-1}\)) was made in ultrapure (or deionized) water previously acidified to pH 2.5 with glacial acetic acid, mixed to dissolve most of the AB, and filtered through 0.22 \(\mu\)m polycarbonate membrane to remove undissolved AB. Two additional AB concentrations were tested: 200 mg L\(^{-1}\), as reported in the original method, and 1600 mg L\(^{-1}\). Curves prepared with AB solutions at these three concentrations were used to verify the saturation threshold of the dye (i.e., the concentrations at which enough dye is present to stain all XG in solution).

Both XG and AB solutions were made the day before they were used and kept at 4 °C overnight. XG solution was made fresh for each curve. AB solution can be used for up to 30 d, unless noticeable AB precipitates form (see Comments and Recommendations section).

The extraction solution (80% sulfuric acid) was prepared according the original method in ultrapure (or deionized) water by slowly mixing ~98% sulfuric acid in water into a 1 L glass flask inside the fume hood and letting it cool down to room temperature.

Blanks

Procedural blanks consisted of triplicate, 1 mL volumes of ultrapure water stained with 0.5 mL of AB solution and then filtered. Procedural blanks were compared with blanks consisting of 0.5 mL AB solution added directly to dry filters mounted onto the filtration tower (“dry” blanks) or to filters through which 1 mL of ultrapure water was passed prior to the application of AB stain (“wetted” blanks). Wetted blanks are the blanks described in the original method (Passow and Alldredge 1995).

Calibration procedure

A serial dilution of the XG stock solution was performed by pipetting 0.125, 0.250, 0.500, 0.750, and 1 mL of XG stock solution, in triplicates, into clean 5-mL polypropylene tubes and bringing the final volume to 1 mL with ultrapure water. The dilution series prepared from a stock solution made at exactly 75 mg L\(^{-1}\) produces a 5-point dilution series containing 9.37, 18.75, 37.50, 56.25, and 75 \(\mu\)g-XG. Procedural blanks and standard solutions were stained by adding 0.5 mL of AB solution (400 mg L\(^{-1}\)) directly into the polypropylene tubes, for a final volume of 1.5 mL, and mixed by manually agitating the tubes for 1 min. The mixing of AB and XG solutions forms blue stringy gels that are visible to the naked eye at the highest XG concentrations. The stained aliquots were poured (not pipetted) directly onto the filtration towers and filtered through 0.22 \(\mu\)m or 0.45 \(\mu\)m polycarbonate filters (25 mm, Isopore, Millipore) at <175 mmHg. Either a standard glass filtration apparatus (tower and frit, Lab A) or a polysulfone filtration apparatus (funnel tower and support screen, Labs B and C) were used. Filtration performed using vacuum at 75, 150, and 175 mmHg did not result in significantly different absorbance values (Supporting Information Fig. S1A). Filters were transferred to clean 20 mL glass scintillation vials with...
polypropylene screw caps. Preparation of filters for extraction takes approximately 30 min. At this point, filters can be frozen at \(-20\,^\circ C\) or \(-80\,^\circ C\) for at least 7 d for later extraction, as absorbance values of frozen filters were not significant different from those from filters extracted on the day of filtration (no freezing, Supporting Information Fig. S1B).

The spectrophotometer was first blanked with ultrapure water. Before initiating the extractions, the absorbance of an aliquot of the acid extraction solution was recorded at 787 nm against ultrapure water to check for consistency and potential contaminations of each extraction solution batch (0.008–0.150, see Comments and Recommendations section). The instrument was then blanked with extraction solution. In a fume hood, 6 mL of extraction solution were added to the filters, and vials were capped immediately. Filters were soaked in extraction solution for 2–20 h and vials were agitated regularly (Passow and Alldredge 1995). Given that sulfuric acid is highly hygroscopic, extraction vials must be capped during extraction. The AB absorbance from filters extracted in 6 mL of extraction solution was read using 1.0 cm polystyrene disposable cuvettes (Fisher-Scientific, Hampton, New Hampshire). Alternatively, filters can be extracted in 3 mL of extraction solution, in which case absorbance must be measured using 0.5 cm cuvettes. Absorbance values (<1.0) at 787 nm were recorded (Passow and Alldredge 1995) using a diode array Agilent 8453 UV-visible spectrophotometer (Lab A), a Molecular Devices SpectraMax M3 (Lab B), or a ThermoFisher Genesys 10S Visible spectrophotometer (Lab C).

**Assessment of stained XG retention efficiency**

Determination of the XG mass is based on stock solution concentration and dilution series, rather than by XG dry weight from a replicate unstained set of filters (original method). To reliably derive XG mass from the stock concentration and dilution factors, it is important to verify the retention efficiency of the stained XG (i.e., whether there is any loss of stained XG during filtration). This was tested by determining AB absorbance both in the retentate (stained XG collected on filter, as described earlier) and in the filtrate (fraction that passed through the filter) (Fig. 2). The filtrate was collected in precombusted glass tubes placed inside the side-arm flask, re-stained with 0.5 mL of AB solution to ensure enough dye was present to bind with any unstained XG, and refiltered. Filters corresponding to the retentate and filtrate fractions were acid extracted and AB absorbance was measured as described earlier (Fig. 2). This assessment of XG retention efficiency was performed with 0.22 \(\mu\)m and 0.45 \(\mu\)m filters.

**Calibration curves and \(r\)-values**

XG mass for each calibration point was calculated as: XG mass (\(\mu\)g) = 1000 \times stock solution concentration (mg L\(^{-1}\)) \times volume of XG stock solution used for each point of the serial
Comparison between original and updated calibrations

To demonstrate that the original and new XG powders were fundamentally different regarding solubility and gelification potential, a calibration curve was performed according to the original protocol using the new XG (purchased in 2017).

Assessment

AB concentration

Curves produced according to the updated calibration method using AB at 200 mg L\(^{-1}\) (original method) showed under-staining of XG, particularly at mass greater than ~37 µg-XG (Fig. 3). No significant difference was observed between absorbance values in curves prepared with AB at 400 and 1600 mg L\(^{-1}\) (Fig. 3), showing that AB must be prepared at 400 mg L\(^{-1}\) or higher concentration, to contain enough dye to bind with all XG in solution.

In the original calibration method, the maximum amount of XG gels retained on filters was reported ~35 µg, even though the stock solution was prepared at the same concentration listed here, due to loss of nongel forming XG through the filter before staining (Passow and Alldredge 1995). Thus, in the original protocol, an AB solution at 200 mg L\(^{-1}\) contained enough dye to bind with all XG gels retained on the filters. In this study, the range of XG mass stained by AB in solution is extended to 75 µg, thus a higher AB concentration is needed.

Blanks

Absorbance values were 0.012 (±0.001) for procedural blanks, 0.014 (±0.004) for “dry” blank filters, and 0.013 (±0.002) for “wetted” blank filters (original method). Blank values reported here from both protocols and “dry” blanks represent the amount of AB trapped by the polycarbonate filter itself, i.e., the amount of dye that stains the filters.

Retention efficiency of AB-stained XG

For the updated method, blank-corrected absorbance values of filters corresponding to stained XG in the filtrate (Fig. 2) were ~0.002 (±0.003) and ~0.001 (±0.002) when 0.45 µm and 0.22 µm filters were used, respectively. These results indicated that 100% of stained XG was retained on the filters.

Calibration curves, f-values, and detection limits

Curves made with the new XG using the updated calibration method across three independent laboratories had f-values of 96, 100, and 76, and MDL of 5.6, 9.0, and 9.0 from labs A, B, and C, respectively. These values are in the range of those reported in the original method (88 and 139, Passow and Alldredge 1995). Similar MDL limits for the original calibration method have been reported previously (5 and 4 µg-XG, respectively, reported by Engel et al. 2009, Wurl et al. 2011) (Fig. 4).

Curves prepared using the new XG and original calibration protocol yielded very low XG mass on the filters (5–16 µg-XG), due to the high solubility of the new material and, consequently, low absorbance values (<0.070) that were not significantly different from one another (Fig. 4). The f-value calculated for that curve was over threefold greater than those for the calibration curves prepared according to the updated protocol.
Calibration against a reference material is a crucial step in any quantitative method based on spectrophotometry in oceanographic research (Lowry et al. 1951; Dubois et al. 1956), and calibration methods must be reproducible across laboratories. However, in the case of TEP calibration, the solubility properties of the XG powder currently available are fundamentally different than those of the XG powder used in the original calibration method (Passow and Alldredge 1995). In this study, we demonstrated the inability of the new XG powder to form gel-like particles in solution, which precludes the reproduction of the original calibration method and this incompatibility generated the need to develop an updated protocol. The updated method described here was shown to be reproducible across three different laboratories. In addition, it eliminates the need to produce a second set of unstained filters to determine XG mass, and thus the inherited error in these measurements, as well as the time to produce, dry, and weigh those filters. Thus, the updated method proposed here is simple, cost- and time-efficient, and provides a way forward for all researchers interested in reliably quantifying TEP in the ocean.

The \( f \)-values calculated for the curves produced with the updated method are within the range of those reported in the original calibration method, indicating that data calibrated using both methods are comparable. However, a literature review showed that few studies reported \( f \)-values for TEP calibration over the years, and those varied widely, from as low as ~40 (Biermann et al. 2015) to as high as 235 (Van Nevel et al. 2012). Variations in \( f \)-values have been previously attributed to different AB batches, thus it is unclear how the changes in XG solubility properties affected the calibration curves and TEP values reported in the literature over the past few years. In this study, we show that the AB solution prepared at a minimum concentration of 400 m L\(^{-1}\) is saturated, thus the effect of variable AB batches in the updated protocol should be minimized. We also demonstrated that curves (and consequently \( f \)-values) obtained at three independent laboratories are consistent, indicating that the updated calibration method provides the community with a robust protocol to calibrate TEP measurements moving forward.

It is important to note that, while the updated calibration method can be performed with the XG currently available on the market, seawater samples for TEP measurements must be filtered before staining. As AB precipitates with dissolved polysaccharides and salts, its direct addition to natural seawater samples may overestimate TEP concentrations (Passow and Alldredge 1995). Rather, TEP gels in seawater samples should be first collected by filtration and subsequently stained with AB solution, as described in the original method. Consequently, the updated calibration method described here should be restricted to the preparation of a XG calibration curve with current available XG powder. The fact that the calibration curves and samples are handled differently regarding the staining procedure (in solutions vs. on filter) is a caveat of the updated calibration method. However, we have demonstrated that the new XG cannot be retained on filters when unstained, thus to continue to use XG as a reference material and unit of equivalence for TEP concentration measurements, the new XG material must be stained prior to filtration.

The updated method proposed here describes a reliable determination of XG mass, consistent absorbance readings, and reproducible calibration curves among different laboratories. This represents an important advancement in the field, as at present, there has been a general inability of researchers to accurately calibrate and quantify TEP measurements due to fundamental differences in XG solubility. Obtaining reproducible calibration curves and, consequently, more reliable TEP
measurements will allow for the better understanding of natural distributions of TEP among distinct environments and conditions and carbon cycling dynamics in the marine environment.

**Comments and recommendations**

While scientific methods must be reproducible, small variability between laboratories can be expected with any multi-step hands-on protocol. In an effort to keep inter-laboratory variability to a minimum, the following checks are recommended:

**XG retention efficiency check**

Potential sources of variability in the retention efficiency of stained XG could be the result of differences in filtration apparatus or the accuracy of the vacuum pump readings. It is worth mentioning that two different types of filtration apparatuses (glass and polysulfone) were used in the work presented here and generated closely matching calibrated curves. Despite this agreement, to maximize the accuracy and comparability of this calibration, each laboratory should conduct a retention efficiency test (Fig. 2) to determine the efficiency of retention of AB-stained XG for the particular lab materials in use. Retention efficiency lower than 100%, but consistent throughout the 5-point calibration curve, must be accounted for in XG mass calculation.

**Absorbance check**

The 80% sulfuric acid solution should be inspected for background absorbance and potential contaminations, particularly if stored in contact with plastic. Sulfuric acid is a strong acid and long-term storage in plastic containers or in contact with plastic parts (e.g., a bottle-top dispenser) can cause slow degradation of the plastic that can lead to yellowing of the solution. This can cause high blanks and interfere with the calibration curve. Thus, the absorbance of the extraction solution at 787 nm should be checked routinely before extraction.

**AB solution storage**

AB solutions can become unstable and form insoluble precipitates over time. As the dye gradually precipitates out of solution over time, older solutions will become less efficient in staining XG. In line with observations by Villacorte et al. (2009), AB solutions filtered once and stored at 4 °C were stable for at least 30 d in our laboratory (data not shown). However, that time frame might be different for different AB batches. Therefore, whenever possible, it is recommended to prepare a fresh AB solution that will be filtered once just before preparing a calibration curve and staining a batch of samples. If not using a fresh AB solution, it is recommended that the solution is checked for the formation or precipitates, both by visually inspecting the solution and by running a blank check to verify whether blank values are significantly higher than those obtained on the day the solution was prepared and filtered. If the solution is older than 30 d and/or precipitates are visible and blank values increase, the solution should be discarded. Refiltering solutions multiple times due to the presence of precipitates will only eliminate the precipitated dye and will not mitigate the decreased staining potential of the solution. Older, weak solutions will likely result in under-staining of XG, thus affecting the reproducibility and reliability of calibration curve and compromising the contextualization of a study in the larger body of literature.

**Reporting f-values**

Of the 192 published manuscripts that utilize the original Passow and Alldredge (1995) TEP calibration method, only seven studies actually report the f-value utilized in their study. Given the inherent variability in AB batches, it is critical that the researchers, who are interested in quantifying TEP, adequately report the details of TEP calibration protocols, particularly f-values and the detection limit for each curve. This will greatly facilitate accurate comparisons between data sets generated by multiple laboratories.

**Abbreviations**

| AB  | Alcian Blue. |
|-----|--------------|
| TEP | transparent exopolymer particles |
| XG  | xanthan gum |

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Acknowledgments
The authors thank A. Stubbins for the use of the spectrophotometer; J. Sweet, T. Jenarewong, and P. Duffy for laboratory assistance; and A. Boyette and L. DeLeo for graphical assistance with Figs. 1 and 2. This work was supported by the National Science Foundation (OCE-1459190 to E.H.; OCE-1459200 and OCE-1537951 to K.D.B., OCE-1658527 to U.P.).

Submitted 3 May 2018
Revised 5 July 2018
Accepted 9 July 2018

Associate editor: Gordon Taylor