Determination of extracellular polymeric substances (EPS) using a modified phenol-sulfuric acid (PSA) assay for sugars

Jody Deming[1], Shelly Carpenter[1]

[1]University of Washington, School of Oceanography

Shelly Carpenter

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We use this protocol and it's working

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ABSTRACT

This described protocol reflects our experience in measuring extracellular polysaccharide substances (EPS), particulate and dissolved, in very cold and salty samples from polar regions. We adapted the protocols from the original phenol-sulfuric acid extraction method of Dubois et al. (1956) in our efforts to find a suitably quantitative measure of EPS (Decho and Gutierrez, 2017). The described protocol reflects successful efforts as published in the papers cited below.

Method:
DuBois, M, Gilles, KA, Hamilton, JK, Rebers, PA, Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28(3): 350–356. https://doi.org/10.1021/ac60111a017

Published examples of method use:
Krembs, C, Eicken, H, Deming, JW. 2011. Exopolymer alteration of physical properties of sea ice and implications for ice habitability and biogeochemistry in a warmer Arctic. *Proceedings of the National Academy of Sciences* 108(9): 3653–3658. https://doi.org/10.1073/pnas.1100701108

Ewert, M, Carpenter, SD, Colangelo-Lillis, J, Deming, JW. 2013. Bacterial and extracellular polysaccharide content of brine-wetted snow over Arctic winter first-year sea ice. *Journal of Geophysical Research: Oceans* 118(2): 726–735. https://doi.org/10.1002/jgrc.20055

Cooper, ZS, Rapp, JZ, Carpenter, SD, Iwahana, G, Eicken, H, Deming, JW. 2019.Distinctive microbial communities in subzero hypersaline brines from rarely sampled cryopegs and the sea ice of the coastal Arctic. *FEMS Microbiology Ecology*95(12): fiz166.DOI: 10.1093/femsec/fiz166

Rivaro, P, Ardini, F, Grotti, M, Vivado, D, Salis, A, Damonte, G. 2021. Detection of carbohydrates in sea ice extracellular polymeric substances via solid-phase extraction and HPLC-ESI-MS/MS. *Marine Chemistry***228**: 103911. https://doi.org/10.1016/j.marchem.2020.103911

EPS review:
Decho, AW, Gutierrez, T. 2017. Microbial extracellular polymeric substances (EPSs) in ocean systems. *Frontiers in Microbiology*8: 922. https://doi.org/10.3389/fmicb.2017.00922

MATERIALS

Materials for sample collection:

- Polycarbonate (PC) membrane filters (Poretics or similar, 0.4-µm pore size, 25-mm or 47-mm diameter) to capture particulate EPS (pEPS) for measurement
- Glass fiber filter (Gelman Type A/E) as backing disk (25-mm diameter; optional)
- Pall polysulfone plastic filter tower/base on a standard filtration manifold or a fritted glass filtration assembly over a filtration flask if also catching filtrate for dissolved EPS (dEPS) measurement (25-mm or 47-mm diameter; 47-mm may be best for high biomass samples, as the larger surface area reduces clogging)
- Standard vacuum pump and tubing (use < 15 inch Hg vacuum pressure, as standard practice to avoid breaking cells)
- Plastic scintillation vials or microcentrifuge tubes for storing filters
- Freezer (–20°C)
- Labels or indelible pen and tape
- Graduated cylinder appropriate for sample measurement (typically 500-ml cylinder)
- Filter forceps
- Bottle or tube (method-dependent) for saving filtrate for dEPS measurement.

Notes: Everything should be washed and rinsed well with 2-um-filtered deioinized (DI) water (e.g., MilliQ water) before a sample is sub-sampled for filtering and further processing.Rinse labware copiously with DI water between samples.Acid-rinsed glassware is ideal, but may not be possible in the field, when copious rinsing with DI water suffices. For cold marine samples, filtration is done in a temperature-controlled room at the nearest *in situ* temperature possible. Always mix (invert manually several times) a bulk sample before sub-sampling it.

Materials for laboratory processing:

Reagents:
(1) Concentrated sulfuric acid (96%), reagent grade conforming to ACS specifications.
(2) Phenol (5% solution) prepared by adding 5 ml of 45°C-melted phenol (Sigma Chemical #P1037) to 95 ml distilled or DI water and store in a tightly sealed glass bottle at room temperature in the dark (foil wrapped).
(3) Glucose standard (Sigma Chemical #G6918)
(4) Filtered (0.2 µm) deionized water (DI)

Materials:
- Spectramax M2 microplate reader or similar
- 30°C oven
- 96-well microplates for spectrophotometer
- Borosilicate glass tubes (12 mmx 75 mm) or similar reaction tube
- Parafilm squares
- Appropriate pipetman and tips

- Eppendorf repeater pipettor #4780 with 2.5 ml Combitips

SAFETY WARNINGS

Consult the Safety Data Sheets (SDSs) for concentrated phenol as well as for concentrated sulfuric acid and make sure those safety guidelines are followed when using these chemicals, including the use of proper PPE and spill protection measures.

Subsampling for EPS

1 Using a DI-rinsed graduated cylinder, sub-sample 300–500 ml of the sample of interest (reduce the volume if a high biomass is known or suspected, as the filter will clog). Record the volume filtered. (Marine examples: for seawater, > 600 ml is typical; for melted bottom sea ice, 25 ml may be adequate if a visible algal band was present.) DI = 0.2-µm-filtered deionized water.

2 Filter volume onto PC membrane filter using a backing disk and DI-water-rinsed filtration tower/base (and filtration flask if dEPS is being collected) using gentle vacuum pressure (<15 inches Hg on the gauge). Record the volume filtered. PC = polycarbonate; dEPS = dissolved EPS.

3 Using forceps fold the filter in half twice (contents inwards) and place the folded filter in a labeled plastic vial or tube and freeze until processed in the laboratory.

4 If sampling dEPS, remove enough filtrate from the filter flask (1–5 ml for organic-rich samples and 125 ml for typical seawater samples) to an appropriate clean container (e.g., sterile 125-mL glass bottle or 50-cc disposable plastic tube) and label/freeze until processed in the laboratory.

Quantitation of pEPS

5 Prepare a glucose standard curve using DI water. (We have compared artificial seawater solutions with DI water and found little difference.).

6 Transfer frozen filters into glass tubes to thaw at room temperature. Add 250 µL of DI water to the tube and mix well by shaking manually.

7 Add 250 µL of 5% phenol to the tube and vortex, then add 1,250 µL of the sulfuric acid (5 x 250 µL increments using the repeater and combitips). Add acid increments cautiously but in quick succession in a fume hood. (This reaction is highly exothermic, gets very hot, and can become frothy or foam over if the sample salt concentration is high, as in winter sea-ice brines; in that case, a temporary loose parafilm cover over the tubes is advised.)

8 Let glass tube sit to cool, then cap with parafilm and mix by vortexing at low speed (cautiously).

9 Pipet 200 µL reaction mixture from the tube into a 96-well microplate (acid is corrosive to the metal pistons of classic pipetmen; use barrier tips for this step.)

10 Incubate the tubes or microplates at 30°C for 30 minutes before measuring absorption at 490 nm.

11 Read using Spectramax M2 Spectrophotometer/Plate Reader

Converting measured OD490 to µg/mL glucose equivalents

12 Obtain the linear function describing the relationship between absorption (OD490) and glucose equivalent (in µg) of the standard curve. Use linear regression, resulting in a standard curve function of y = mx + b. Account for the reaction volume to convert µg glucose/mL to µg glucose. OD = optical density.

13 Using the function established above, convert from OD490 (y) to glucose equivalents (x) as follows: x = (y – b) / m.

14 If measuring pEPS (x), account for the volume of sample filtered (Vf). The formula therefore becomes: x = (y – b) / (m * Vf).

15 For pEPS analyses, give some thought to carbohydrates contributed by cells captured on the filter.By estimating cell volume and C content and accounting for cell numbers in our samples, we have found the intracellular C contribution of EPS to be negligible.

Quantitation of dEPS

16 Measure 250 µL of thawed sample filtrate directly, following the pEPS protocol above but without the addition of DI water.If no EPS is detected, then the sample can be concentrated for a second analysis, if desired; see Ewert et al. (2013) in protocol description.