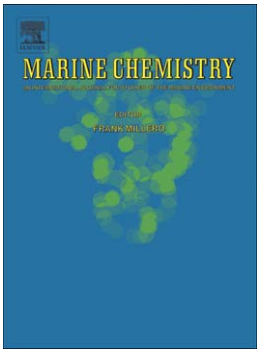

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PII: S0304-4203(11)00101-0
DOI: doi: 10.1016/j.marchem.2011.09.004
Reference: MARCHE 2891

To appear in: *Marine Chemistry*

Received date: 8 October 2010
Revised date: 12 September 2011
Accepted date: 15 September 2011

Please cite this article as: Engel, Anja, Händel, Nicole, A novel protocol for determining the concentration and composition of sugars in particulate and in high molecular weight dissolved organic matter (HMW-DOM) in seawater, *Marine Chemistry* (2011), doi: 10.1016/j.marchem.2011.09.004

A novel protocol for determining the concentration and composition of sugars in particulate and in high molecular weight dissolved organic matter (HMW-DOM) in seawater

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Running head: analysis of carbohydrates in marine samples

Key words: combined carbohydrates, acidic sugars, neutral sugars, amino sugars, HPAEC-PAD, membrane dialysis

Abstract

A method is described to simultaneously determine the neutral, amino, and acidic sugar content of combined carbohydrates in high molecular weight (HMW, >1kDa) dissolved organic matter and in particles from seawater samples. Monomeric sugars are determined after acid hydrolysis and neutralization through acid evaporation using high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD). The separation of sugars during chromatography is achieved in two steps, an isocratic elution (18 mM NaOH) followed by a gradient course of two mobile eluent phases (NaOH and CH_3COONa). HPAEC-PAD has previously been applied to measure neutral and amino sugars in marine samples. Since salt anions interfere with the measurement, some of the earlier studies used ion exchange resins for seawater desalting. Thereby, variable losses of neutral and amino sugars, and the complete removal of acidic sugars have been reported. Here, we show that desalting by membrane dialysis (1kDa) is an efficient alternative to ion exchange resins and yields recoveries of >90% for HMW carbohydrates. We conducted several tests to determine the accuracy and reproducibility of the method. Sugar concentrations determined with our protocol were compared to results obtained with the colorimetric TPTZ-method, and with earlier HPAEC-PAD protocols using cation/ anion exchange resins. Applications of our protocol to field samples indicated that acidic sugars can comprise a substantial fraction (30-50%) of HMW dissolved carbohydrates in seawater. The simultaneous analysis of the three classes of sugars appears promising to detect a larger fraction of marine combined carbohydrates, and thus to improve our understanding of organic matter cycling in the ocean.

Acknowledgements

We thank H.-P. Grossart, H.-J. Mohr, J. Szlosek and J. Koch for fruitful discussion on operation of the Dionex ICS, and R. Weinert (ICBM, Oldenburg) and J. Roa for technical assistance. J. Piontek and C. Borchard are gratefully acknowledged for providing field and culture samples for polysaccharide analysis. The staff of the research vessel Belgica and L. Chou are gratefully acknowledged for their support in obtaining field samples from the Bay of Biscay. This study was supported by the Helmholtz Association contract no HZ-NG-102.

Introduction

The vast majority of organic compounds in the ocean eludes chemical analysis (Wakeham et al. 1997, Hedges 2002, Lee et al. 2004), and thus impedes the exploration of organic matter reactivity and cycling in marine ecosystems. A large fraction of the characterized marine dissolved and particulate organic matter is comprised by carbohydrates, i.e. sugars that are released from polysaccharides and other biopolymers during hydrolysis (Benner 2002). Carbohydrates in seawater include neutral sugars (e.g. glucose, galactose, mannose, rhamnose, arabinose, xylose, ribose and fucose), aminosugars (e.g. galactosamine and glucosamine), and acidic sugars, mainly uronic acids, (e.g. glucuronic acid and galacturonic acid), phosphorylated and sulphated sugars (Mopper 1977, Kaiser and Benner 2000, Leppard 1995). Combined carbohydrates (CCHO) serve as structural components and for energy storage in marine organisms, and comprise about 17-40% of particulate organic matter dry weight (Parsons et al. 1983). Through exudation from phyto- and bacterioplankton cells, herbivorous grazing, microbial degradation and lysis of cells, CCHO are released into seawater, where they represent the largest dynamic carbon source in the ocean (Carlson 2002, Benner 2002). In the ocean, CCHO represent the largest characterizable pool (50-70%) of high molecular weight (HMW) dissolved organic carbon (DOC) (Benner et al. 1992; Pakulski and Benner 1994, Benner 2002, Kaiser and Benner 2009), which is the fraction of DOC that is retained on a membrane with a 1kDa cut-off. HMW-DOC largely represents material freshly produced and consumed by plankton organisms (Amon and Benner 1994, 1996) and is typically observed in highest concentration in the surface ocean (Benner 2002). Carbohydrates contained in HMW-DOC have been suggested to contribute to vertical carbon export in the ocean, as these substances can accumulate in surface waters during vernal times and are mixed into depths during winter

overturning (Carlson et al. 1994, Hansell and Carlson, 2001). Hence, a better knowledge on the dynamics of carbohydrates in HMW-DOC can provide valuable insights to biological carbon cycling, with typical timescales of days to one year.

Carbohydrates comprise between 1 and 2% of Low Molecular Weight (LMW, <1kDa) DOC in surface waters and < 1% in deep waters (Kaiser and Benner 2009, Skoog and Benner 1997). In general, the fraction of LMW- substances that can be biochemically characterized is small (<2%) (Benner 2002, Kaiser and Benner 2009). LMW-DOC is suggested to be diagenetically altered and of low bioavailability, with typical turn-over times of centuries to millennia (Amon and Benner 1996, Hansell et al. 2009).

In seawater, CCHO are primarily polysaccharides; i.e. polymeric sugars that are joint by glycosidic bonds and contain more than ~10 monomer units, equivalent to a molecular weight of >1.5 kDa. Heteropolysaccharides that contain acidic sugars are termed acidic polysaccharides (Decho 1990, Leppard 1995). Acidic polysaccharides include commercially important polysaccharides such as pectin and alginic acid, also known as mucopolysaccharides. Extracellular acidic polysaccharides have gathered much attention in marine biogeochemical research over the last decades. Advances in trace metal biogeochemistry for example, have highlighted their role in trace element cycling, i.e. Fe and Zn, or for removal of the particle reactive tracer ^{234}Th (Quigley et al. 2002, Guo et al. 2002). Several studies also demonstrated that acidic polysaccharides participate in gel particle formation, e.g. the formation of transparent exopolymer particles (TEP) (Mopper et al. 1995, Zhou et al 1998, Chin et al. 1998, Kerner et al. 2003, Verdugo et al. 2004, Engel et al. 2004). Gels establish a bridge between DOM and particulate organic matter (POM), and influence the biogeochemical composition of POM qualitatively (Verdugo et al. 2004). Engel et al (2002), for instance, demonstrated that TEP production results in a selective enrichment of

POC during diatom blooms. Micro- and macrogels can aggregate with each other, and with other organic and mineral material, leading to the formation of fast settling marine snow that accelerate carbon export to the deeper ocean. Recent studies also indicated that the production of acidic polysaccharides is sensitive to changes in seawater CO_2 concentration (Engel, 2002; Engel et al., 2004, Mari 2008). Hence, increasing anthropogenic CO_2 concentration may affect the concentration and molecular composition of organic matter in the ocean. Consequences for future particles dynamics, trophic interactions and marine biogeochemistry are to be expected (Arrigo 2007).

Despite their suggested important role in organic matter fluxes, element cycling, and ecosystem dynamics, little is known about the seasonal and spatial variability of the concentration and composition of CCHO, and in particular of acidic polysaccharides, in the ocean. This can largely be attributed to analytical difficulties in detecting sugars, especially against a high background of sea salt. Several different approaches have been followed to quantify CCHO in marine samples (see Panagiotopoulos and Sempéré 2005, for review); most of them require the hydrolytic cleavage of the polymer into its monomeric components prior to analysis. The monosaccharides are then determined either as bulk carbohydrates after derivatisation (e.g. Burney and Sieburth 1977; Myklestad et al. 1997, Hung and Santschi 2001), or as individual sugars using gas or liquid chromatography (e.g. Cowie and Hedges 1984, Walters and Hedges 1988, Mopper et al. 1992). A high precision method for the direct determination of carbohydrates without prior derivatisation is the high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD). This method has been shown to give reproducible results for neutral sugars from seawater samples at the nanomolar level (e.g. Mopper et al 1992, Skoog and Benner 1997, Borch and Kirchmann 1997). Later, Kaiser and Benner (2000) introduced HPAEC-PAD to determine amino sugars in marine organic matter.

Applications of HPAEC-PAD for sugar analysis in seawater require a desalting step prior to chromatography, because salt anions compete with the sugar moieties for the anion-exchange sites of the column. Salt ions thus interfere with the chromatographic separation, which affects sugar retention times and the reproducibility of measurements. Seawater desalting has frequently been conducted using ion exchange resins, such as the anion exchange resin AG2-X8 and the cation exchange resin AG50W-X8 (BioRad) (e.g. Mopper et al. 1992). These resins, however, also remove acidic sugars. As a consequence, acidic sugars have not been included in most marine studies applying HPAEC-PAD so far. In order to include acidic sugars in HPAEC-PAD, Wicks et al. (1991) tested a variety of resins to desalt seawater. The six resins that they tested (amino, cyano, diol, aromatic sulfonic, quaternary amine and polyethyleneimine resins) yielded an average recovery of neutral, amino and acidic sugars ranging on from only 6.5-19.4% with large differences for individual sugars.

Here, we report a novel protocol for the analysis of sugars in particles, and in HMW-DOM from seawater samples. Our protocol uses seawater desalting by membrane dialysis instead of resins. We optimized hydrolysis conditions and modified HPAEC-PAD settings to include different groups of natural heteropolysaccharides, i.e. neutral, amino and acidic sugars, in a single chromatographic run. The aim of this protocol is to advance and simplify the analysis of HMW carbohydrates from marine samples and to include the detection of acidic sugars during field and laboratory applications. Several tests were conducted to determine the accuracy and reproducibility of the method and the recovery of individual carbohydrates during the dialysis, hydrolysis and neutralization steps. Furthermore, we report results obtained from application of the method during field studies.

Materials and Procedures

The HPAEC-PAD system

HPAEC-PAD was conducted to directly determine and quantify neutral, amino and acidic sugars from seawater samples. HPAEC-PAD takes advantage of the weakly acidic property of carbohydrates (pK=12-13), which at high pH (> pH 13) are partially ionized and can thus be separated with a strong anion exchange stationary phase. Single carbohydrates are then detected by measuring the electrical current generated by their oxidation at the surface of a gold electrode. Pulsed amperometric detection (PAD) employs a repeating sequence of four potentials (E): E1 a potential to detect the current from carbohydrates oxidation, E2 a high negative potential to clean the surface of the electrode, E3 an activation potential to retain an active potential and E4 an oxide reduction potential to reduce the oxide formed in E3 (Rocklin et al. 1998).

Here, we used a Dionex 3000 ion chromatography system for detection of sugar monomers by HPAEC-PAD. This system is equipped with an ICS-3000 SP gradient pump, a thermal compartment (ICS-3000 TC) for precise temperature control of columns from 5°C to 85ºC, a temperature controlled autosampler (AS 50), a detector compartment containing a set of chromatographic columns and an electrochemical detector (ED40) for pulsed amperometric detection (PAD). The detector cell includes a gold working electrode and a pH reference electrode (Ag/AgCl). The following detector settings were used: E_1 = 100 mV (T=0.4 s), E_2 = -2000 mV (T=0.42 s), E_3= 600 mV (T=0.43 s) and E_4 = -100 mV (T=0.5 s). The system control and data acquisition were carried out with Chromeleon CHM1 software (Dionex). Separation of neutral, amino and acidic carbohydrates was achieved using a Dionex CarboPac PA10 analytical column (2x250 mm) coupled with a Dionex CarboPac

PA10 guard column (2 x 50mm). The CarboPac PA10 consists of 10µm polystyrene/ divinyl benzene cross-linked pellicular substrate agglomerated with 460 nm MicroBead latex (Dionex). The capacity of the CarboPac PA10 is approximately 100 µeq per column. The injection of sample was performed using a 25 µl loop. The retention of carbohydrates on exchange columns, and thus the reproducibility of results are highly sensitive to changes in temperature (Panagiotopolus et al. 2001, Yu and Mou 2006). For our system, best resolution of sugars was obtained at 25 °C and therefore applied constantly during all analyses. In order to minimize degradation of samples before analysis, the temperature in the autosampler was kept at 4 °C.

Operation procedure

Separation of neutral and amino sugars was achieved during isocratic elution of a mobile eluent phase (eluent I= NaOH, 18 mM), followed by a gradient course of two mobile eluent phases (eluent I= NaOH, 18-100 mM; eluent II= CH_3COONa, 200 mM) for separation and detection of acidic sugars (Table 1). The columns were flushed with 18mM NaOH for 19 min to equilibrate the system before injection of the first sample and between samples. In order to remove carbonates and other organic and inorganic contaminants, the columns were rinsed with 200 mM NaOH for 30 min after every seventh run and equilibrated to 18 mM NaOH for 45 min. The flow rate of eluent stream was 0.250 ml min^{-1} throughout operation. All samples were analyzed in duplicates, whereas the standard solutions for calibration were measured in triplicates.

For eluent I, a 250 mM NaOH solution was prepared by diluting low-carbonate NaOH solution (50% w/w, Fluka) in filtered Milli-Q water, which had been degassed with helium (5.0, Airliquide) for at least 20 min. For eluent II, a 1 M sodium acetate (CH_3COONa) solution

was prepared by dissolving an aliquot of sodium acetate (99%, anhydrous, Fluka) in Milli-Q water. The sodium acetate solution was filtered through a 0.2 μm nylon membrane and degassed for at least 20 min. All eluent containers were closed and pressurized with helium to 0.15 MPa to minimize the uptake of atmospheric carbon dioxide. Changes in concentration of these two eluents were obtained by dilution with high-degree de-ionized water from a Milli-Q system (18 MΩ, ELSA), vacuum filtered through a 0.2 μm nylon membrane and degassed with helium for at least 20 min.

The system was calibrated with a mixed sugar standard solution (Table 2). The solution was prepared with Milli-Q water, divided into aliquots and stored at -20 °C in precombusted (500 °C for 8 h) glass vials until required. The mixed sugar standard included a) the neutral sugars: fucose (4.6 μM, Fuc), rhamnose (3.1 μM, Rha), arabinose (2,0 μM, Ara), galactose (2.4 μM, Gal), xylose/ manose (3.1 μM, Xyl/ Man), glucose (2.4 μM, Glc), b) the amino sugars: galactosamine (2,0 μM, GalN), glucosamine (2.8 μM, GlcN), and c) the acidic sugars: galacturonic acid (2.8 μM, Gal-URA), gluconic acid (5.1 μM, Glu-Ac), glucuronic acid (3.0 μM, Glc-URA) and muramic acid (1.9 μM, Mur-Ac). Regular calibration was performed by injecting 12.5 μl, 15.0 μl, 17.5 μl and 20 μl of mixed standard solution. Linearity of the calibration curves of individual sugar standards was verified in the concentration range 10 nM-10 μM. Therefore, the standard mixture was diluted 10, 20, and 50 fold with Milli-Q water. Injection volume for samples and for the blank was 17.5 μl. To check the performance of carbohydrate analysis and stability of the HPLC-PAD system, a 17.5 μl standard solution was analyzed after every second sample. The detection limit was 10 nM for each sugar with a standard deviation between replicate runs of <2%. Milli-Q water was used as blank to account for potential contamination during sample handling. Blanks were treated and analyzed in the same way as the samples. Blank concentration was subtracted from sample concentration if above the detection limit.

The performance of the chromatographic separation between two sugars can be described by the resolution factor (R_s):

(1) $$R_s = 2\frac{t_{Ri-1} - t_{Ri}}{w_{i-1} + w_i}$$

Where t_{Ri-1} and t_{Ri} are the retention times of a sugar (*i*), and of the preceding sugar (*i-1*); w_i and w_{i-1} are the associated peak widths. Here, we determined real peak widths from intersects between the peak slopes and the baseline. Table 2 shows the retention times and resolution of standard sugars used for calibration at the above described settings. Peak widths of standard sugars never exceeded 2min, and peaks were fully separated, except for Man and Xyl. Concentrations of Xyl and Man were therefore quantified together. Overlapping peaks of Xyl and Man have frequently been reported (Jørgensen and Jensen 1994, Keherve et al. 1995, Borch and Kirchman 1997, Engbrodt 2001, Kirchman et al. 2001, Goldberg et al. 2009). According to Skoog and Benner (1997) the yield of Man and Xyl in seawater samples is about equal. The full chromatogram of the standard sugar mixture as well as chromatograms of standard sugars detected in seawater samples, and in the blank sample is shown in Figure 1.

Assessment

Several tests were conducted to determine the accuracy and reproducibility of the method, the recovery of individual carbohydrates during the dialysis, hydrolysis and neutralization steps as well as the comparability to bulk carbohydrate measurements.

I. Dialysis

Prior to analysis, seawater samples were desalted by membrane dialysis to remove ions, which interfere with the chromatographic separation and detection. Desalting of seawater was achieved by membrane dialysis using a dialysis tube with a molecular weight

cut-off (MWCO) of 1kDa (Spectra Por 7, Spectrum Laboratories). The 1kDa cut-off membrane has widely been used to determine HMW dissolved carbohydrates, i.e. polysaccharides, in seawater (Benner et al. 1992, Aluwihare et al. 1997, Kaiser and Benner 2009). To remove the sodium azide preservative, the dialysis tube was rinsed before and after a 30 min soaking step with Milli-Q at 20°C. Afterwards, the tube was pre-rinsed with 2 ml of sample. Then, the dialysis tube was filled with 10 ml of seawater sample and was sealed tightly at both ends using two dialysis tubing closures (Spectra Por). During dialysis, the tube was kept floating in a 1L beaker filled with Milli-Q, which was continuously mixed using a magnetic stirrer and changed two times during desalting. Dialysis was conducted in a temperature controlled room at 0-4°C. In order to minimize adsorption of macromolecules onto the dialysis membrane, the tube was placed in an ultrasonic bath for 5 min prior to sample removal. The time required for desalting of the seawater sample and the recovery of sugars after dialysis was evaluated during several tests with seawater of different salinity.

The recovery of carbohydrates after dialysis was evaluated during several tests using cellulose and laminarin as standard polysaccharides, and Milli-Q or NaCl solution (34%) as solvents. Cellulose ($(C_6H_{10}O_5)_n$, with n=500-5000 and MW>10kDa) is the major structural plant polysaccharide on earth, containing β(1→4) linked D-Glc units and insoluble in water. Laminarin (MW>3kDa) is a natural polysaccharide of brown algae containing β(1→3) and β(1→6)-linked D-Glc units. It is a linear polysaccharide, with a β(1→3):β(1→6) ratio of 3:1 and in contrast to cellulose water-soluble. Each standard was split into triplicates of 3ml and of 10ml. Triplicates of 3 ml were directly hydrolyzed at a concentration of 0.8 M HCl at 100°C for 20h. Triplicates of 10 ml were dialyzed for 4h as described above. The dialyzed triplicates were hydrolyzed in the same way as the non-dialyzed references. After hydrolysis, all samples were neutralized by evaporation using N_2 and subsequently analyzed as described in the following sections.

To determine the presence of salt residues after dialysis, or of contaminants interfering with the IC analysis, we run several replicate Milli-Q blanks and procedual blanks, i.e. blanks prepared with Milli-Q and 34 g L^{-1} combusted NaCl (8h at 500°C), treated and analyzed in the same way as the samples.

II. Hydrolysis and neutralization

In order to cleave combined carbohydrates into their monomeric components, we conducted an acid hydrolysis, followed by neutralization of the acid through evaporation of the liquid with N_2. Optimal conditions for the acid hydrolysis of dissolved CCHO (dCCHO) and of total CCHO (tCCHO), including particulate CCHO (pCCHO) and dCCHO, were determined during several tests using natural and standard CCHO. All samples and blanks were dialyzed for 240 min at <5°C as described above, and hydrolyzed in precombusted (500 °C, for 8 h) sealed glass ampoules for 20h at 100°C.

Natural CCHO were collected from continuous cultures of the coccolithophore *Emiliania huxleyi*. For analysis of dCCHO, samples were filtered through 0.45 µm syringe filters (GHP membrane, Acrodisk, Pall). Prior to filtration, the syringe filters were rinsed with several ml of Milli-Q water first and seawater sample thereafter. For tCCHO, the unfiltered sample was analyzed. Prior to dialysis, salinity (S=34) of samples was reduced to 17 by dilution with Milli-Q water (1:1) to shorten the dialysis procedure. After, dialysis several replicates of 3 ml of each filtered and unfiltered samples were treated with hydrochloric acid to reach concentrations of 0.1 M, 0.2 M, 0.4 M, 0.8 M, 2 M, 4M and 8 M HCl and hydrolyzed in precombusted (500 °C, for 8 h) sealed glass ampoules for 20h at 100°C. In addition to natural CCHO, the hydrolysis efficiency of chitin (Sigma) and pectin (polygalacturonic acid, Sigma) exemplifying two standard polysaccharides was tested in a saline matrix (NaCl 15 g L^{-1}, combusted for 8h at 500°C). Both chitin and pectin are sparingly soluble in water. Therefore, the dry powder (200 mg L^{-1}) of each standard was first suspended in saline

solution and then filtered through 0.45 µm syringe filters (Acrodisc). The dissolved chitin and pectin samples were dialyzed, and subsequently hydrolyzed using 0.2, 0.4, 0.8, 1 and 2 M HCl.

After cooling, the liquid of the acidified sample was evaporated under N_2 atmosphere at 50 °C. Evaporation was completed after 4h. Then, 0.2 ml of Milli-Q were added to the dry residue, and the evaporation repeated for 30 min. Browning of samples during the evaporation procedure was never observed. The dry residue was re-suspended with Milli-Q water. In case of natural CCHO, 1.5ml Milli-Q, equivalent to half of the sample volume subjected to evaporation, was used to compensate for the dilution step prior to dialysis. In case of standard CCHO, 3ml Milli-Q was used for re-suspension. In principle, using a volume of Milli-Q smaller than the initial sample volume allows for a modest enrichment of CCHO in the sample. This is especially advantageous for detecting low CCHO concentration in field samples or rare sugars. We found that the minimum volume needed for efficiently re-suspending the dry pellet and for smooth autosampler operation is 0.5ml, equivalent to a three-fold enrichment of CHO, and a minimum detection limit of 3nM final concentration.

All samples were treated with a vortex homogenizer for 3 min before filling into the autosampler vials.

III. Test using TPTZ method

It has been shown that the quantity of sugars determined in seawater strongly varies between the different analytical procedures (Panagiotopoulos and Sempéré 2005). One of the methods frequently used to measure total dissolved mono-and polysaccharides in marine samples is the TPTZ (2,4,6-tripyridyl-s-triazine) method described in detail in Myklestad et al. (1997). Here, monosaccharides, and non-reducing sugars and polysaccharides made reducing by hydrolysis of glycosidic bonds, are oxidized with ferricyanide and determined colorimetrically after condensation with the chromogen TPTZ. The absorbance of the colored

complex $Fe(TPTZ)_2^{2+}$ is standardized with D-glucose. In principle, all reducing sugars, including uronic acids, can be determined, but the efficiency of the reaction with TPTZ relative to glucose varies between different carbohydrates; e.g. 103% for fructose and 51% for mannuronic acid (Myklestad et al. 1997).

We compared dCCHO concentration obtained with our HPAEC-PAD method to results yielded with the TPTZ-method in samples taken from a continuous culture of the polar marine diatom *Fragilariopsis cylindrus*. Therefore, 100 mL of culture sample were filtered through 0.45 µm syringe filters (GHP membrane, Acrodisk, Pall). Replicate 10ml subsamples of the filtrate were dialyzed (1 kDa MWCO) and subsequently hydrolyzed with 1ml 0.8 M HCl at 100°C for 20h. Neutralization was done by evaporation under N_2-gas as described above. For the TPTZ-method, an additional set of samples was prepared, neutralizing the samples by addition of 1ml of 0.8 M NaOH, because base addition was also used in the original protocol of Myklestad et al. (1997).

Analysis of dCCHO by HPAEC-PAD was performed as described above. The analytical TPTZ-procedure was conducted as described in Myklestad et al. (1997) using replicate 1ml aliquots and D-glucose as standard sugar. The amount of monosaccharides (MCHO) was determined from non-hydrolyzed aliquots. The concentration of dCCHO, was calculated by subtracting MCHO from total dissolved carbohydrates determined in the hydrolyzed fraction. The detection limit for carbohydrates with the TPTZ method at our lab was 0.4 µM with a precision of 3%.

IV. Test using cation/anion resins for desalination

We compared the sugar concentration of samples that were desalted according to our dialysis protocol with those that were desalted using the AG2-X8 and AG50W-X8 (BioRad) ion-exchange resins after Mopper et al. (1992) (Figure 2). One of the differences between

the two procedures is that desalination is conducted for the non-hydrolyzed sugar fraction >1kDa in case of dialysis, and for monomeric sugars in case of resin desalination. Hence, when using resins, combined carbohydrates are hydrolyzed prior to desalination; free monomeric sugars can also be determined. Because deoxyribose is completely destroyed during hydrolysis, it has often been used as an internal standard to account for losses during resin desalination. Here, we added 1 μM of 2-deoxyribose as internal standard to the samples after the hydrolysis/neutralization step, and prior to running the samples through the resins.

Analyses of natural dCCHO and tCCHO were performed for samples of a continuous culture of *E. huxleyi* having a salinity of 34. For all samples, acid hydrolysis (0.8 M HCl for 20h) and neutralization by evaporation under N_2 were conducted, following the procedures described above. Free monomeric sugars were below detection in the non-hydrolyzed sample that was desalted by the resin.

V. Application to field samples

We applied our protocol to determine tCCHO and dCCHO in seawater samples that were collected with Niskin bottles at 8 different depths (5-100m) at a station in the northern Bay of Biscay in 2006, during a cruise with the research vessel *Belgica*. For dCCHO, samples were filtered through 0.45 µm syringe filters (GHP membrane, Acrodisk, Pall) into 20 ml combusted (500°C for 8h) glass vials. Prior to filtration the syringe filters were rinsed with several ml of Milli-Q water first and seawater sample thereafter. For tCCHO, the unfiltered sample was filled into combusted 20 ml glass vials. Duplicate samples were stored frozen (<-20°C), and thawed immediately before analytical procession. Results for dCCHO were compared to concentrations of DOC, determined from duplicate 20 ml samples, filtered

through combusted GF/F, and stored frozen (<-20°C) until analysis on a Shimadzu TOC-VCSH using the HTCO method (Qian & Mopper, 1996).

Results

I. Dialysis

The dialysis procedure was introduced to remove ions, which interfere with the chromatographic separation and detection. The duration of desalting procedure was determined during two experiments with seawater of low salinity (15), sampled at the Baltic Sea close to Kiel Bight, and of high salinity (34), sampled at the North Sea close to Helgoland Island. The decrease of seawater salinity within three replicate dialysis tubes was determined with a refractometer (Krüss Optronic, Germany) after 60 min, 180 min, 240 min and 300 min (Figure 3). Desalting was completed for seawater with salinity of 15 after 240 min of dialysis at a temperature of <5 °C, whereas it took more than 300 min to reduce salinity of 34 to <1. As a consequence of these results, and in order to keep the dialysis procedure feasible, we decided to dilute seawater samples of high salinity with Milli-Q water prior to dialysis using a volume ratio of sample to MilliQ water of 1:1. This dilution of sample is compensated during the sample neutralization step, when the residue is taken up with Milli-Q water equivalent to half of sample volume exposed to evaporation.

Recovery of standard polysaccharides after dialysis was >90% during all tests (Table 3). No contamination with sugars (>10 nM) was detected in the Milli-Q blanks. In the NaCl blanks, a small amount of Glc averaging 5.5 nM with a standard deviation of 9.9 nM (n=5)

was observed. Since Glc was absent in 3 out of 5 replicates, this result may be due to contamination during sample preparation.

A potential loss of polysaccharide during dialysis may occur if sugars adsorb onto the dialysis membrane. During the development of the dialysis procedure, we in fact observed a lowed yield of sugars in samples without sonification. Thus, sonification proved to be important for the effective recovery of macromolecular carbohydrates after dialysis. Another potential loss may occur in the course of the dialysis procedure, if enzymes, such as present in natural samples, would hydrolyze polysaccharides into subunits small enough to pass through the 1kDa membrane. In order to minimize enzymatic cleavage, the temperature during dialysis was kept <5°C. We also tested for this potential loss of combined carbohydrates during the dialysis procedure by adding 10 ml of an aged and non-axcenic culture of the diatom *Fragilariopsis cylindrus* (DOM_{FCYL}), filtered through 0.45 µm syringe filters (GHP membrane, Acrodisk, Pall) to a standard solution of laminarin. Because natural polysaccharides were also present in DOM_{FCYL}, we dialyzed and analyzed the laminarin standard with and without DOM_{FCYL} addition as well as DOM_{FCYL} solely. This yielded 709±7.2 nM Glc for the laminarin standard, 446 ± 0.9 nM Glc for combined carbohydrates contained in DOM_{FCYL} and 1187 ± 12 nM Glc for the 1:1 mixture. Hence, the mixture yielded a slightly higher value (2.8 %) as the numerical sum of its components. Given a standard variation of 1.7 % for the sum of the two individual components, the slightly higher value of the mixture was within a range of acceptable variability. A significant loss of combined carbohydrates during dialysis due to the presence of enzymes was clearly not observed. It is of course difficult to generalize from here to all potential applications, and including a test for enzymatic cleavage with individual samples is recommended for applications with supposedly high enzymatic activity, e.g. sewage, biofilms, aggregate and sediment pore water.

II. Hydrolysis and neutralization

In accordance with previous findings (Mopper 1977, Borch and Kirchman 1997), we observed that individual sugars responded differently to the HCl treatment (Figure 4a). For tCCHO, the yield of several sugars, including Glc, Gal and Gal-URA was highest at 0.8 M HCl. A strong decrease, equivalent to >50% of monosaccharide concentration, was observed for HCl concentrations >2.0 M. Highest yield of dissolved uronic acids was achieved at 0.2 M HCl. Dissolved uronic acid concentrations decreased moderately between 0.2 and 0.8 M (37% on average) and sharply beyond 0.8 M.

The yield of the most abundant monosaccharide Glc increased until 2.0 M for both, dCCHO and tCCHO, and declined rapidly at higher HCl concentration. The amino sugars GlcN and GalN as well as the two acidic sugars Mur-Ac and Gluc-Ac were not detected during these tests. The absence of amino sugars in *E. huxleyi* cultures has been reported earlier (de Jong et al. 1976). However, in order to also determine the cleavage of rare sugars such as Mur-Ac, Glu-Ac, GalN, and GlcN, we repeated the procedure with different samples of *E. huxleyi* cultures using HCl concentrations between 0.1 and 2.0 M. We indeed detected GalN, Glc-Ac and Mur-Ac in one culture, likely due to the presence of bacteria in the sample. The response of these sugars to acid hydrolysis showed a slight decline of concentration between 0.1 and 0.8 M and a more pronounced loss beyond 0.8. M (Figure 4b).

The hydrolysis efficiency of chitin increased only slightly with acid concentration, yielding maximum differences for GlcN of 4 % between 0.2 and 2.0 M HCl (table 4), and 2% difference between 0.8 and 2.0 M HCl. Hence, the release of GlcN from chitin appeared to be little sensitive to acid concentration within the tested range. This result is in accordance with findings of Kaiser and Benner (2000), who reported that the release of GlcN from different natural CCHO varied between 5 and 30% depending on acid strength and

concentration (3M and 6M HCl, 1.2 M H_2SO_4). They also showed that the same hydrolysis condition could yield either higher or lower recovery of amino sugars depending of the source of the material, i.e. the producing organism. In our test, the hydrolysis yield for Gal-URA as released from Pectin was highest at 0.8 M, and decreased with increasing acid concentration by about 20% (Table 4).

We also examined a potential loss of individual sugar monomers during the hydrolysis and neutralization steps described above. Therefore, we compared the monosaccharide yield of a mixed standard solution with and without application of the hydrolysis and neutralization procedures. We included cellobiose in the standard mixture. Cellobiose is a disaccharide containing two Glc units combined by a β-1,4-glycosidic bond. Two aliquots of the standard monosaccharide mixture were measured in duplicate directly, and served as reference. The other aliquot was split into two replicates of 3 ml, acidified with 0.8 M HCl, hydrolyzed for 20 h, neutralized as described above and measured in duplicates, respectively. Compared to the reference values, few changes were observed in the acidified and neutralized samples yielding average recoveries between 90 and 107 % for all sugars except for Rha and Gal-URA yielding only 81 %. Fructose was completely destroyed during hydrolysis, as observed before (Borch and Kirchman 1997, Jørgensen and Jensen 1994). Cellobiose totally disappeared after hydrolysis, adding two times its concentration to the Glc pool. This indicated a complete hydrolysis of the disaccharide without loss of its monomeric components. In total, we recovered 82% of all sugars contained in the standard mixture. These results compare well to earlier findings that report recoveries of monomeric sugars after the hydrolysis and neutralization steps in the range of 75-92% (Borch and Kirchman 1997). Thereby, a higher loss of sugars has been attributed to the neutralization step by some authors (Engbrodt 2001, Mopper 1977).

III. Comparison with the TPTZ method

Using our HPAEC-PAD method, we determined a concentration of 0.99±0.04 µM for dCCHO in the *F. cylindrus* culture sample. The TPTZ method yielded 1.55±0.02 µM for dCCHO samples neutralized by evaporation, and 0.88±0.01 µM, when samples were neutralized by base addition (Figure 5). A higher concentration yield with the TPTZ method was expected, because TPTZ determines all reducing sugars, while HPAEC-PAD is restricted to standard sugars. We also observed that neutralization by evaporation results in a higher recovery of sugars when using the TPTZ-method. Hence with our HPAEC-PAD protocol, we determined 64-112 % of TPTZ sugars, depending on the way of neutralization.

IV. Comparison with cation/anion resins for desalination

Comparing samples that had been desalted by cation/anion exchange resins to those desalted by membrane dialysis indicated sugar specific losses during cation/anion-exchange desalination between 28 and 100% (Table 5). Losses were generally higher for dCCHO than for tCCHO, probably due to lower absolute concentrations in dCCHO. As observed earlier, acidic sugars were completely removed by the resins. Total concentration of neutral and amino sugars in samples subjected to resin desalination was about 33% of those desalted by dialysis. We also assessed sugar losses during resin desalination by adding 2-deoxyribose (0.1 ml of a 10mM stock solution) as internal standard to the samples after the neutralization step and prior to running the samples through the resins. Because 2-deoxyribose is completely destroyed during hydrolysis, its final concentration in the sample can be used to calculate how much of added standard was lost in the resins. For our samples, recovery of 2-deoxyribose was about 30-33%, and therewith in the same range as obtained through direct comparison with the dialyzed samples.

V. Application to field samples

The phytoplankton community in surface waters (salinity 35.6) of the Bay of Biscay during June 2006 was dominated by coccolithophores (Harlay et al. 2009). All three groups of sugars, i.e. neutral, amino and acidic sugars, were detectable at each depth. As an example, sugar concentrations are given for one station (7.5°E, 48°N) sampled on 6. June 2006. DOC concentration at this site varied between 65 and 127 µmol L^{-1}. Highest POC concentration was observed at the surface with 26 µM, and declined to 2.6 µM at 100m depth (Harley et al. 2009). In dCCHO, concentration ranged from 500-992 nM for neutral sugars, 280-530 nM for uronic acids and 19-42 nM for amino sugars. For pCCHO, determined by subtracting dCCHO from tCCHO, concentrations ranged from 32 to 2300 nM for neutral sugars, from <10 to 451 nM for uronic acids and from <10 to 44 nM for amino sugars. Hence, acidic sugars were more abundant in DOM than in POM and contributed substantially to the dissolved sugar pool. The molar percentages of sugar monomers in dCCHO and tCCHO are presented in table 6, the contribution of the three sugar classes to the pool of DOC in figure 6.

Discussion

HPAEC-PAD is a state of the art method to determine sugar monomers and has frequently been applied for seawater, freshwater and sediment samples. Given the high analytical precision of HPAEC-PAD, the necessary pretreatment of marine samples, e.g. to desalinate seawater and to cleave complex carbohydrates into their monomeric components, is a critical step, and may distort the absolute concentration as well as the proportions of individual sugars.

Here, we showed that membrane dialysis is a simple and robust method for desalting seawater, and an adequate pretreatment of HMW-carbohydrates to be subsequently analyzed by HPAEC-PAD. The suggested dialysis procedure yielded desalination of seawater within a reasonable time and without significant contamination or loss of sugars. Our protocol includes desalting of seawater using a 1kDa membrane. Assuming a molecular weight of 180Da for a sugar monomer (e.g. Glc), combined sugars with a molecular weight of >1kDa contain more than 5 monomers. Thus, our dialysis approach will principally include all polysaccharides and even some oligosaccharides. Smaller oligomers and free monomers will pass through the membrane and can thus not be determined. This may limit the applicability of our approach for microbiological studies that aim to investigate the turn-over of free sugars that are directly taken up by microorganisms. However, it is known that turn-over rates of free sugars in seawater are very high, and that their concentration in marine samples are thus often at or below the detection limit of HPAEC-PAD anyway (Engbrodt 2001, Benner 2002, Kaiser and Benner 2009). Oligosaccharides, however, can comprise a significant fraction of neutral sugars. Skoog and Benner (1997) showed that the majority of neutral aldoses was contained in LMW-DOM (<1kDa) in samples collected at the equatorial Pacific, although neutral aldoses accounted for only 10-20% of total carbohydrates in their study. Conducting dialysis with smaller MWCO-membranes, e.g. 100-500 Da (Spectra Por), could allow for including oligosaccharides and hence a larger fraction of LMW-dCCHO while using our protocol.

Several studies previously applied ion-exchange resins to desalt seawater prior to HPAEC-PAD and reported a wide range of recoveries for neutral sugars, i.e. 50% to 90% (Borch and Kirchman 1997, Skoog and Benner 1997, Engbrodt 2001, Kirchman et al. 2001, Goldberg et al. 2009). Higher recoveries were obtained for solutions of sodium chloride spiked with sugar standards; i.e. 72-100% (Sempéré et al., 2008). Sugar specific losses

during resin extraction have been reported (Sempéré et al., 2008), and were also observed during this study. A preferential adsorption of individual monomers during resin elution may potentially bias polysaccharide composition towards sugars that are less adsorbed. The advantage of dialysis clearly is that monosaccharides that were combined in carbohydrates larger than the cut-off size of the membrane are retained in the samples after desalination, and can be determined by HPAEC-PAD after hydrolysis and neutralization. Dialysis can therefore reduce the risk of a selective loss of sugars during desalination.

The mixture of monomers released during hydrolysis includes neutral, amino and acidic sugars. The performance of the acid hydrolysis of combined carbohydrates has been reported to vary with the kind and source of material, with the strength and type of acid used, as well as with the duration and temperature during hydrolysis (for review see Panagiotopolous and Sempéré 2005). In general, strong acids may be more effective in breaking stable bonds, but are more likely to destroy sensitive sugars. The use of HCl for hydrolysis has been shown to yield comparable results to the hydrolysis with H_2SO_4 for neutral sugars (Borch and Kirchman 1997) and for amino sugars (Kaiser and Benner 2000), and has previously been applied for seawater samples (e.g. Grossart et al. 2007, Sempéré et al. 2008). Moreover, HCl is volatile and can be neutralized by N_2 evaporation, whereas H_2SO_4 requires neutralization by mineral precipitation, which may provide substrate for sugar adsorption and subsequent loss (Walters and Hedges 1988). An alternative procedure to remove HCl and H_2SO_4 using ion retardation resins has been applied in some studies (Kaiser and Benner 2000). We chose 0.8 M HCl for the hydrolysis of natural heteropolysaccharides in HMW-DOM and POM to optimize for the simultaneous recovery for a large variety of sugars.

First results of the application of our method to field samples showed that naturally low concentrations of combined carbohydrates can be detected. Thereby, concentrations of dissolved combined neutral and amino sugars in field samples were comparable to previously published values (Kaiser and Benner 2000, 2009). Acidic sugars were determined in dCCHO and tCCHO during a coccolithophore bloom, and represented 30-50% of the dissolved and 21-40% of the total carbohydrate pool respectively. So far, acidic sugars have largely been neglected in HPAEC-PAD protocols for seawater analysis, due to their elimination during sample desalting by ion-exchange resins. Hung et al. (2001) determined total uronic acids in HMW-DOM in the estuarine area off Galveston Bay, and yielded concentrations in the range 100-900 nM. Their data compare well to uronic acid concentration observed for the Bay of Biscay during this study (280-530 nM). Acidic sugars in CCHO, specifically uronic acids, are suggested to play an important role in biogeochemical cycling. They also are contained in gel particles, such as transparent exopolymer particles (TEP); a group of particles that gathered much attention due to their role in particle aggregate formation and recently also for their contribution to a gelatinous sea surface microlayer (Cunliffe and Murrell 2009). Although not included in this study, the application of our protocol for other acidic sugars, such as phosphorylated and sulphated sugars, should principally be feasible. These sugars are important components of mucus substances released by marine algae, especially macrophytes (McCandless and Craigie 1979). Similar to uronic acids, sulphated sugars are supposed to facilitate coagulation of polysaccharides (Leppard 1995).

Our proposed method allows for the simultaneous determination of neutral, amino and acidic sugars contained in HMW-DOM or in POM. The advantage of a simultaneous determination of the three sugar classes over their individual analysis is primarily a gain in efficiency and comparability. First, the amount of time required for sample preparation,

hydrolysis, neutralization, and for the rinsing and equilibration of the columns is reduced as these steps have to be conducted only once. Moreover, the combined handling and use of one mixed standard solution can help to minimize methodological errors. This benefits especially large samplings, e.g. field surveys, time-series and extensive experiments. We chose 0.8 M HCl for the hydrolysis of natural heteropolysaccharides in HMW-DOM and POM to optimize for the simultaneous recovery for a large variety of sugars. Certainly, a joint analysis of several sugars has to compromise individual requirements in hydrolysis conditions, eluent concentration and flow rate, at the potential expense of precision in the detection of individual components. Applications that aim to investigate a particular sugar component will have to optimize our procedure for the specific purpose.

Our method shall provide an opportunity to gather more information on quantities and total composition of sugars in HMW-DOM and in particles, including gels, and may thus be helpful to advance the understanding of the reactivity, size partitioning and cycling of organic matter in the ocean. The protocol can easily be adopted in laboratories conducting HPAEC-PAD, as all materials used are commercially available. In contrast to ion-exchange resins that need to be purified, activated and regenerated, the application of disposable dialysis membranes is less laborious, and the performance of dialysis membranes is more standardized and less sensitive to handling.

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Legends:

Figure 1: Chromatograms of sugars detected by HPAEC with integrated PAD at a flow rate of 0.250 ml min^{-1} and an injection volume of 17.5 µl. a) Full chromatographic run of the mixed sugar standard. Below: Elution of neutral and amino sugars during the isocratic course of NaOH (eluent I) (left panel), and elution of acidic sugars during the gradient course of NaOH (eluent I) and NaAc (eluent II) (right panel) b) mixed sugar standard c) dCCHO (10-100nM) of a full marine seawater sample (Bay of Biscay), d) tCCHO (20-400 nM) of a full marine seawater sample (Bay of Biscay), e) Blank sample. Identification: 1 Fuc, 2 Rha, 3 GalN, 4 Ara, 5 GlcN, 6 Gal, 7 Glc, 8 Man/ Xyl, 9 Glu-Ac, 10 Mur-Ac, 11 Gal-URA, 12 Glc-URA, A acetate peak,

Figure 2: Flow chart of the sample treatment performed prior to the analysis of monomeric sugars by HPAEC-PAD. Protocol using cation/anion exchange resins to desalinated marine samples (left flow chart) and protocol described in this study using membrane dialysis (right flow chart). Both procedures were compared for recovery of sugars produced by the marine phytoplankton species *Emiliania huxleyi* (see text).

Figure 3: Desalting of seawater of low salinity (15, circles) and of high salinity (34, triangles) during the dialysis procedure with a 1kDa MWCO membrane at 4°C.

Figure 4a, b: Effects of different concentrations of HCl (0.1 M, 0.2 M, 0.4 M, 0.8 M, 2 M, 4M and 8 M) applied during hydrolysis on the yield of sugars contained in total (tCCHO; open circles) and dissolved (dCCHO, solid circles) combined carbohydrates produced by the marine coccolithophore *E. huxleyi*. a) abundant sugars, b) rare sugars. Concentrations of

dGlc-Ac, dGalN and dGlcN were below detection (<10nM). Data are averages ± 1 SD for two replicate dCCHO samples and three replicate tCCHO samples.

Figure 5: Comparison of dCCHO concentrations determined from a marine diatom culture (*Fragilariopsis cylindrus)* by using the novel protocol for HPAEC-PAD, and the TPTZ-method after Myklestad et al. (1997). For the TPTZ-method, samples were neutralized either by evaporation under N_2 or by addition of NaOH.

Figure 6: Depth distribution of neutral sugars (NS, black circles), amino sugars (AS, open circles) and uronic acids (URA, black triangles) in dCCHO >1kDa as fractions of dissolved organic carbon (DOC) determined at a station in the Bay of Biscay (June 2006).

Tables

Table 1: Concentrations of the eluent I sodium hydroxide (NaOH), and eluent II sodium acetate (NaAc) applied during HPAEC-PAD.

Time (min)	Eluent I NaOH (mM)	Eluent II NaAc (mM)
0.00	18	-
20.00	18	-
21.00	100	-
25.00	100	200
35.00	100	200
40.00	75	-
45.00	75	-
46.00	18	-
55.10	18	-
65.20	18	-

Table 2: Chromatographic performance of HPAEC-PAD sugars analysis. Average retention times were calculated from 19 runs and are given ± 1 SD.

Monosaccharide (Brand)	Retention time (min)	Peak width (min)	Resolution factor R_s
Fuc (Sigma, 98%)	5.25 ± 0.01	0.41	
Rha (Sigma, 99%)	8.99 ± 0.02	0.46	8.5
GalN (Sigma, 99%)	9.53 ± 0.03	0.57	1.0
Ara (Sigma, 98%)	10.37 ± 0.03	0.53	1.5
GlcN (Sigma, 99%)	11.22 ± 0.03	0.76	1.3
Gal (Sigma, 99%)	13.36 ± 0.04	0.87	2.6
Glc (Fluka, 99%)	14.64 ± 0.04	0.86	1.5
Man (Sigma, 99%)	16.29 ± 0.05	1.34	1.5
Xyl (Sigma, 99%)	16.29 ± 0.05	1.34	0.0
Glu-Ac (Sigma, 99%)	28.60 ± 0.00	0.11	16.9
Mur-Ac (Sigma, 95%)	29.13 ± 0.01	0.13	4.4
Gal-URA (Fluka, 97%)	29.81 ± 0.01	0.14	5.0
Glc-URA (Fluka, 97%)	30.26 ± 0.01	0.08	3.9

Table 3: Recoveries of standard sugars after dialysis using 1 kDa membrane-tubes were obtained by comparison with non-dialyzed aliquots of same initial concentration. ND: non-dialyzed, D: dialyzed. Averages and standard deviations were calculated from n=3 for each sample and n=5 for each blank; bd=below detection.

Test	Sample		Solvent	Concentration (µM)		Recovery (D:ND; %)	
				Avg.	SD	Avg.	SD
1	Cellulose	ND	MilliQ	117.9	6.7		
	Cellulose	D	MilliQ	115.8	2.9	98.2	2.0
2	Laminarin	ND	MilliQ	51.7	2.4		
	Laminarin	D	MilliQ	50.1	1.9	96.9	4.8
3	Laminarin	ND	MilliQ	85.3	1.3		
	Laminarin	D	NaCl (34%)	79.3	1.1	93.0	3.0
4	Blank	ND	MilliQ	bd	-	-	-
	Blank	D	MilliQ	bd	-	-	-
	Blank	D	NaCl (34%)	0.0055	0.0099	-	-

Table 4: Average yields of CHO from standard polysaccharides after hydrolysis for 20h at 100°C using different concentrations of HCl. Yields were normalised to 2M hydrolysis, n=4.

HCL	Chitin		Pectin	
(M)	GlcN	total CHO	Gal-URA	total CHO
0.2	0.96	0.93	0.94	0.78
0.4	0.98	0.97	1.07	0.82
0.8	0.98	0.97	1.18	0.90
1	0.99	0.98	0.94	0.81
2	1.00	1.00	1.00	1.00

Table 5: Concentrations (nM) of dCCHO and tCCHO detected in samples of *E. huxleyi* after desalting using either membrane dialysis (1kDa MWCO) or cation/anion exchange resins. Concentrations are given as averages (x) of triplicate samples ± 1 SD. The ratio R/D indicates the percentage of sugars detected after resin desalting compared to those after dialysis. bd: below detection limit (10 nM) in each replicate.

	Resin desalting				Dialysis desalting				R/D	R/D
	dCCHO		tCCHO		dCCHO		tCCHO		dCCHO	tCCHO
	x	SD	*x*	SD	*x*	SD	*x*	SD		
Fuc	4	6.7	24	1.6	14	0.2	56	1	29	43
Rha	bd	-	bd	-	68	2	187	9	-	-
Ara	15	0.7	87	1.3	64	17	163	13	23	53
Gal	31	1.5	681	23	43	2	1077	47	72	63
Glc	83	2.3	381	5.4	704	114	2148	131	12	18
Man/ Xyl	25	0.4	152	6.9	107	32	340	52	23	45
Gal-URA	bd	-	bd	-	40	6	756	54	-	-
Glc-URA	bd	-	bd	-	153	8	424	29	-	-
Total	158	5.7	1324	38	1194	181	5151	334	13	26

Table 6: Mol percentages of carbohydrates determined in HMW-DOM >1kDa (dCCHO) and in total carbohydrates (tCCHO), including dCCHO and particulate carbohydrates. Samples were collected during a cruise to the Bay of Biscay in 2006. Concentrations of Mur-Ac and Glu-Ac were below detection. Bd: below detection limit of 10nM.

depth (m)	GlcN Mol%	GalN Mol%	Fu Mol%	Rha Mol%	Ara Mol%	Gal Mol%	Glc Mol%	Man/Xyl. Mol%	Gal-URA Mol%	Glc-URA Mol%	sum CCHO µM
dCCHO											
5	2	2	4	4	4	8	31	11	30	5	1.05
10	2	1	3	3	2	7	34	9	35	3	1.02
20	1	2	3	3	4	8	41	9	31	bd	0.96
30	1	1	2	2	3	4	54	4	27	4	1.47
40	2	1	2	2	4	4	28	8	42	7	1.08
60	2	2	3	3	3	3	39	8	29	9	0.85
80	1	1	2	2	2	3	48	8	31	4	0.81
100	2	1	2	2	2	3	47	7	28	8	0.87
tCCHO											
5	1	1	8	13	7	14	24	11	17	4	3.90
10	1	1	6	19	6	13	17	8	24	4	2.94
20	1	1	4	10	8	9	27	13	23	5	2.32
30	1	1	3	5	11	6	14	20	34	5	1.63
40	1	1	2	6	13	7	11	23	31	5	1.76
60	1	1	5	4	13	6	19	27	20	5	2.62
80	1	1	2	1	12	1	22	20	26	5	2.04
100	1	2	2	2	20	2	32	8	25	6	0.88

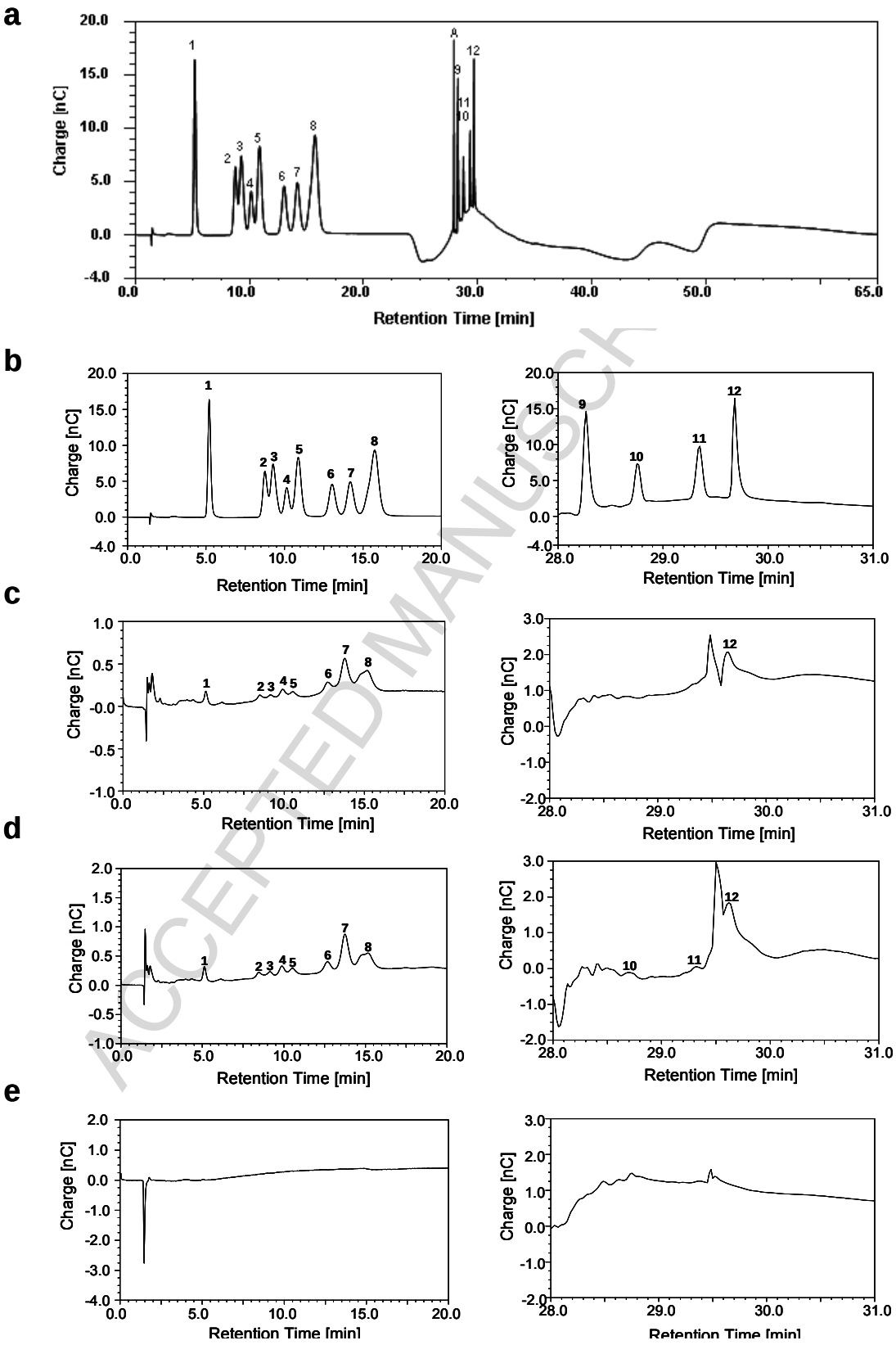

Figure 1a-e

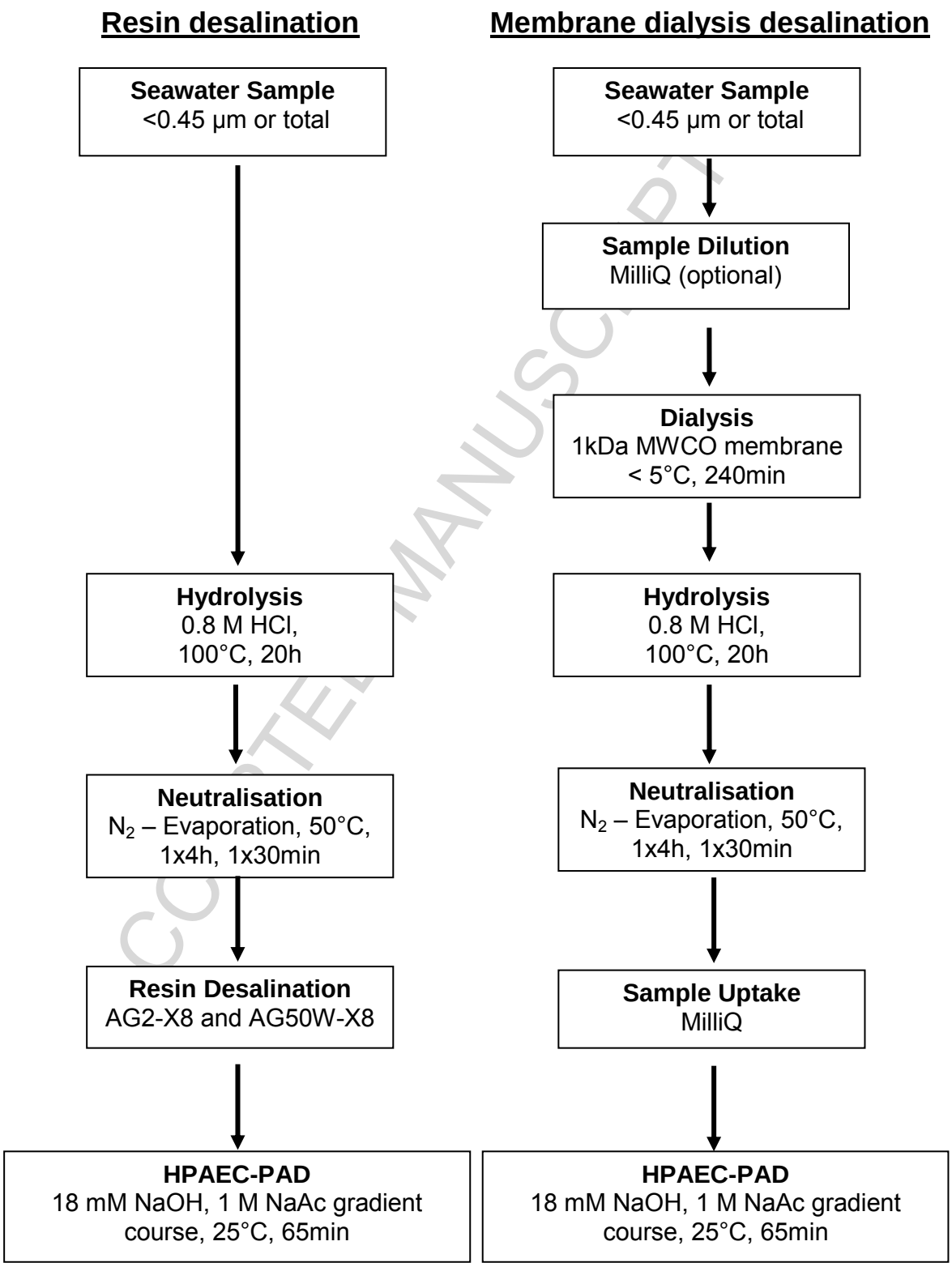

Figure 2

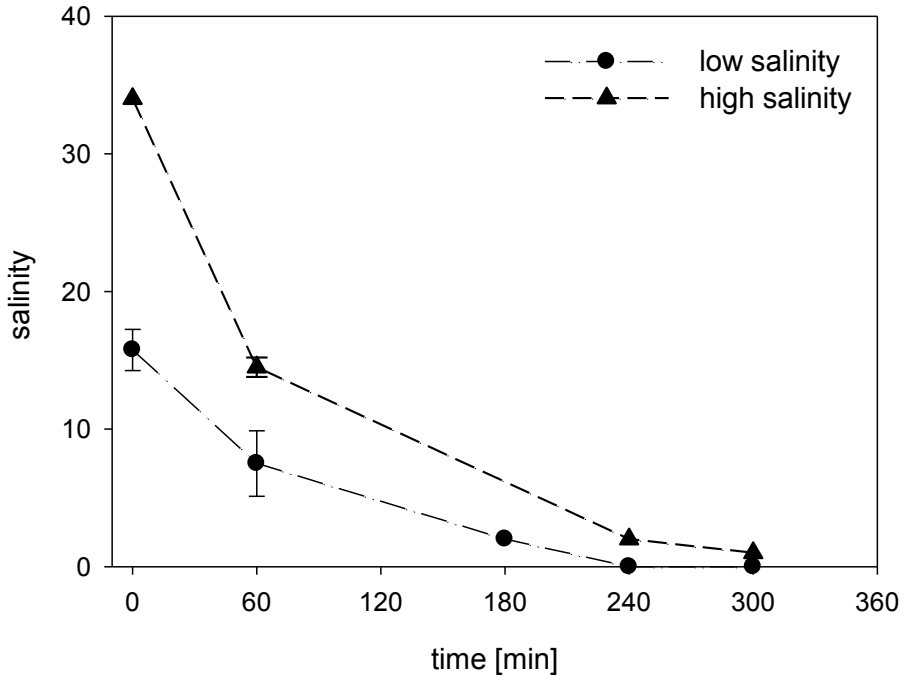

Figure 3

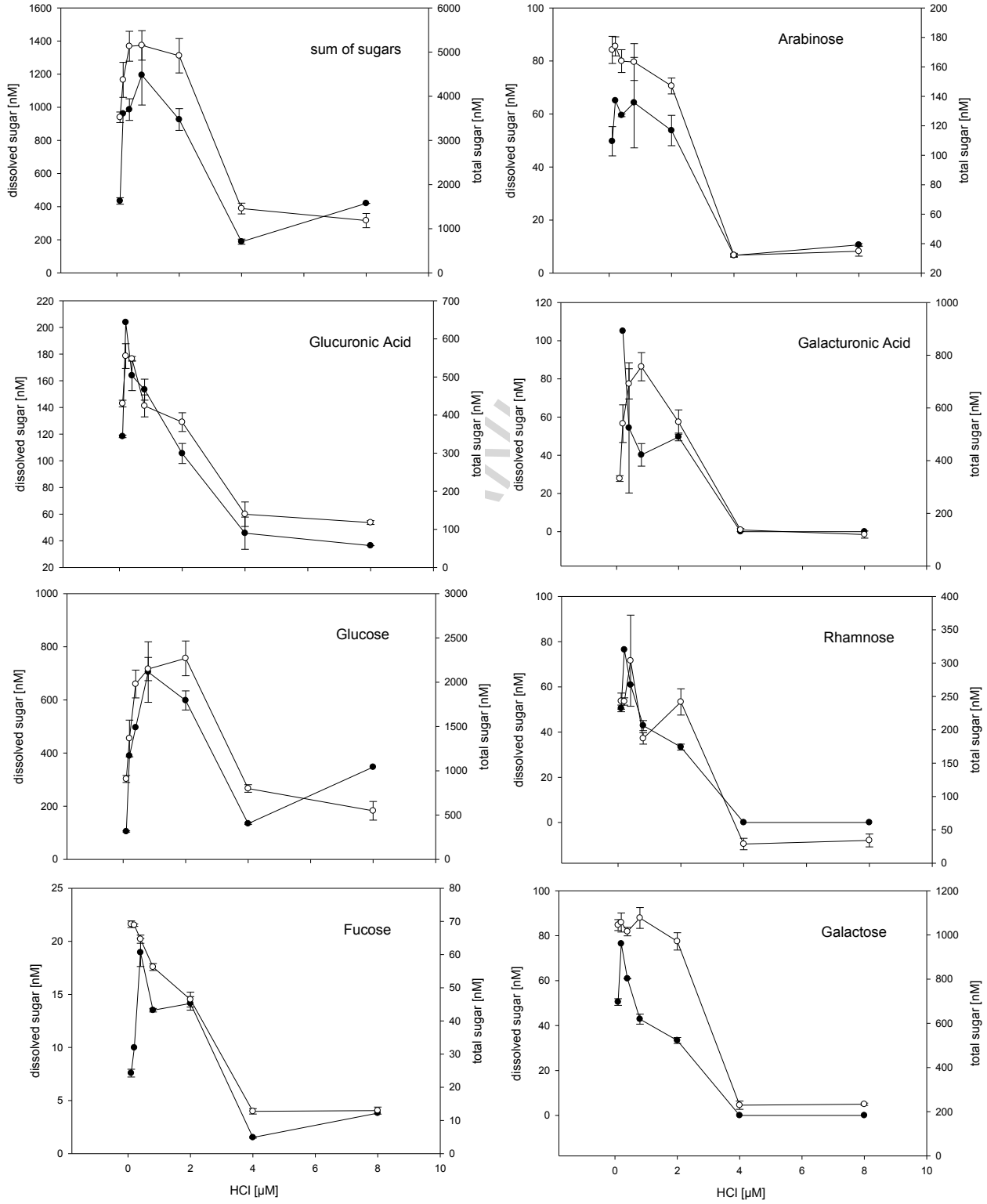

Figure 4a

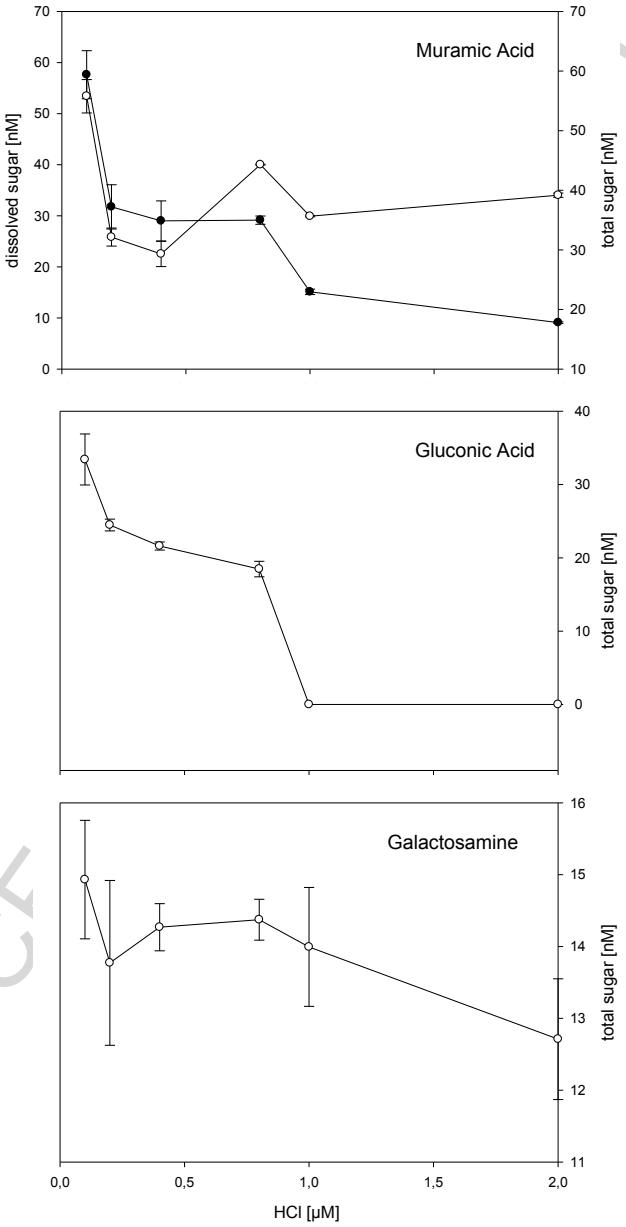

Figure 4b

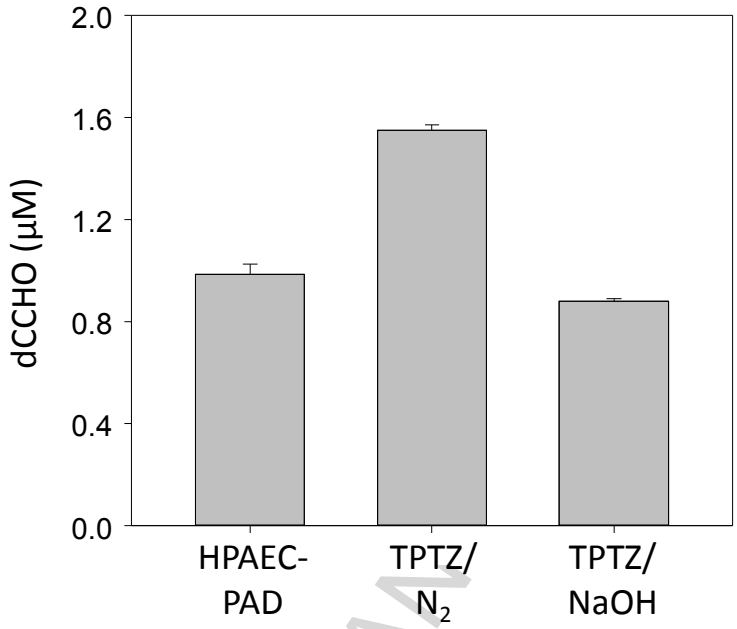

Figure 5

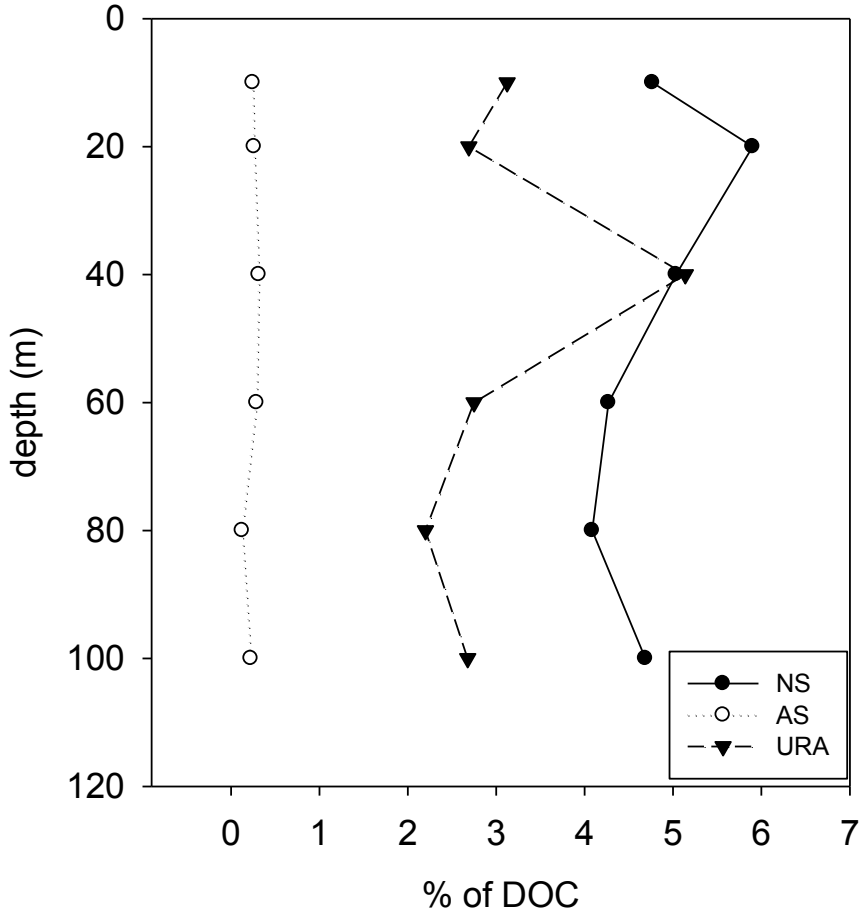

Figure 6

Research highlight

Novel protocol for HPAEC-PAD

Simultaneously determination of neutral, amino, and acidic sugars of HMW organic matter in seawater

Membrane dialysis (1kDa) proposed as a simple and efficient pretreatment for seawater samples

Acidic sugars comprise up to 50% of dissolved marine combined carbohydrates