LIMNOLOGY
and
OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 2023

doi: 10.1002/lom3.10558

Quantitative and qualitative comparison of marine dissolved organic nitrogen recovery using solid phase extraction

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Abstract

Marine dissolved organic carbon and nitrogen (DOC and DON) are major global carbon and nutrient reservoirs, and their characterization relies on extraction methods for preconcentration and salt removal. Existing methods optimize for capturing and describing DOC. Here, we report an optimized analytical strategy to recover marine DON for subsequent molecular characterization. Retention efficiencies between 5% and 95% are reported for seven solid phase extraction (SPE) sorbents, with PPL recovering 23% of marine DON compared to 95% recovered with C_{18}. Additional comparisons of the effect of varying sample volumes and elution speed, and the resulting molecular composition of DON extracts, were investigated using C_{18} and PPL sorbents. Sample volumes > 200 mL decreased DON retention efficiency independent of SPE sorbent, and gravity elution recovered 1.7- to 4.2-fold more DON compared to vacuum elution. Characterization of extracted DON by negative-ion electrospray ionization Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS) highlights compositional differences between DON species recovered by each method. DON isolated with optimized methods includes low molecular weight (< 600 Da) peptide-like compounds with low O:C ratios (0.2 to 0.5) that are not detected by other SPE sorbents (e.g., PPL). The majority of additional DON isolated with this approach was undetectable by direct infusion negative mode FT-ICR MS analysis.

The largest reservoir of reactive carbon in the ocean is dissolved organic matter (DOM), contributed primarily by photosynthesis occurring in the sunlit surface ocean (Carlson 2002). Nitrogen-containing compounds (dissolved organic nitrogen, DON) comprise a fraction of DOM, which includes a wide range of poorly characterized molecules that are thought to result from the release by and degradation of microbes and phytoplankton generally (Bronk and Glibert 1991; Bronk et al. 1994; Bronk and Ward 1999; Arakawa et al. 2017), and in particular by dinitrogen fixing microbes (Capone et al. 1994; Glibert and Bronk 1994; Bonnet et al. 2016; Knapp et al. 2016). DON is the prevalent form of bioavailable nitrogen (N) throughout the low-latitude surface ocean and has been reported to fuel export in oligotrophic gyres (Letscher et al. 2013, 2016; Knapp et al. 2018), highlighting the need for further characterization of the more bioavailable fractions of this heterogenous pool (Koch et al. 2008; Kujawinski et al. 2009; Hertkorn et al. 2013).

Due to low concentrations in surface waters (50–80 μM dissolved organic carbon (DOC); 4–6 μM DON), characterizing the composition of marine DOM relies on effective concentration and salt removal from seawater. One approach isolates high molecular weight (HMW, 1–100 nm) DOM using ultrafiltration (McCarthy et al. 1996; Benner et al. 1997), from which carbohydrates, carboxylic rich aliphatic material, lipids, and proteins have bee identified as important components of HMW DOM (Aluwihare et al. 1997). Within the marine HMW DON pool, amine and amide bonds are particularly common (Aluwihare et al. 1997; McCarthy et al. 1997), with different amide functionalities accumulating in the surface vs. deep ocean HMW DON pools (Aluwihare et al. 2005). However, the HMW fraction represents 20–35% of the total DOM pool (Benner et al. 1997; McCarthy et al. 1997), leaving the majority of marine DON uncharacterized by such techniques.

Characterization of low molecular weight DOM (LMW, 50–2000 Da) routinely employs solid-phase extraction (SPE) of

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Author Contribution Statement: A.N.K., R.B., A.M.M., and C.M. designed the research. C.M. and A.N.K. carried out DON extractions. C.M. and A.M.M. carried out FT ICR MS analysis. R.B., C.M., and A.M.M. carried out FT ICR MS data analysis. All authors contributed to the writing of the manuscript.

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marine DOM onto various sorbents, with the DOM then eluted off the SPE sorbent with a solvent for analysis, especially by nuclear magnetic resonance spectroscopy and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS; Hertkorn et al. 2007, 2013; Kujawinski et al. 2009; Kujawinski 2011). FT-ICR MS coupled with electrospray ionization (ESI) provides ultrahigh resolving power and mass accuracy that enables elemental composition assignment from mass-to-charge ratios (*m*/*z*) that can be attributed to specific elemental compositions, although isomeric separation requires additional separation, isolation, and fragmentation experiments that are challenged by DOM complexity. Studies of marine DOM composition couple negative-ion ESI with FT-ICR MS to selectively ionize acidic species and identify a highly complex, polyfunctional, polydisperse organic mixture that contains carboxyl-rich alicyclic molecules across a wide molecular weight range ($150 < m/z < 1200$; Hertkorn et al. 2006).

However, the extraction efficiency and composition of DOM recovered by SPE methods is influenced by both the sorbent and the elution solvent protocol, with routine methods primarily optimized for DOC capture and recovery and subsequent ESI FT-ICR MS characterization (Dittmar et al. 2008; Li et al. 2016). While prior work has described extraction efficiencies of ~30–60% of marine DOC by SPE (Dittmar et al. 2008), little attention has focused on the efficacy of these techniques for isolating DON from seawater. Previous studies report low recoveries of marine DON compared to DOC with SPE, with low DON recoveries indicated by the elevated C:N ratios of extracted material (19:1 to 33:1; Dittmar et al. 2008; Green et al. 2014), compared to bulk DOM values (10:1 to 14:1; Benner 2002). Since DON represents a small fraction of the DOM pool, optimal isolation techniques may differ for DON compared to DOC.

With the majority of oligotrophic surface ocean DON originating from the subsurface (Carlson 2002), low extraction of DON by SPE methods increase the likelihood that efforts to characterize marine DON composition may miss the small but dynamic fraction of the DOM pool that affects nutrient cycling in the surface ocean. Here, we compare retention and elution efficiencies of marine DON using different SPE sorbents, sample volumes, and elution rates, as well as how these methodological differences may affect the chemical characterization of DON using FT-ICR MS. We report an improved, quantitative method for DON extraction, and describe qualitatively the chemical composition of portions of the DON pool not captured by prior methods.

Methods

Seawater collection

Surface seawater was collected in the oligotrophic Gulf of Mexico via Teflon tubing deployed to 2 m depth and with a peristaltic pump (with platinum-cured silicone tubing in the pump head) through a Whatman Acropack 0.2 μm polyethersulfone capsule filter into acid-washed, sample-rinsed 1 liter amber polycarbonate bottles that were immediately frozen at −20 °C until further processing on land, although we note that freezing and thawing of samples may influence recovery.

DON extraction

Extraction of DON by SPE sorbents followed previously described protocols (Dittmar et al. 2008) unless otherwise noted. Immediately prior to extraction, thawed seawater was acidified to pH 2.5 using trace metal grade hydrochloric acid (HCl, 1 mL L^{-1} sample). Seven commercially available SPE sorbents were tested, including: Agilent Bond Elut PPL (styrene-divinylbenzene polymer modified with a proprietary nonpolar surface; 3 mL, 100 mg, Agilent part #12105004), Agilent ENV (1 mL, 100 mg, Agilent part #12105013), Agilent C_{18} (3 mL, 200 mg, Agilent part #12102025), Agilent Mega Bond Elut PSA (12 mL, 2000 mg, Agilent part #12256055), Agilent Bond Elut Diol 2OH (6 mL, 1000 mg, Agilent part #12256007), Agilent Bond Elut Plexa (6 mL, 500 mg, Agilent part #12259506), and Phenomenex Strata CN (6 mL, 1000 mg, Phenomenex part #8B-S007-JCH). Each SPE cartridge was rinsed with three cartridge volumes of LC–MS grade methanol, followed by three volumes of acidified (pH = 2 with HCl) ultra-pure (i.e., 18.2 MΩ cm) water. Subsequently, 0.2 liters of seawater was transferred through acid-rinsed Teflon tubing over the SPE cartridge at a flow rate of 1 mL min^{-1} using a gentle vacuum. The SPE cartridge was then rinsed with three volumes of acidified ultra-pure water to remove salts using a gentle vacuum (1 mL min^{-1}), as well as using gravity (0.1 mL min^{-1}) for C_{18} and PPL sorbents. Comparison with automated extraction/elution techniques (Singer et al. 2010; Lucci and Núñez 2014) was outside the scope of this analysis. The DOM sample was then eluted off the SPE cartridge using 500 μL LC–MS grade methanol and collected into a 2 mL borosilicate glass scintillation vial with PTFE-lined cap. Additionally, the extraction efficiency of the C_{18} and PPL sorbents were evaluated with seawater volumes ranging from 0.2 to 0.5 liters for PPL, and up to 8.0 liters for C_{18}. PPL and C_{18} sorbents were evaluated due to their common use (Dittmar et al. 2008) and high retention efficiency (Table 1), respectively. The volumes investigated span the range of sample volumes commonly evaluated in culture and environmental samples (Dittmar et al. 2008; Kujawinski et al. 2009; Boiteau and Repeta 2015).

Quantification of DON retention and elution efficiencies

Quantification of DON retention efficiency was determined by comparing the concentration of DON in the bulk, surface seawater sample (4.9 μM) with the concentration of DON in the sample water after it passed through each SPE cartridge. The nitrate + nitrite (NO_3^-+NO_2^-) concentration in this oligotrophic surface water sample was < 0.1 μM. The concentration of the DON not retained on the SPE sorbent was

Table 1. Comparison of rentention efficiency of 0.2 liter surface ocean DON on different SPE sorbents.

SPE type	Structure	Target molecules	Volume (mL)	Bed mass (mg)	Retention efficiency (%) (± 1 S.D.)	*n*
Strata CN	Silica based	Polar, hydroxyl groups	6	500	5.1 ± 1.3	4
ENV	Polystyrene-divinyl benzene	Polar	1	100	11.1 ± 1.2	3
PPL	Modified polystyrene divinyl benzene	Polar	3	100	22.9 ± 2.5	4
PSA	Alkylated amine	Polar	12	2000	29.5 ± 2.0	2
Diol (2OH)	Similar to unbonded silica	Polar and nonpolar	6	1000	66.6 ± 1.5	2
Plexa	Hydroxylated surface, polystyrene-divinyl benzene core	Nonpolar	6	500	92.7 ± 0.2	2
C_{18}	Octadecyl bonded phase, silica-based	Nonpolar	3	200	95.0 ± 0.2	4

evaluated by collecting 12 mL of sample after it passed over the SPE cartridge into a muffled (450 °C) glass vial (DWK part #244746) and adding 1.5 mL potassium persulfate and sodium hydroxide reagent and autoclaving at 121 °C, which quantitatively oxidizes DON to NO_3^- (Knapp et al. 2005). Each batch of DON samples included reference materials to monitor oxidation efficiency, including caffeine (IAEA-600) and glutamic acid (USGS-40), as well as reagent blanks. The resulting NO_3^- concentration was determined by adding 250 μL of the persulfate oxidized sample to a heated, acidic vanadyl sulfate solution which quantitatively reduces NO_3^- to nitric oxide (NO), which was then measured using chemiluminescence (Braman and Hendrix 1989). The typical standard deviation associated with this DON concentration analysis is ±0.3 μM. Additionally, to determine what fraction of the DON could be eluted off of the SPE column, the methanol-extracted DON was dried down and then re-dissolved in 40 mL of ultra-pure water. Subsequently, the DON and DOC concentrations were determined by high temperature combustion, with a typical standard deviation of ±0.6 μM (Hansell et al. 1993).

Fourier transform-ion cyclotron resonance mass spectrometry analysis

Samples were diluted 1:1 in methanol (LC–MS grade, Sigma-Aldrich Chemical Co.; St. Louis, MO) and analyzed by direct infusion ESI in negative ion mode at a flow rate of 500 μL min^{-1}. Extracted samples were analyzed with a custom-built 9.4 T FT-ICR mass spectrometer at the National High Magnetic Field Laboratory, equipped with a horizontal, 220 mm bore diameter operated at room temperature (Kaiser et al. 2011), and a modular ICR data station (Predator; Blakney et al. 2011) facilitated instrument control, data acquisition, and data analysis. Ions generated by negative ESI at atmospheric pressure were accumulated in an external linear quadrupole ion trap for 300–700 m s and transferred by rf-only quadrupoles (2.0 MHz and 255 $V_{p\text{-}p}$ amplitude) to the ICR cell (Kaiser et al. 2014). ICR time-domain transients were collected from a seven segment open cylindrical cell with capacitively coupled excitation electrodes (Kaiser et al. 2011) based on the Tolmachev configuration (Tolmachev et al. 2008, 2011). One hundred individual transients of 5.8–6.1 s duration collected for crude extracts were averaged, apodized with a Hanning weight function, and zero-filled once prior to fast Fourier transformation. For all mass spectra, the achieved spectral resolving power approached the theoretical limit over the entire mass range, for example, average resolving power, m: $\Delta m_{50\%}$, in which $\Delta m_{50\%}$ is mass spectral peak full width at half-maximum peak height was ~1,000,000–1,300,000 for absorption mode at m/z 500 for all mass spectra.

Due to increased complexity at higher m/z, broadband phase correction (Xian et al. 2010, 2012) was applied to the entire mass spectrum for the SPE extracts to increase resolution of isobaric species as previously described (Beu et al. 2004). ICR frequencies were converted to ion masses based on the quadrupolar trapping potential approximation (Grosshans et al. 1991; Shi et al. 2000). Each m/z spectrum was internally calibrated with respect to an abundant homologous alkylation series whose members differ in mass by integer multiples of 14.01565 Da (mass of a CH_2 unit) confirmed by isotopic fine structure based on the "walking" calibration equation (Savory et al. 2011). Experimentally measured masses were converted from the International Union of Pure and Applied Chemistry mass scale to the Kendrick mass scale (Kendrick 1963) to identify homologous series for each heteroatom class (i.e., species with the same $C_cH_hN_nO_oS_s$ content, differing only by their degree of alkylation). Mass spectral peaks detected six times above the baseline root mean square noise level for each fraction were assigned elemental compositions, grouped by Kendrick mass defect, and grouped based on type and number of heteroatoms (e.g., oxygen and N). Peak assignments and data visualization were performed with PetroOrg software by Kendrick mass defect analysis as previously described (Hughey et al. 2001; Wu et al. 2004; Corilo 2018). Elemental compositions were assigned to formulae of elemental composition $C_{4\text{-}100}H_{4\text{-}200}O_{2\text{-}30}\,N_{0\text{-}9}S_{0\text{-}1}$ as the deprotonated and chlorine adduct forms (^{35}Cl and ^{37}Cl), with a maximum double bond equivalent (DBE) of 15, O:C ratio of 0.8, and mass error of 0.2 ppm, a threshold selected based on mass error plots (Fig. S2). Tabulated assigned peaks are publicly-available at: https://osf.io/dcu3z/?view_only=a6461c07c6994b39bec1e11d1132083a. For each elemental composition, the heteroatom class, type (DBE = number of rings plus double bonds involving carbon) and carbon number, *c*, were tabulated for subsequent generation of heteroatom class relative abundance distributions and graphical abundance-weighted van Krevelen diagrams. While some positively charged nitrogen-containing species (e.g., amines) may be preferentially detected in positive mode, the direct infusion spectra contain many salt as well as ammonium adducts that make it difficult to assign molecular formula with a high degree of confidence. For this reason, negative mode ionization is the most commonly used for FT-ICR MS analysis and was selected for this study, with the acknowledgment that this approach captures a fraction of the whole DON pool. We note that previous studies have found similarly low N:C ratios of molecules within DOM detected by ESI whether using positive or negative ion mode (He et al. 2021; Roth et al. 2022).

Results and discussion

Retention efficiencies of 0.2 liter seawater samples on different SPE sorbents

We compared the retention efficiency of 0.2 liter volumes of Gulf of Mexico surface seawater DON using seven SPE sorbents to identify the sorbent that yielded maximum retention for further study. Retention was measured by comparing the concentration of DON in seawater prior to and after passing through a solid phase extraction (SPE) column, with DON concentrations before and after passing the SPE column measured using persulfate oxidation (see section "Quantification of DON retention and elution efficiencies" above). We observed a range of retention efficiencies from 5 ± 1.3% for the Phenomenex Strata CN (*n* = 4) to 95 ± 0.2% for Agilent Bond Elut C_{18} (*n* = 4; Table 1). While less work has been done comparing retention and elution efficiencies of SPE cartridges for DON (Stücheli et al. 2018), previous studies have reported optimal extraction of marine DOC (43–62%) using Agilent Bond Elut PPL (Dittmar et al. 2008). Given the C:N ratio of recovered marine DOM from these studies (21:1 to 33:1) compared to natural seawater (10:1 to 14:1), the estimated efficiency of DON recovery is between 20% and 40%. Another recent study found that PPL retained 34% of surface and 39% of deep ocean DON (Broek et al. 2017). These results are comparable to the findings here, which indicate that the PPL sorbent retained 23 ± 2.5% (*n* = 4) of DON from Gulf of Mexico surface seawater (Table 1).

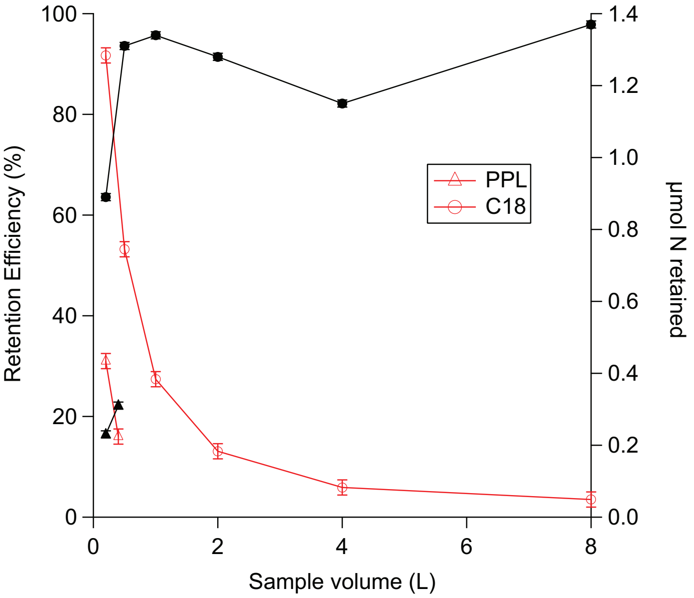

Fig. 1. Comparison of PPL (100 mg bed mass; triangles) and C18 (200 mg bed mass; circles) SPE sorbent retention efficiency (red open symbols) and μmols N retained by sorbent (black filled symbols) as a function of sample volume. Error bars correspond to the standard deviation of μmol N retained given $a \pm 0.3$ μM standard deviation associated with DON concentration analysis, and the corresponding change in calculated retention efficiency.

In addition to the high retention efficiency of DON onto the C_{18} sorbent, the Plexa sorbent also retained 93 ± 0.2% (*n* = 2) of the marine DON sample. Both the C_{18} and Plexa sorbents target nonpolar analytes. This suggests that much of marine DON in the analyzed samples is lipophilic. Indeed, SPE sorbents designed to retain polar molecules were the least effective at retaining marine DON (Table 1). These results are consistent with prior work that found that the C_{18} sorbent was effective at retaining marine chromophoric DOM (Amador et al. 1990) and extracting estuarine DOM (Simjouw et al. 2005). We note that while the SPE sorbents used to test the retention and extraction efficiencies had different total bed masses, the bed mass was not correlated with retention efficiency of a 0.2 liter sample (Table 1). For example, 500 mg of Strata CN SPE only retained 5 ± 1.3% of the DON in a 0.2 liter sample, whereas 200 mg of the C_{18} SPE retained 95 ± 0.2% of the 0.2 liter seawater sample, indicating that the SPE sorbent chemistry plays an important role in retaining marine DON.

Comparison of SPE retention based on sample volume

In addition to testing the retention efficiencies of different SPE sorbents, the effect of varying sample volumes on retention efficiency for the C_{18} and PPL sorbents was evaluated (Fig. 1). SPE sorbents have a finite loading capacity that is dependent on the compatibility of the binding properties of the SPE sorbent and the nature of the chemical being extracted, before breakthrough and incomplete retention is observed. Prior studies have found that high mass loadings (e.g., > 0.5 mg DOC per 100 mg PPL resin) discriminate against highly oxygenated compounds (Li et al. 2016). For marine waters, little breakthrough of DOC has been previously observed for loadings of up to 50 liters of deep seawater per 5 g PPL sorbent (Dittmar et al. 2008). The results presented here suggest that the loading capacity for marine DON on SPE sorbents appears lower than for DOC. DON extraction efficiencies decreased significantly with volumes > 0.2 liters for both C_{18} (200 mg) and PPL (100 mg) sorbents (Fig. 1). While the total mass of DON captured on the C_{18} sorbent was higher with a 0.5 liter seawater sample (1.3 μmol N) compared to a 0.2 liter seawater sample (0.9 μmol N), the fractional retention of a 0.5 liter DON sample was only 53% compared to the 92% for a 0.2 liter sample (Fig. 1). These results imply that surface seawater loadings above 0.2 liters per 200 mg C_{18} sorbent discriminate against DON molecules. Furthermore, sample loading volumes ≥ 0.5 liter at best resulted in incremental increases in the total amount of DON recovered when the tradeoff between retention and increased sample volume was favorable (Fig. 1). For example, 4 liters of the 4.9 μM DON sample passed over a C_{18} sorbent only retained 5.9% of the DON in the sample, capturing 1.16 μmols N, or less N than was retained by either the 2 or 8 liter sample, given the tradeoffs in sample volume vs. retention efficiency (Fig. 1). Similarly, because of the decrease in sample retention when a 0.5 liter sample was passed over a PPL sorbent compared to a 0.2 liter sample (i.e., 16% vs. 23%, respectively) only a modest increase in μmols N retained in the larger sample (i.e., 0.31 vs. 0.23 μmols N) was observed (Fig. 1). Consequently, larger sample volumes were not evaluated for the PPL sorbent. Thus, increasing sample volumes may not necessarily result in increased mass of DON recovered for further characterization. Given the potential for preferential retention of certain compound classes on SPE sorbents and “washout” of others with higher sample volumes (see section “Comparison of FT-ICR MS analysis of 0.2 liter surface eluted using a gentle vacuum vs. gravity” below), we chose a sample loading volume of 0.2 liter for further experiments.

SPE elution efficiency

While the C_{18} SPE sorbent *retained* the largest fraction of a 0.2 liter marine DON sample, DON recovery also relies on effective elution. The most common DOM SPE methods elute samples off of SPE sorbents using methanol and a gentle vacuum, extracting the solvent at < 2 mL min^{-1} (Dittmar et al. 2008). Here, we evaluated the elution efficiencies of a 0.2 liter marine DOM sample from the PPL and C_{18} sorbents, selected because PPL is commonly used for marine DOM extraction and C_{18} given its superior ability to retain marine DON. Using a gentle vacuum (1 mL min^{-1}) to pull 1 mL of methanol through the sorbent yielded 39% of the total DON sample from the PPL sorbent. This is a relatively low elution efficiency, but problematically, this elution efficiency exceeded the retention efficiency for the PPL sorbent (23%), which violates mass balance. Gentle, vacuum-assisted elution yielded only 22% of the DON from C_{18}, a minor fraction of the 95% of the DON sample retained by the C_{18} sorbent. In contrast, gravity elution of methanol through the PPL and C_{18} sorbents at 0.1 mL min^{-1} yielded 67% and 92% of the DON sample, or 1.7- and 4.2-fold higher recovery of DON compared to gentle vacuum elution, respectively. We expect that the low yield of DON from C_{18} with vacuum relative to gravity elution results from the superior retention of DON by the C_{18} sorbent, with the longer exchange times using gravity elution providing more time for DON to enter the mobile phase than using gentle vacuum elution. While for C_{18} these elution efficiencies do not violate mass balance, for PPL both vacuum and gravity elution results indicate that more DON was eluted from the sorbent than was retained by it.

We hypothesize that this apparently higher elution than retention efficiency for the PPL sorbent results from the potentially significant blank introduced by the procedures used to quantify the elution efficiency. Direct measurement of DON concentration in the methanol-extracted samples by persulfate oxidation, as was used for quantifying DON retention, is not possible, as the persulfate oxidation reaction involves free-radical reactions, which are poisoned by even trace amounts of methanol (Bartlett and Cotman 1949). Consequently, it was necessary to analyze eluted DON concentrations by high-temperature combustion, for which the methanol was evaporated under an N_2 stream and the sample was dissolved in a larger volume of ultrapure water. We consider the redissolving of the extracted sample in ultrapure water to be the largest contributor to this apparent blank, as ultrapure water has nonzero DON concentrations, commonly 1 to 2 μM in our lab at the time of analysis. Given that 40 mL of ultrapure water was added to the dried extract, this could contribute 80 nmols N to the 230 nmols N retained by the PPL sorbent, which would correspond to a 35% elution efficiency, similar to the observed 39% elution efficiency. Additionally, the high-temperature combustion analysis employed to measure the DON concentration in eluted samples has higher standard deviations than persulfate oxidation, typically ±0.6 μM. We expect that these issues contributed significant contaminant N and uncertainty that bias the measured recoveries of eluted DON from the PPL and C_{18} sorbents. Since the PPL retained less DON than the C_{18} sorbent (i.e., 230 vs. 930 nmols, respectively), the 80 nmols DON contamination from the ultrapure water would represent a larger fraction of the eluted DON in the PPL than the C_{18} extract. Consequently, while the absolute elution efficiency is uncertain, the results are still instructive.

Most importantly, we find that gravity elution yields more DON than gentle-vacuum assisted elution from either the C_{18} or PPL sorbents, all of which had the same quantity of ultrapure water added to their dried extracts. Additionally, the C:N ratios of gravity eluted samples were more similar to bulk DOM measurements than gentle-vacuum eluted samples. Acknowledging that some DOC and DON in the eluted samples likely originates in the ultrapure water, but that this will

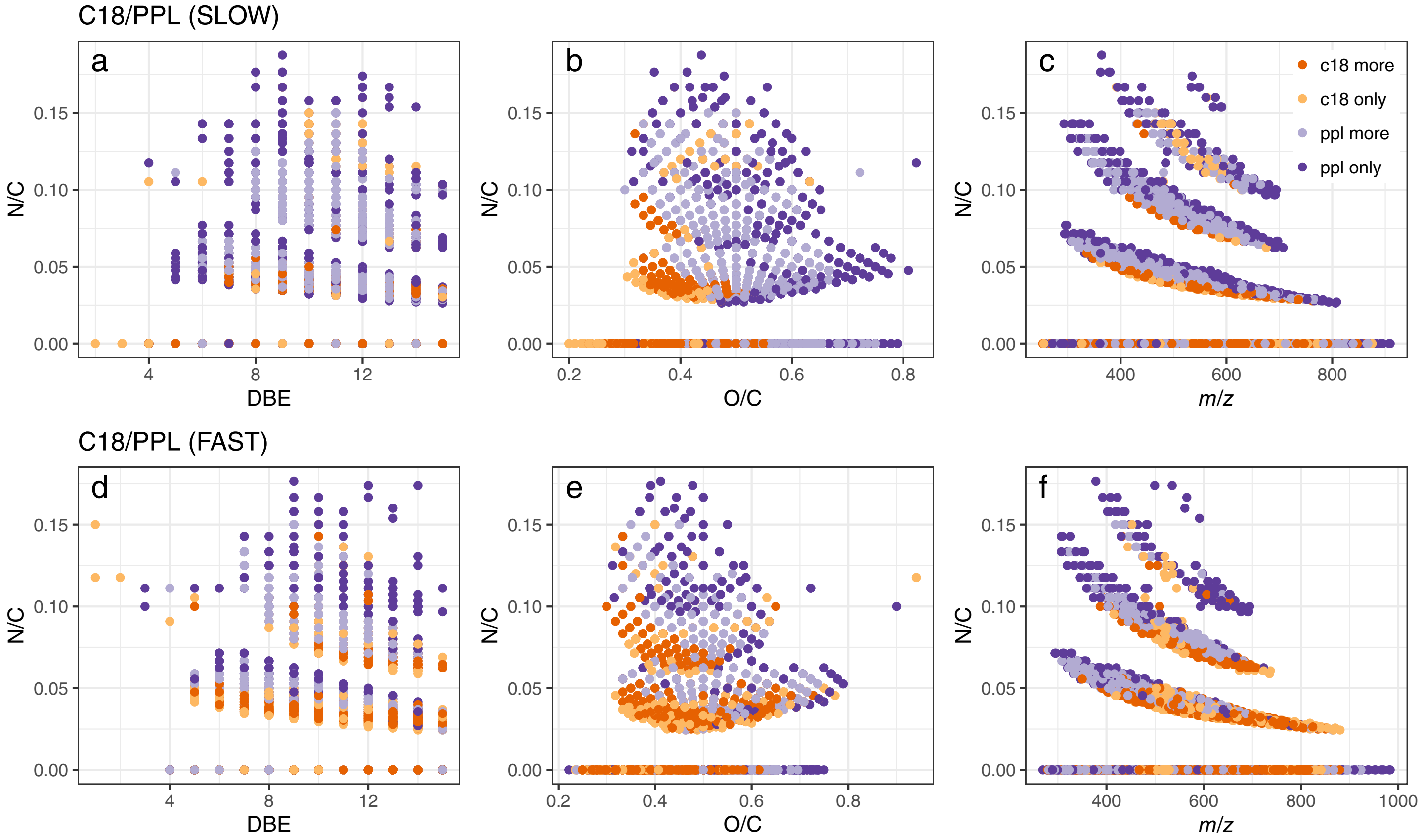

Fig. 2. N/C diagrams of molecular formulae attributed to FT-ICR MS spectra from 0.2 liter surface seawater samples eluted by gravity (slow) (**a**–**c**) and gentle vacuum (fast) (**d**–**f**), including characterization of double bond equivalents (DBE) (a, d), O:C ratio (b, e), and mass to charge ratio (*m/z*) (c, f). The color scale corresponds to the relative detection of molecular species in samples extracted with C_{18} vs. PPL sorbent. "C_{18} only" and "PPL only" correspond to molecular ions that were only detected under one extraction condition. "C_{18} more" and "PPL more" correspond to ions detected under both conditions where the intensity ratio (C_{18}/PPL) was, respectively, > 1 or < 1.

represent a larger source of bias for PPL than C_{18} extracted samples, the C:N ratios of DOM extracted using gentle vacuum elution were 39:1 on the PPL and 24:1 on the C_{18} SPE sorbents, respectively. Prior work has similarly reported elevated C:N ratios for marine DOM when extracted using gentle vacuum elution from PPL and C_{18} SPE sorbents (Dittmar et al. 2008; Broek et al. 2017). However, gravity elution of DOM yielded C:N ratios of 14:1 using PPL and 12:1 using C_{18} SPE sorbents, respectively. These results are consistent with the C_{18} SPE sorbent both retaining and yielding the majority of a marine DON sample when it is extracted using gravity elution. Thus, the higher yield of DON using gravity vs. gentle vacuum elution of solvent provides a promising method adaptation to recover a greater proportion of a marine DOM sample from SPE sorbents.

FT-ICR MS characterization of DON using different elution methods and SPE sorbents

The experiments and mass balance calculations described above establish that: (1) for a 0.2 liter sample, C_{18} SPE sorbents are 4.2-fold more effective at retaining marine DON than PPL SPE sorbents; (2) for sample volumes > 0.2 liter per 200 mg resin the fraction of DON retained by SPE sorbents decreases; and, (3) gravity elution of solvent over a SPE sorbent extracts 1.7- to 4.2-fold more DON than gentle vacuum filtration. Given the variability in the application of SPE methods for characterizing environmental DOM, especially the use of different SPE types and sample volumes, we ask whether these differences in methodological protocols are reflected in the chemical composition of the organic matter extracted from SPE sorbents. Here, we use FT-ICR MS to evaluate this question.

First, we address a fundamental limitation of FT-ICR MS characterization of DON by ESI, which is the wide range of ionization potentials that comprise the polydisperse DOM pool. Ion suppression results from the presence of less volatile compounds that can change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector. Complex, polydisperse and polyfunctional samples (e.g., DOM, petroleum) contain many thousands of compounds that span a wide range of ionization potentials in

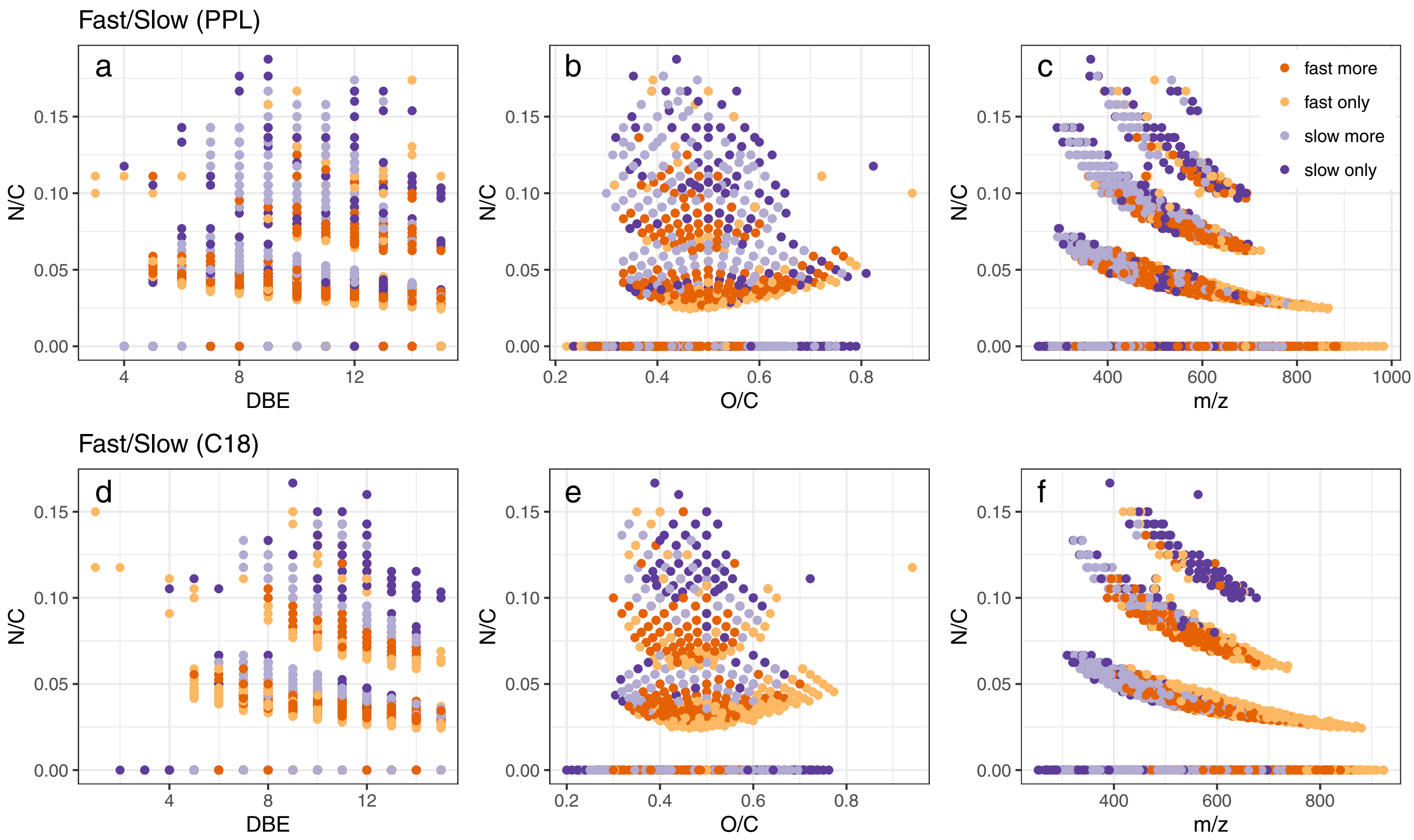

Fig. 3. N/C diagram of molecular formulae attributed to FT-ICR MS spectra from 0.2 liter surface seawater samples extracted with PPL (**a–c**) vs. C_{18} (**d–f**) sorbents, including characterization of double bond equivalents (DBE) (a, d), O:C ratio (b, e), and mass to charge ratio (*m/z*) (c, f). The color scale corresponds to the relative detection of molecular species in samples extracted with gentle vacuum (fast) vs. gravity (slow) elution. "Fast only" and "Slow only" correspond to molecular ions that were only detected under one extraction condition. "Fast more" and "slow more" correspond to ions detected under both conditions where the intensity ratio (fast:slow) was, respectively, > 1 or < 1.

negative-ion ESI, and thus detection of each compound class is affected by sample matrix related ion suppression (Annesley 2003). DOM contains ~50% oxygen-containing compounds that consist of multiple oxygen-bearing functional groups that span a wide range of ionization efficiencies, thus limiting the detection of DOM compounds by any ESI mass spectral techniques to the most efficiently ionized compounds, often oxygen-rich carboxylic acids. Therefore, N-rich compounds, which are already a minor fraction of the total organic matter, are suppressed in negative ion ESI by more efficiently ionized compounds that are higher in signal magnitude. Consequently, the challenge in molecular characterization of DON from marine waters is multifaceted. First, routinely applied SPE techniques recover a lower percentage of DON than DOC, and the more abundant oxygen classes suppress the ionization of the small fraction of DON that is recovered. This study takes the first step of improving marine DON characterization by FT-ICR MS by increasing the extraction efficiency of DON using optimized SPE methods to increase the fraction of marine DON available for molecular characterization. Here, we compare how these improved extraction methods result in differences in chemical composition as determined using FT-ICR MS.

Comparison of FT-ICR MS analysis of 0.2 liter surface seawater extracted with C_{18} vs. PPL sorbents

Negative-ion ESI FT-ICR MS analyses of 0.2 liter surface seawater isolated with PPL and C_{18} SPE sorbents and eluted using gravity (0.1 mL min^{-1}) and a gentle vacuum (1 mL min^{-1}) were compared to qualitatively characterize DON compositional differences between samples extracted with the two sorbents. Despite the large differences in total DON extracted, the FT-ICR MS spectra of the PPL and C_{18} extracted DOM were overall very similar (Figs. S3, S4). Subtle differences in the composition of DON extracted with the two different resins largely followed trends observed for bulk DOC (Fig. S7, discussed in Supporting Information). The most intense N-containing ions incorporated only 1–2 N atoms per molecule (Fig. S3). Those with O:C ratios < 0.5 tended to be more abundant or uniquely detected in the C_{18} extract, while ions with greater O:C ratios tended to be more abundant in the PPL extract (Fig. 2). These results are consistent with the chemical selectivity of more polar material by PPL vs. C_{18}. Yet, these molecules are at most minor components of the overall DON pool.

Since the overall N:C ratio of the extracted DOM measured by high-temperature combustion (section "SPE elution efficiency") exceeded 0.08 under the highest recovery conditions, there must be a major component of DON-comprised of molecules with elevated N:C stoichiometries that was preferentially extracted by C_{18} over PPL. However, no molecular formula with N > 4 could be attributed to any of the detected FT-ICR MS peaks in these samples. Furthermore, very few molecular features were observed with N:C ratios > 0.1, most of which were more abundant or only detected in the PPL extracted samples. In the slowly eluted C_{18} sample, there was a group of ions with *m/z* ranging from 500 to 600 that contained N:C ratios between 0.1 and 0.15, O:C ratios between 0.4 and 0.5, DBE values between 10 and 14, and relative abundance up to 6%, which is within the stoichiometric range of peptides (Rivas-Ubach et al. 2018). This outcome suggests that most of the DON in these samples was not present as small, easily ionized molecules that could be detected. This can include very small or large molecules below the mass range of the FT-ICR MS. DON components that have been quantified in seawater include urea and free amino acids (5–10% of DON) that are smaller than the FT-ICR MS mass window, as well as HMW proteins and amino-sugars (~30%; Sipler and Bronk 2015). It is also possible that negative mode ESI may not effectively ionize and detect the N-containing molecules that are selectively recovered by C_{18}. This may have been in part due to the suppressive effect of other non-N containing ions that were uniquely detected in the C_{18} extracts (Fig. S5). Indeed, these ions may at least in part explain why some low abundance DON molecules were detected only in the PPL extracted samples (see Supporting Information methods).

Comparison of FT-ICR MS analysis of 0.2 liter surface eluted using a gentle vacuum vs. gravity

Next, we compared the same FT-ICR MS analysis of a 0.2 liter surface seawater sample extracted onto a C_{18} resin and eluted using a gentle vacuum (1 min mL^{-1}) with a 0.2 liter sample similarly extracted but eluted by gravity (0.1 mL min^{-1}; see section "SPE elution efficiency"; Figs. 3, S3, S6, and S7). The differences in the spectra indicate that elution speed resulted in a shift in relative compound abundance toward those with higher N:C ratios and lower molecular weight (< 500 m/z) and O:C ratios (< 0.6) in samples that were eluted by gravity compared with vacuum-assisted elution (Fig. 3). Together with the increased DON and DOC recovery in the gravity vs. vacuum-assisted elution, this suggests that the slower elution yields a greater fraction of tightly retained, small peptide-like molecules, which may require more time to equilibrate between the stationary and mobile phases. Given the increased abundance and presence of ions with N:C ratios > 0.1 in the slow-eluted samples (Fig. 3), it seems that this may account for at least some of the improvement in DON extraction efficiency observed when using the gravity vs. vacuum-assisted sample elution. However, it is also likely that other DON components were not detected by negative mode ESI FT-ICR MS for the reasons described above.

Conclusion

We report a SPE method designed to extract DON from marine samples, with improved yields of DON resulting from using the C_{18} sorbent, relatively small sample volumes, and using gravity instead of a gentle vacuum to elute the sample. The results described here emphasize the challenge in developing and applying methods to chemically characterize the bulk marine DOM pool. While prior methods have been optimized to extract and have successfully characterized many features of the marine DOC pool, here we show that modifications of these methods are necessary to analyze marine DON. Specifically, we find that the C_{18} SPE sorbent retains 4.2-fold more, and gravity elution from C_{18} yields up to 4.2-fold more DON, compared to gentle-vacuum elution of a sample from the commonly used PPL SPE sorbent. Additionally, the retention of DON onto SPE sorbents decreases with increasing sample volume, indicating that SPE sorbent sites may become saturated above > 0.2 liters of sample, with preferential loss of N-containing compounds. These results are consistent with previous observations that lower proportions of highly oxygenated polar compounds are lost from complex DOM at high mass loadings (Li et al. 2016), resulting in samples biased toward more nonpolar molecules. This is consistent with our finding that in addition to the differences in the quantity of DON extracted we also find differences in the chemical composition of DON depending on the protocols used. Comparisons of the molecular composition of DOM extracted using these different methods by FT-ICR MS indicates that some of the previously missing portion of the marine DON pool is in part composed of small, peptide-like N containing molecules. However, most of this missing DON is not detected in negative-ion direct infusion ESI FT-ICR MS, and further analytical development is warranted to fill this gap.

These findings that different extraction protocols yield quantitatively and qualitatively different portions of the bulk DON pool underscores the importance of optimizing methods to target specific DOM pools. We also note that evaluation of extraction efficiency still provides only an upper, and not lower, bound for the fraction of a bulk sample evaluated by FT-ICR MS. Ionization efficiency, ion suppression, and molecular weight constraints also prevent all of the DOM that is introduced to the FT-ICR MS from being characterized. We expect that the methods described here may be further optimized by considering sample elution with a series of different solvents or using chromatography as a means of fractionating the organic matter to reduce ion suppression and adduct formation (Boiteau et al. 2019; Kim et al. 2019; Han et al. 2021;

Qi et al. 2021; Rowland et al. 2021). Furthermore, a larger number of transients across several narrower mass windows may further improve the signal-to-noise ratio of spectra acquired by FT-ICR MS, and thus achieve more comprehensive characterization of DON (Bercovici et al. 2022). Complementary approaches such as small molecule (< 200 Da) quantification and ultrafiltration may be employed to constrain the fraction of extracted DON that is outside of the mass window of FT-ICR MS.

Data availability statement

Data presented in this manuscript are available at: https://osf.io/dcu3z/.

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Acknowledgments

A.N.K. gratefully acknowledges NSF-OCE 1537314 and NSF-OCE 2148989, which funded this work. We thank Taylor Broek for conversations. We also thank Markus Huettel for running extraction efficiency samples on his high temperature combustion machine for DON concentration analysis, and Huan Chen for assistance in data collection. A.M.M. was partially supported by the Gulf of Mexico Research Initiative, and funding for R.B. was from NSF-OCE 2149091. A portion of this work was performed at the Ion Cyclotron Resonance User Facility at the National High Magnetic Field Laboratory, which is funded by the National Science Foundation Division of Chemistry and Division of Materials of Research through NSF DMR 16-44779, and the State of Florida.

Conflict of Interest

None declared

Submitted 30 August 2022
Revised 05 May 2023
Accepted 22 May 2023

Associate editor: Hayley Schiebel