

COMMENTARY

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Key Points:

- While C emission rates are widely understood to be substrate controlled, characterizing organic matter quality remains a challenge
- Optical measurements are widely used, but they may not be the best tool for assessing organic matter quality
- NOSC provides a thermodynamically relevant measure, which could be used as a more rigorous metric of organic matter quality

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## Advanced Molecular Techniques Provide New Rigorous Tools for Characterizing Organic Matter Quality in Complex Systems

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**Abstract** Carbon flux rates are widely understood to be substrate controlled; however, characterizing substrate quality continues to be a challenge. We suggest that, while optical measurements have their place, they are not the only, or the best, tool for characterizing organic matter *quality*. Nominal oxidation state of the carbon provides a thermodynamically relevant measure, which could be used as a metric of organic matter quality. Calculating nominal oxidation state of the carbon requires a suite of advanced complementary analysis but is then trivial to calculate from the resulting data sets.

### 1. Introduction

Wetlands represent one of the largest natural sources of CH<sub>4</sub> flux to the atmosphere (Bridgman et al., 2006). Despite this, the uncertainties in predicting wetland CH<sub>4</sub> fluxes under a warming climate remain large (Shuang et al., 2017). Experimental attempts to address this uncertainty have largely focused on assessing organic matter quality as a control on CO<sub>2</sub> and CH<sub>4</sub> flux (e.g., Chanton et al., 2008; Cunada et al., 2018; Tfaily et al., 2013). This is logical since, in these largely anaerobic environments, organic C is primarily degraded by soil and sediment microorganisms to CO<sub>2</sub> and CH<sub>4</sub>, which is ultimately emitted to the atmosphere. Radiocarbon analyses across multiple wetlands have highlighted that subsurface dissolved organic matter (DOM) drives microbial CO<sub>2</sub> and CH<sub>4</sub> production (Chanton et al., 1995, 2008; Chasar et al., 2000; Tfaily et al., 2014; Wilson et al., 2016). Thus, many studies attempt to link DOM quality with C fluxes (e.g., Cunada et al., 2018). In practice, bulk optical measurements are often used to make inferences about DOM quality across habitats, with environmental disturbance, or with changing inputs (e.g., Broder et al., 2017; Coble, 1996; Cunada et al., 2018; Downing et al., 2009; Jane et al., 2017; Saraceno et al., 2009; Spencer et al., 2014; Tfaily et al., 2013). We suggest, however, that optical measurements do not provide the best metric for measuring organic matter quality. For example, the specific ultraviolet absorbance at 254 nm normalized to dissolved organic carbon concentration (i.e., SUVA<sub>254</sub>) is frequently reported as a measure of organic matter aromaticity which is then often considered to be synonymous with organic matter recalcitrance (e.g., Broder et al., 2017). However, many aromatic compounds are bioavailable even under anaerobic conditions (Keiluweit et al., 2016) and may actually play an important role in microbial CO<sub>2</sub> and CH<sub>4</sub> production (Valenzuela et al., 2017; Wilson et al., 2017). Instead, we suggest that calculation of the nominal oxidation state of the carbon (NOSC; Keiluweit et al., 2016) provides a thermodynamically relevant metric for assessing organic matter quality and is, therefore, more appropriate than bulk optical measures. NOSC is directly related to the thermodynamic driving force (LaRowe & Van Cappellen, 2011) making it possible to calculate the energy provided by the oxidation of a particular compound given the available electron acceptors and environmental conditions (Keiluweit et al., 2016). This is ultimately what is meant by organic matter quality. NOSC can be calculated simply from molecular formulae:

$$\text{NOSC} = -(( -z + 4(\#C) + (\#H) - 3(\#N) - 2(\#O) + 5(\#P) - 2(\#S) ) / (\#C)) + 4$$

(where z is the net charge).

Thus, this metric provides a measurable, calculable quantity that can be used to make quantitative comparisons of organic matter quality across sites, studies, and even disciplines. Finally, this approach provides a valuable measure for modelers to predict responses in organic matter cycling—and ultimately C emissions—to changing global temperatures, organic matter inputs, or availability of electron acceptors (e.g., O<sub>2</sub>

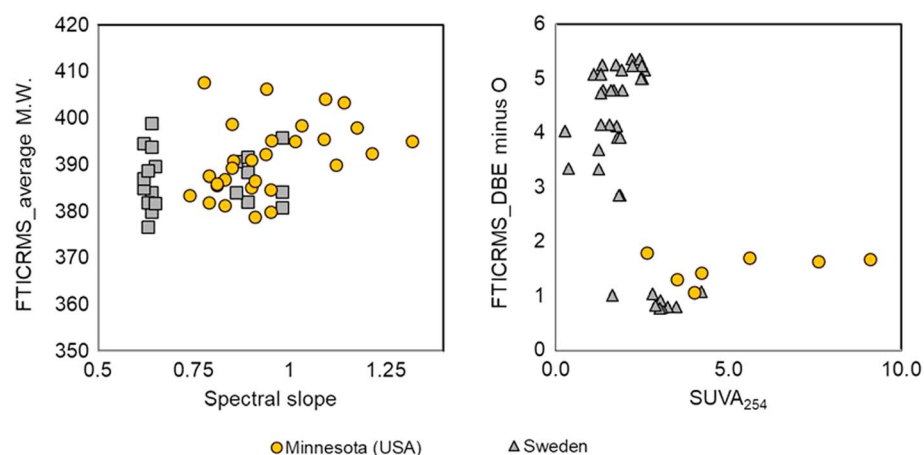
due to climate induced drying or flooding). However, NOSC cannot be calculated from the traditional optical measurements typically used to infer organic matter quality; it requires a more detailed analysis available only through advanced molecular techniques. Herein, we argue that, while optical measurements have their place, assessing the quality of organic matter is better accomplished by these more advanced analytical methods.

### 1.1. DOM Quality

Optical measurements have been used to infer organic matter quality through correlations with bulk molecular weight (Helms et al., 2008), aromaticity, and humic content in numerous studies (e.g., Cunada et al., 2018; McKnight et al., 2001; Weishaar et al., 2003). The term humic is operationally defined in terms of the fraction of organic matter, which forms insoluble precipitates at alkaline pH. Although this characterization was criticized more than 80 years ago (e.g., Waksman & Cordon, 1936), the complexity of organic matter prevented a rigorous analysis of its composition (e.g., Haworth, 1971) and humic substances remained a mysterious fraction of organic matter for some time. Over a decade ago, nuclear magnetic resonance (NMR) analysis showed that all of the detectable signals in humic material can be assigned to known chemical classes (e.g., carbohydrates, lignins, and proteins; Kelleher & Simpson, 2006), but the idea of humic substances in organic matter continues to persist in the literature (e.g., Broder et al., 2017; Ejarque et al., 2017; Valenzuela et al., 2017). Using current techniques, it is possible to correctly characterize and, in some cases, quantify many of these humic substances (Kelleher & Simpson, 2006), which represent only a small fraction of the total organic matter pool (Schmidt et al., 2011) and cover a broad range of oxidation states that are more or less bioavailable (Lehmann & Kleber, 2015).

Fluorescence measures, particularly from excitation-emission matrix spectroscopy (EEMS), have been used to differentiate between terrestrial, microbial protein-like, and (where appropriate) marine organic matter inputs (e.g., Coble, 1996; McKnight et al., 2001). These optical measurements were a boon to researchers wishing to characterize DOM. They are inexpensive and fast, allowing high throughput so that high-resolution sampling in both space and time is possible. With proper standardization fluorescence, in particular, can allow quantification. Optical data can be collected in situ and may be used by remote sensing allowing high-frequency, real-time data collection for understanding ecosystem properties (reviewed in Aiken, 2014; Ruhala & Zarnetske, 2017). There are limitations, which are too often ignored in studies utilizing these techniques. For example, optically active DOM makes up only a fraction of the total organic matter pool (Cory et al., 2011); thus, important components may be missed by this technique altogether. Fluorophores are sensitive to interactions in solution and behave nonlinearly in complex mixtures such as DOM. This behavior is rarely taken into account when inferring DOM quality or quantifying changes across habitat types. Further, the information-packed spectra produced by EEMS are typically analyzed via parallel factor analysis or a similar statistical technique, which, by definition, is a data reduction technique that reduces the spectra into the smallest number of excitation/emission pairs, which can be recombined to reproduce the original data. Thus, the components identified by parallel factor analysis and other statistical analyses are not actual fluorophores but rather an excitation/emission pair that may represent an amalgamation of overlapping compounds complicating their interpretation. For example, many compounds overlap in the protein-like region of the EEMS spectrum; further, the optically active tyrosine and tryptophan moieties with which this region is classically identified are typically quenched in proteins and therefore may not be contributing to the fluorescence in that region at all (Aiken, 2014). To complicate things further, not all compounds in DOM are fluorescence active—Thus, some changes in the DOM may not be spectrally detected causing important information to be missed or the changes that are detected may comprise a trivial fraction of the overall DOM pool emphasizing some feature that may ultimately contribute little to the question under investigation.

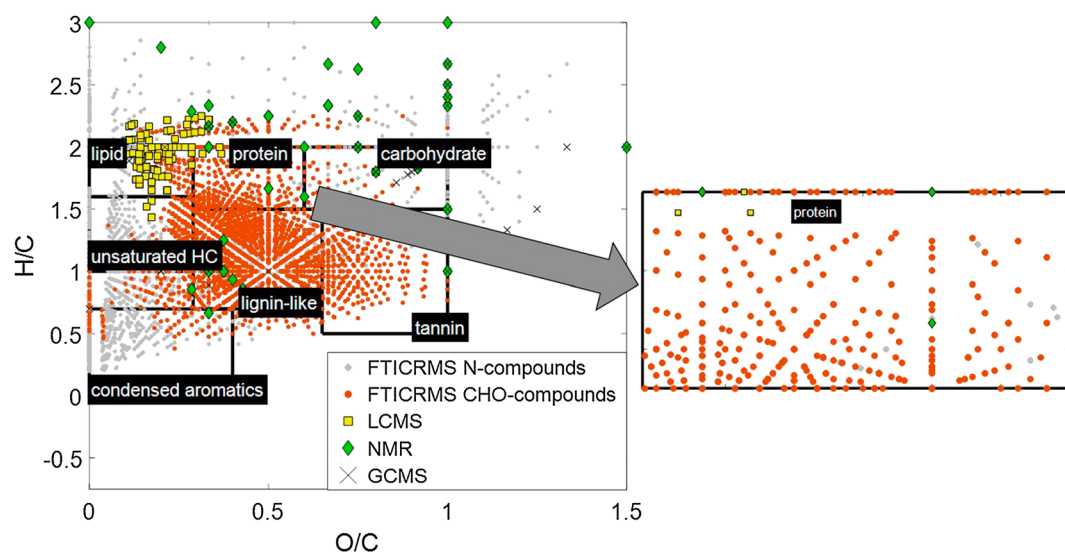
Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) has emerged as an important tool for identifying molecular formula comprising DOM (Kim et al., 2003; Stenson et al., 2002, 2003). This technique is capable of simultaneously identifying, to molecular formula, thousands of compounds making it ideal for identifying compounds in complex mixtures and providing more detail about the molecular characteristics than bulk optical measurements. Comparisons between FTICRMS results and optical measurements ( $SUVA_{254}/EEMS$ ) reveal that some of the assumptions about how optical properties vary with molecular weight or aromatic content may be incomplete or, in some cases, erroneous (Figure 1). This could potentially be due to the fact that either not all molecules are ionizing during electrospray ionization (ESI) FTICRMS



**Figure 1.** Regressions of average molecular weight (M.W.) based on FTICRMS (left panel) versus the optical proxy spectral slope and double bond equivalences (DBE minus O; right panel) versus the optical proxy  $SUVA_{254}$  for pore water samples collected from a *Sphagnum*-dominated bog and *Carex*-dominated fen site in Stordalen mire, Sweden, and a Spruce (*Picea* sp.) bog in Minnesota (USA) by these authors (unpublished data). The FTICRMS-based average molecular weight of the dissolved organic matter was not significantly correlated with the optical proxy for molecular weight: spectral slope ( $p > 0.05$ ). Double bond equivalences (DBE minus O) were not significantly correlated with the optical proxy for aromaticity ( $SUVA_{254}$ ). The apparent separation in the Sweden samples along spectral slope corresponds closely with habitat, the *Sphagnum*-dominated bog has lower spectral slope, while the *Carex*-dominated fen has higher spectral slope. These two habitats, however, do not differ in average molecular weight ( $t = 0.095$ ,  $p > 0.05$ ), suggesting that while optical properties are indicating differences in bulk dissolved organic matter, they may not be fully capturing the desired features. FTICRMS = Fourier transform ion cyclotron resonance mass spectrometry.

(considering both negative and positive ionization) or because optical measurements are mainly focused on aromatic/conjugated compounds and are therefore missing much of the DOM pool.

While a powerful tool when used correctly, FTICRMS is not always used to its full potential. For example, van Krevelen diagrams (van Krevelen, 1950), which plot O/C content versus H/C content (Figure 2), are used almost ubiquitously to make inferences about organic matter quality, reaction pathways (Kim et al., 2003), or changing compounds classes. However, van Krevelen diagrams (vK) have several limitations, they do not capture mass-dependent information, many compounds can have similar O/C versus H/C ratios meaning that each point is not necessarily unique on the vK, and any heteroatom content information (which can be large in some DOM samples) is lost (Reemtsma, 2010). Further, regions of the vK, which have historically been identified as tannin like, carbohydrate like, lignin like, or protein-like, may be inadequately defined. For example, oftentimes, compounds from natural DOM samples that plot in the protein region of the vK do not even contain any N in their assigned molecular formulae, making their identification as protein-like doubtful (Figure 2). Further, when used alone, FTICRMS may be missing critical changes in C cycling. This is because, in most cases, FTICRMS is limited to molecular formula of at least 100 Da and the resulting data are semiquantitative. Recent advances in coupling FTICRMS with separation techniques (e.g., LC; e.g., Veličković et al., 2017) have reduced concerns about ion competition and improved confidence in inferring relative abundances from FTICRMS spectral intensities. One limitation of FTICRMS that remains is that many critical metabolites, like glucose, sucrose, and pyruvate are not only too small to be detected by FTICRMS but may be poorly ionized by ESI. Positive-mode atmospheric pressure photoionization is more efficient at ionizing N-containing compounds than negative-mode thus both (+) and (−) ESI atmospheric pressure photoionization must be used for characterizing DOM samples, which contain important fractions of N-rich compounds (Osborne et al., 2013). In comparison, gas chromatography mass spectrometry (GCMS) is capable of identifying relatively low molecular weight compounds including metabolically important ones like glucose and sucrose and provides information about their relative abundance, which can be used to infer changes with perturbation or across habitats. NMR takes things a step further by providing quantitative information on even hard to analyze, critically important metabolites such as acetate. By integrating all of these techniques we can gain a more holistic view of the DOM (Figure 2). One caveat with both GCMS and NMR is that they require a



**Figure 2.** Van Krevelen diagram (H/C versus O/C, ratio) with boxes outlining the traditional assignments for compound classes identified by FTICRMS (circles), LCMS (squares), NMR (diamonds), and GCMS (crosses) from a sample of dissolved organic matter collected from peat in northern Minnesota, USA (Wilson & Tfaily, 2018). Circles representing O/C and H/C of formula identified by FTICRMS are color coded to reflect nitrogen-containing formula (gray dots) and nonnitrogenous compounds (red dots). The inset zooms in on the region traditionally identified as protein like, highlighting the large number of red (non-N compounds) in this region whose identification as protein-like is dubious. FTICRMS = Fourier transform ion cyclotron resonance mass spectrometry; NMR = nuclear magnetic resonance.

database against which to identify and quantify compounds; therefore, novel compounds remain a challenge and are best identified via FTICRMS and related emerging techniques (e.g., Veličković et al., 2017).

LC MS/MS analysis of organic matter composition using orbital ion-trap and quadrupole time of flight MS has been employed to identify potential structure and chemical formula of organic compounds through comparison to library mass spectra, mainly in marine systems (Kujawinski et al., 2017; Longnecker & Kujawinski, 2017). These instrumentations have lower resolving power compared to the FTICRMS and are characterized by significantly higher scan speeds due to the coupling with liquid chromatography. Unlike direct injection FTICRMS, which makes use of its superior mass resolution to assign molecular formulae, only around 1% of organic compounds could be annotated through publicly available mass spectral libraries (such as massBank, Horai et al., 2010; National Institute of Standards and Technology (NIST), Stein, 2014; METLIN (<http://metlin.scripps.edu>), Guijas et al., 2018; MetFrag, Wolf et al., 2010). Thus, FTICRMS remains superior in identification of novel compounds. Ongoing development of new tools and new data analysis pipelines will facilitate integration of additional instrumentation techniques to better understand the complexity and composition of organic matter. NOSC provides a metric for standardizing with regard to organic matter quality across the various techniques.

## 1.2. Integrating Techniques for a Complementary Analysis

Unlike optical measures, which are typically used to characterize only the DOM, these techniques can be used to characterize both DOM and solid organic matter following an appropriate extraction protocol (e.g., Tfaily et al., 2015, 2017). Each of the proposed analytical techniques has its own complement of strengths and weaknesses; by integrating them in a combined approach we can leverage their strengths to obtain the most comprehensive analysis of organic matter in a system. The first step of this integration would involve identification of all of the compounds present in a sample. The high complexity of natural organic matter makes it necessary to leverage multiple analytical techniques. Depending on the question under investigation, the resulting compounds identified by each of the techniques may simply be combined into a single comprehensive list. NOSC could be calculated for each formula in this master list. Since the techniques provide a range of quantitative (e.g., NMR) and semiquantitative (GCMS, liquid chromatography mass spectrometry (LCMS), and FTICRMS) results, a standardization approach may be needed to make comparisons across samples. One approach is to identify redundant compounds among the various techniques and then use those

compounds to standardize responses across various techniques. For example, phosphocholine ( $C_5H_{15}NO_4P$ ) is detectable by FTICRMS, LCMS, and NMR. If responses for all compounds are normalized relative to the phosphocholine response for that particular analytical technique, then a relative abundance is generated that is semiquantitative and cross comparable. In some cases, the various techniques may not necessarily need to be combined into a single coherent matrix but each technique may be applied in a targeted approach to investigate complementary fraction of the organic matter. For example, NMR and GCMS may be used to explore small substrates and energetic intermediates, while FTICRMS may be used to infer changes in larger molecular weight secondary metabolites. In short, natural organic matter is a highly complex mixture requiring a combination of techniques to fully characterize. Collectively, these analyses can all be used to identify molecular formula from which it is possible to calculate a bulk NOSC value. A complementary approach integrating all of these methods in a rigorous analysis of both the small and large metabolites will be critical for reconstructing the subsurface C cycling pathways, which ultimately lead to C flux. NOSC provides the framework for integrating these molecular techniques to calculate a thermodynamically relevant metric of organic matter quality, which encompasses the most comprehensive view of naturally occurring organic matter.

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