

Ionic Liquid Extraction Unveils Previously Occluded Humic-Bound Iron in Peat Soil Pore Water

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Globally, peatland ecosystems store tremendous amounts of C relative to their extent on the landscape, largely owing to saturated soils which limit decomposition. While there is still considerable uncertainty regarding CO₂ production potential below the water table in peatland ecosystems, extracellular Fe reduction has been suggested as a dominant pathway for anaerobic metabolism. However, colorimetric methods commonly used to quantitate Fe and partition between redox species are known to be unreliable in the presence of complex humic substances, which are common in peatland pore water. We evaluated both the standard *o*-phenanthroline (*o*-P) Method and an ionic liquid extraction (ILE) Method followed by quantitation with inductively coupled plasma optical emission spectrometry (ICP-OES) to compare total Fe recovery and Fe²⁺/Fe³⁺ ratios in four distinct peatland ecosystems, ranging from rich fen to bog. While total Fe concentrations measured with ILE and *o*-P were correlated, the ILE method proved to be superior in both total Fe quantitation and in separately quantifying ferric (Fe³⁺) and ferrous (Fe²⁺) iron. In peat pore water, the *o*-P Method underestimated Fe³⁺ by as much as 100%. A multivariate approach utilizing fluorescence- and ultraviolet (UV)-visible (Vis) spectroscopy identified indices of dissolved organic matter (DOM) humification and redox status that correlated with poor performance of the *o*-P Method in peat pore water. Where these interferences are present, we suggest that site-specific empirical correction factors for quantitation of total Fe by *o*-P can be created from ILE of Fe, but recommend ILE for accurate appraisals of iron speciation and redox processes.

Abbreviations: DI, deionized; DOC, dissolved organic carbon; DOM, dissolved organic matter; EEMs, excitation-emissions matrices; FI, fluorescence index; H_p, index of humification; ICP-OES, inductively coupled plasma optical emission spectrometry; ILE, ionic liquid extraction; *o*-P, *o*-phenanthroline; PARAFAC, parallel factor analysis; PCA, principal component analysis; RI, index of redox dissolved organic matter status; TEA, terminal electron acceptor; UV, ultraviolet; Vis, visible.

Core Ideas

- Dissolved organic matter character should be evaluated when selecting Fe quantitation methods.
- The *o*-phenanthroline Method cannot consistently speciate Fe in bogs and fens.
- Ionic liquid extraction yields superior Fe quantitation over *o*-phenanthroline.

Peatlands represent critical terrestrial C stores that are preserved by a combination of hypoxic and anoxic soil environments. These conditions present an energetic challenge for microbial consortia in anaerobic environments which utilize alternative (to oxygen) terminal electron acceptors (TEAs) during heterotrophic metabolism. In anaerobic conditions, microbes preferentially reduce several alternative TEAs for respiration, with thermodynamic yield declining in the order: NO₃⁻, Mn⁴⁺, Fe³⁺, SO₄²⁻, and ultimately CO₂ (see review by Megonigal et al., 2004). Although there is wide agreement that alternative TEA reduction is important for anaerobic decomposition, variation in the magnitudes of anaerobic CO₂ production across studies suggests that there is high variability in facultative and obligate anaerobic processes that occur below the water table (Morris et al., 2011; McLaughlin and Webster, 2014), particularly in the expected sequen-

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tial reduction of TEAs (Vile et al., 2003; Dettling et al., 2006; Keller and Bridgham, 2007; Knorr and Blodau, 2009; Deppe et al., 2010; Kane et al., 2013). A growing body of literature draws attention to a missing pool of electron acceptors responsible for relatively high C mineralization rates in saturated peatland soils (Keller and Bridgham, 2007; Estop-Aragones et al., 2012; Gupta et al., 2013). Adding to this complexity, humic substances are redox-active and the microbial reduction of humic substances can facilitate electron transfer to other redox-active functional groups and metals in anaerobic environments (Klüpfel et al. (2014), and references therein). Humic substances, organic matter, and organometallic complexes in particular have been nominated as important electron acceptors in northern peatlands (Lipson et al., 2010, 2012; Lau et al., 2015).

Underestimation of Fe, and specifically Fe^{3+} , could account for the “missing” pool of TEAs in peatlands because Fe^{3+} is difficult to measure accurately in peat pore water with common colorimetric analyses. Peat pore water is rich in DOM consisting largely of an array of humified products of plant decomposition and microbial activity (Thurman, 1985; D’Andrilli et al., 2010; Tfaily et al., 2013). Humic and fulvic acids form stable coordination complexes with Fe^{3+} (Maranger and Pullin, 2003; Antunes et al., 2007; Catrouillet et al., 2014; Fujii et al., 2014). These complexes are effective extracellular electron acceptors in anaerobic environments, including peat (Benz et al., 1998; Bauer et al., 2007; Lipson et al., 2010). Therefore, it is likely that complexed Fe and humic substances are important electron acceptors in peatlands, but unfortunately common spectrophotometric methods for estimating Fe^{3+} (U.S. EPA, 1980) are known to be unreliable in the presence of humic and fulvic acids. For example, Skogerboe and Wilson (1981) drew attention to uncertainty surrounding complete development time of the spectrophotometric o -P- Fe^{2+} complex when fulvic acids were present. More recently, Fujisawa et al. (2011) demonstrated that, in the presence of humic acids, o -P was only able to quantitate the unbound fraction of Fe^{2+} and that the reaction between DOM-bound Fe and o -P was governed by the dissociation rate of the DOM-Fe complex rather than the amount of o -P. The dissociation rate of DOM complexed Fe exposed to o -P was approximately six orders of magnitude slower than the experimentally derived dissociation rate for a simple organic ligand (lactate; Fujisawa et al., 2011). Incomplete reaction with o -P may lead to skewed estimates of Fe concentrations in systems rich in DOM (Fadrus and Maly, 1975; Pepper et al., 2010, and references therein). Because Fe^{3+} is hypothesized to be a significant TEA in the mineralization of C in these systems, method biases in measurement of total Fe, Fe^{2+} , or Fe^{3+} could manifest as an unbalanced energy budget in peatland metabolic studies. This is an important consideration because a large body of wetland research has depended on this method and other spectrophotometric assays to quantitate total Fe, Fe^{2+} , and Fe^{3+} (Pentrák et al., 2013).

Caveats about matrix interferences in spectrophotometric determination of total Fe and Fe^{2+} have been raised in the past (Skogerboe and Wilson, 1981; Anastácio et al., 2008), but the

problem is not universal because environmental samples can vary widely in their matrix components (Perdue, 1998). Additionally, there is no consensus as to whether the effectiveness of different Fe quantitative methods varies with matrix characteristics, particularly for samples containing highly humified, complex DOM. For example, Pentrák et al. (2013) recommended that o -P is applicable for Fe quantitation in minerotrophic wetland (fen) sediments and soil pore water, based on the quantitative recoveries of Fe compared with another Fe assay, the ferrozine method. Anastácio et al. (2008) compared the performance of o -P with that of the ferrozine method and also recommended o -P across a wide range of ecosystems, including systems with clay-sorbed amorphous Fe oxides. Lipson et al. (2010) reported total Fe recoveries in Arctic peat soil using o -P that were not significantly different from direct quantitation using ICP-OES Methods. Iron redox status has been estimated in natural systems using the ferrozine assay (Lovely and Philips, 1986; Pepper et al., 2010; Keller and Takagi, 2013), which demonstrated accurate results in DOM-rich matrices. Despite these reports of reliable results, both assays have been shown to experience interference in the presence of both model DOM compounds and environmental DOM, including humic acids (Guo et al., 2007; Yamamoto et al., 2010; Fujisawa et al., 2011). It is likely that changes in DOM character exert varying interference effects on spectrophotometric determination of Fe species in different peatland ecosystems, such as in bogs vs. fens. For example, changes in water residence time and connectivity with groundwater in different peatlands influence DOM character (Hribljan et al., 2014), which likely interacts with changes in alkalinity in governing Fe speciation (Boomer and Bedford, 2008). Therefore, to accurately appreciate the role Fe plays in anaerobic metabolism in bogs and fens it is important to critically evaluate spectrophotometric determination of Fe in the challenging matrix of peat pore water and to identify conditions where spectrophotometric approaches are not suitable.

Here we demonstrate an alternative means of iron extraction and subsequent direct quantitation in humic-rich matrices. The method employs an ILE with an acid organophosphorous compound, following methods described by Pepper et al. (2010). This method has long been used in industrial hydrometallurgy (e.g., Belkouché et al., 2005; Guezzen and Didi, 2012 and references therein), and is reported to quantitatively and selectively extract Fe^{3+} independent of dissolved organic C (DOC) concentration (Pepper et al., 2010). The extraction portion of the ILE method proceeds via a three-step mechanism. At low pH, H^+ ions donated to humic-Fe complexes cause the release of both Fe^{2+} and Fe^{3+} ions. Ferric iron is then chelated by three *bis*-2-ethylhexyl phosphoric acid molecules. Formation of the Fe^{3+} -tris(*bis*-2-ethylhexyl phosphate) complex is kinetically preferable to reformation of organo-ferrate complexes in a wide range of model organic matrices (Pepper et al., 2010), and the affinity of *bis*-2-ethylhexyl phosphate for Fe^{3+} is strongly favored over complexation with Fe^{2+} . Phase separation using *n*-heptane ensures optimal partitioning of the Fe^{3+} -tris(*bis*-2-ethylhexyl

phosphate) complex into the organic phase, leaving Fe²⁺ behind in the aqueous phase. The extracted Fe³⁺ is then back-extracted into aqueous media with strong acid for analysis and both aqueous Fe³⁺ and Fe²⁺ are independently quantitated using ICP-OES. Total Fe is separately measured on an un-extracted sample. Given the evidence for reliable quantitative performance in other highly chelating matrices, ILE may present a way of tracking Fe redox species (Fe²⁺ and Fe³⁺) in the challenging peat pore water matrix. Here, we evaluated the quantitation of Fe²⁺ and total Fe in peat pore water from a variety of peatland environments, from rich fen to bog. Performance of the *o*-P method was compared with the ILE Method in complex environmental matrices and in deionized water. In this study, we address three questions.

1. Is the *o*-P Method suitable for Fe quantitation and oxidation state speciation in northern peatlands?
2. Can the ILE Method provide improved quantitation and Fe speciation in these systems?
3. Can UV-Vis and fluorescence spectroscopy be used to identify DOM characteristics that predict poor performance of the *o*-P Method in peat pore water?

MATERIALS AND METHODS

Study Sites and Sampling Protocol

Study sites consisted of four locations encompassing a wide gradient of pore water chemistry and DOM content and character (Table 1), including a rich sedge fen (mean pH of 6.2) from interior Alaska, USA (Kane et al., 2010), a rich cedar fen peatland (mean pH of 6.5) and a water table manipulation experiment in a bog mesocosm (mean pH of 4.5) in northern Michigan, USA (Potvin et al., 2015). Sampling at the bog mesocosm site was divided equally into plots with unaltered and lowered water table positions.

We collected pore water samples from a depth of 20 cm below the peat surface through established piezometers into airtight, headspace-free containers. All piezometers were purged before collection, and were outfitted with 120- μ m Nitex

mesh (Dynamic Aqua Supply LTD.) to avoid sampling coarse particulate matter. In the rich sedge fen peatland, pore water samples were drawn up from the peat with a syringe, purged, injected into pre-evacuated vials, and then shipped overnight on ice to the USDA Forest Service Northern Research Station in Houghton, MI for analysis the following day. Samples from all other sites were harvested in the same manner, but were analyzed within 2 h of collection.

To evaluate discrete interference effects of increased humification and reduction of DOM over time, pore water samples from the bog site (two from the high water table treatments, two from the low) were collected as above and transferred under N₂ into serum vials, which were sealed and incubated in the dark at ambient temperature (total number = 16 bottles for high and low water table treatments). Subsets of these samples ($n = 4$) were removed for analysis on Days 0, 7, 21, and 48 of the experiment for Fe quantitation and organic matter characterization following the same methods as described for field samples.

In the lab, sealed samples were placed in a positive-pressure glovebox (Model 60245DG, TDI International), and the atmosphere was exchanged five times or more with zero-grade N₂. As the samples equilibrated to laboratory temperature, extraction reagents were degassed and prepared under N₂. After reaching ambient temperature, all samples were uncapped and filtered through 0.45- μ m sterile nylon membrane filters (AQ Brand Microsolv) and divided into two aliquots each, which were transferred to glass vials fitted with Teflon-faced septa while in the glovebox. Samples were then analyzed using the two different Fe quantitation methods.

Iron Quantitation Quality Control

To compare the performances of *o*-P and ILE in the absence of DOM and to quantify any inadvertent oxidation during sample handling, duplicate quality control standards consisting of distilled-deionized water (DI H₂O ≥ 18.2 M Ω cm⁻²) at

Table 1. Physico-chemical parameters for samples used in method comparison. Bog samples represent a mean value ($n = 5$ samples each).

Sample	SUVA ₂₅₄ [†] L mg-C ⁻¹ cm ⁻¹	E2 [‡]	E4 [§]	DOC [¶] mg L ⁻¹	TDN [#] mg L ⁻¹	DOC/TDN	H _p ^{††}	RI ^{‡‡}	FI ^{§§}	PARAFAC ^{¶¶}					%Ca:%Cc ##
										%C1	%C2	%C3	%C4	%C5	
Rich Sedge Fen	4.07	4.45	14.88	58.28	2.5	23.31	15.74	0.77	1.16	0.36	0.17	0.25	0.19	0.027	1.44
Rich Cedar Fen	2.89	4.61	3.41	32.55	1.7	18.82	13.49	0.68	1.17	0.39	0.19	0.24	0.14	0.037	0.53
Bog High WT	3.93	4.21	8.10	81.98	3.1	30.41	13.07	0.63	1.15	0.43	0.19	0.23	0.10	0.05	0.89
Bog Low WT	3.75	4.50	19.82	146.75	4.1	35.78	16.89	0.67	1.11	0.44	0.14	0.27	0.13	0.03	0.62

[†] SUVA₂₅₄ Specific Ultraviolet absorbance at 254 nm. Described in Weishaar et al. (2003).

[‡] E2 Unitless. A₁₂₅₄:A₁₃₆₅ Index of aromaticity (UV-Vis). Described in Worrall et al. (2002).

[§] E4 Unitless. A₁₄₆₅:A₁₆₆₅ Index of oxygen content (UV-Vis). Described in Worrall et al. (2002), Osborne et al. (2007).

[¶] Dissolved organic carbon.

[#] Total dissolved nitrogen.

^{††} H_p Unitless. Index of humification (fluorescence). Described in He et al. (2013).

^{‡‡} RI Unitless. Redox index (fluorescence). Described in Miller et al. (2006).

^{§§} FI Unitless. Fluorescence index. Described in Johnson et al. (2011).

^{¶¶} %C1–5 Parallel Factor Analysis scores. Fraction of total modeled fluorescence; this work.

^{##} %C_a/%C_c Ratio of %C2/%C3. Described in Kothawala et al. (2012).

either 50:50, 80:20, 85:15, or 90:10 Fe[II]:Fe[III] at 1, 1.5, 2, and 3 mg L⁻¹ total Fe were prepared. Duplicate 5-mL aliquots of each quality control standard were transferred to glass vials fitted with Teflon-faced septa for analysis using the two different Fe quantitation methods.

Ionic Liquid Extraction

The ILE procedure was identical to that described by Pepper et al. (2010), except that the volumes of sample and reagents were increased to accommodate our instrument requirements and the time allotted for forward extraction was extended to 2 h to ensure maximal mixing of this larger volume. Thus, 5 mL of sample was transferred to an extraction vial, to which 15 mL of 0.67 M HCl (ACS grade; Fisher Scientific) was added, followed by the addition of 20 mL of 0.1 M *bis*-2-(ethylhexyl) phosphoric acid (97% Sigma Aldrich) in *n*-heptane (HPLC grade, Acros Organics). A reagent blank consisting of only DI water was also included to correct for trace Fe found in the HCl solution. Laboratory trials in DI water and environmental matrices both confirmed that extraction vessel geometry and resulting liquid turbulence influenced the rate at which this mass transfer-limited extraction proceeds, and that effective mixing is essential for results to be reliable. In our case, optimal forward extraction of Fe³⁺ into the organic phase was accomplished by laying extraction vials on their sides to maximize the organic-aqueous interface, and shaking at 500 rpm on a shaker table for 2 h. Following forward extraction of Fe³⁺, resulting emulsions were allowed to clear, and a 10-mL aliquot of the aqueous phase containing the Fe²⁺ fraction was transferred into a high-density polyethylene centrifuge tube (BD Falcon) for storage and analysis. A 10-mL aliquot of the organic phase was transferred to a vial containing 10 mL of 4.0 M HCl, and was shaken for 20 min at 500 rpm to back-extract Fe³⁺ into the aqueous phase. The resulting aqueous phase in the back-extraction vial was similarly transferred to a high-density polyethylene centrifuge tube for storage and analysis. Quantitation of extracted Fe²⁺ and Fe³⁺ and un-extracted total Fe were performed on an ICP-OES (PerkinElmer Optima 7000DV) in the Michigan Technological University Stable Isotope Laboratory. The values reported are the average of five injections per sample, and instrument error was calculated to be ±10% of the mean.

Colorimetric Iron Quantitation

For comparison, total Fe and Fe²⁺ were also measured using the *o*-P Method (Hach Co., Loveland CO, USA; Methods 8146 and 8008, respectively) under ambient atmosphere at laboratory temperature. Spectrophotometric methods were scaled for use with a 96-well plate reader (see Sinsabaugh et al., 2000). Samples and corresponding matrix spikes were diluted 1:10 with DI water and were reacted with 0.16% *o*-P for Fe²⁺ (Hach Method 8008; Hach Co., Loveland CO, USA), and 0.16% *o*-phenanthroline *p*-toluenesulfonic acid salt supported by a reducing agent consisting of sodium thiosulfate, sodium hydrosulfate, sodium citrate, and sodium metabisulfite for total Fe (Hach Method

8146), corresponding with the beginning of ILE. The method detection limits of these colorimetric assays were 0.01 mg L⁻¹ in the 1:10-diluted samples. Reaction progress was monitored by reading the absorbance ($\lambda = 510$ nm) of standards and samples at 15 min, 45 min, 60 min, 3 h, and 24 h using a microplate reader (Spectra-Max M2; Molecular Devices Corp., Sunnyvale, CA, USA). Laboratory trials indicated that the 3-min reaction time recommended by the manufacturer was insufficient for samples from peatland matrices to reach completion, though 15 min was sufficient to allow absorbances of matrix-free standards to stabilize. Dissolved organic matter-rich samples required 3 h for stable color formation, and all results from *o*-P Methods reported are from the 3-h measurement. During the time between readings, plates were kept wrapped in foil to prevent photochemical reactions (Pentrák et al., 2013). We analyzed reagent-free samples simultaneously with reacted samples to correct for any background absorbance. To also compare ILE to ferrozine, a colorimetric method similar to *o*-P, Fe²⁺ and total Fe quantitation were performed on a subset of bog samples ($n = 4$) following the methods outlined in Viollier et al. (2000). Standards used to build the calibration curves for the colorimetric methods were submitted to the Michigan Tech Stable Isotope Laboratory for concentration verification by ICP-OES, and the spectrophotometric calibration curves were built from these actual concentrations.

Pore Water Matrix Characterization

All matrix characterizations occurred in concert with Fe quantitation. Filtered aliquots of each sample were analyzed for organic acid and anion content on a Dionex ICS 2000 ion chromatograph (Dionex Corp.). Ultraviolet-visible absorbance and fluorescence spectra (EEMs, Excitation-Emission Matrices) were both measured simultaneously with a Horiba-Jobin Yvon Aqualog C (Horiba Corp.). Run parameters were Excitation: 240 to 600 nm by 3-nm increments; Emission: 212 to 608 nm by 3 nm bandpass; integration time = 0.25 s. Samples with absorbance greater than 0.6 at λ_{254} ($A_{254} > 0.6$) were diluted with DI H₂O, such that they were $0.20 < A_{254}(\text{sample}) < 0.6$ to satisfy the assumption of detector linearity required by modeling (Stedmon and Bro, 2008; Lawaetz and Stedmon, 2009). Absorbance, excitation, and emission corrections were performed simultaneously with measurement against an integrated reference detector. Resulting UV-Vis data were later corrected with a scalar absorbance correction factor (the inverse of diluted concentration) for data analysis, as laboratory trials indicated that A_{254} and other indices of interest vary linearly in these sample matrices. Preliminary exploration found that the measured fluorescence intensities of DOM in these systems also vary linearly with dilution within this range, thus the same scalar correction factor was applied to EEMS fluorescence intensities. Further preprocessing steps followed the scheme presented in Lawaetz and Stedmon (2009) and Singh et al. (2013). The resulting EEM fluorescence intensities were normalized to the integral of the water Raman peak (Ex 350/Em 371–428) of the

sample. We normalized the resulting fluorescence intensities to an external, ultrapure Raman peak water standard (Starna Cells, Atascadero, CA, USA), integrated similarly to the sample Raman peak and collected daily. Excitation-emissions matrices were then analyzed for fluorescence index (FI; Johnson et al., 2011), an index of humification (H_p ; He et al., 2013), for an index of DOM redox status (RI) derived from Miller et al. (2006), and for a second index of DOM redox status (C_a/C_c ; Kothawala et al., 2012) using the area in a 10 nm × 10 nm window under the component peak maxima (as a % total EEM fluorescence).

Statistical Analysis

Linear regressions of ILE and *o*-P total Fe recovery versus total Fe determined directly by ICP-OES were performed using SAS (SAS Institute Inc.) to evaluate the performance of either method in both DI water ($n = 5$) and environmental matrices ($n = 20$). All regression analyses were considered significant at $\alpha = 0.05$ and departure from $y = x$ (recovery by ICP-OES as independent variable) was used as an indicator of method efficacy.

Multivariate approaches were used to characterize variation in sample matrices. Parallel Factor Analysis (PARAFAC) was used to decompose 121 fluorescence EEMs from a variety of peatlands into discrete fluorescence components following the methods of Stedmon and Bro (2008) and Kothawala et al. (2014). Principal Component Analysis (PCA) was used as a means of arranging sample chemical matrices for qualitative exploration (Kothawala et al., 2012; Bro and Smilde, 2014) incorporating a suite of spectrophotometric variables reflecting pore water character (cf. Table 1) and including % total Fe recovered by *o*-P. Partial Least Squares analysis (PLS) was performed to identify correlative factors (antagonistic matrix interferences) that best explain *o*-P method performance using the same variables as for the PCA model, but constraining the y -block to be percentage of total Fe recovered by *o*-P or the fraction of Fe^{2+} relative to total Fe (by *o*-P). Partial least square models were selected that optimize \times block cumulative variance captured and R^2 while minimizing Q residuals and maximizing Hotelling's T^2 . Parallel factor analysis, PCA, and PLS were performed using PLS_Toolbox (Eigenvector Research, Inc., Wenatchee, WA, USA) in Matlab R2014a.

RESULTS

The *o*-P method routinely underquantified total Fe in peatland pore water (Fig. 1; paired t test $p < 0.001$) but remained quantitative in DI water (data not shown—not significantly different from ICP-OES; paired t test $p = 0.20$). In contrast, ILE results were not significantly different from ICP-OES in either DI water (data not shown; paired t test $p = 0.45$) or in environmental matrices (Fig. 1; paired t test $p = 0.23$). In the worst cases *o*-P reported more Fe^{2+} than total Fe, most notably in samples from the bog ecosystem (Fig. 2a-2b; average across the dataset for Fe^{2+} : Total Fe =

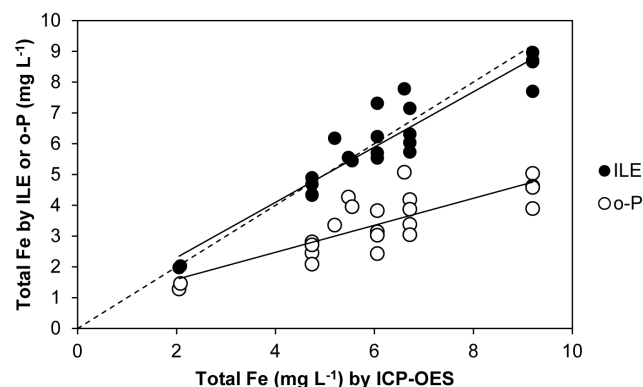


Fig. 1. Ionic liquid extraction (ILE) yielded quantitative results (vs. inductively coupled plasma optical emission spectrometry [ICP-OES] derived total Fe) in DOM-rich matrices ($y = 0.91 \pm 0.07x + 0.36 \pm 0.45$, $R^2 = 0.90$, $F = 169.76$, $p < 0.001$) whereas *o*-phenanthroline experienced reduced accuracy ($y = 0.45 \pm 0.07x + 0.55 \pm 0.43$, $R^2 = 0.72$, $F = 46.93$, $p < 0.001$; standard errors of coefficients are presented). Dashed line is $y = x$.

1.098). Because the *o*-P method estimates Fe^{3+} from total Fe by mass balance, it reported approximately 87% less Fe^{3+} than ILE in samples across all study sites (range = 53–100% less; Fig. 2). Quality control standards did not indicate any inadvertent oxidation during the ILE extraction procedure (average difference = 0.6%, paired t test $p = 0.50$), indicating that anoxic conditions persisted throughout each extraction. Ferrozine recovered ~30% less total Fe than ILE in the samples assayed (68.9–69.7% recovery), which is lower than that recovered by *o*-P in the same subset of samples (81.5–88.5% recovery).

The pore water samples used in this study encompassed a range in chemical composition. Dissolved organic C concentrations ranged from 32.6 to 172.3 mg L⁻¹ and there was

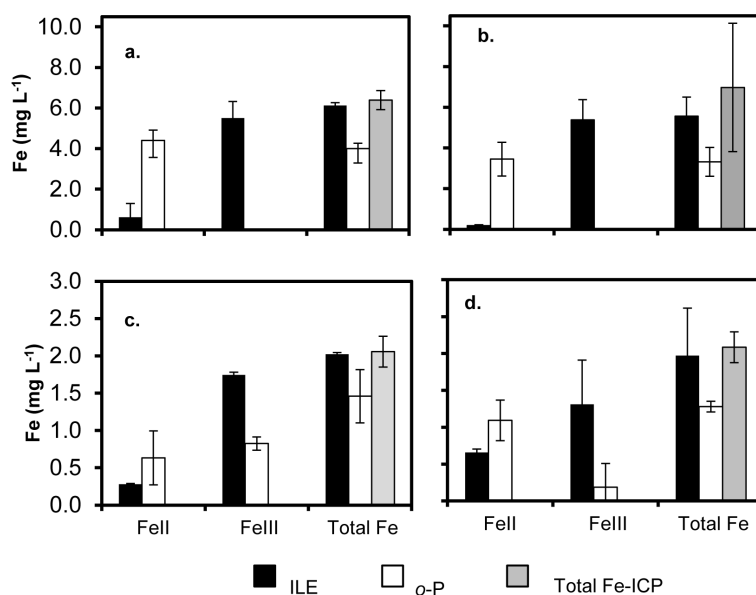


Fig. 2. *o*-phenanthroline (*o*-P) underestimated total Fe, leading to estimates of Fe^{3+} 53 to 100% less than ionic liquid extraction (ILE) in four distinct peatlands (a.) Experimental *Sphagnum* bog with high water table; (b.) Experimental *Sphagnum* bog with low water table; (c.) Rich cedar fen; (d.) Rich *Sphagnum*/*Carex* peatland. Error bars depict 1 standard deviation of replicate measurements for ILE ($n = 10$), *o*-P ($n = 6$), and Total Fe-ICP ($n = 10$).

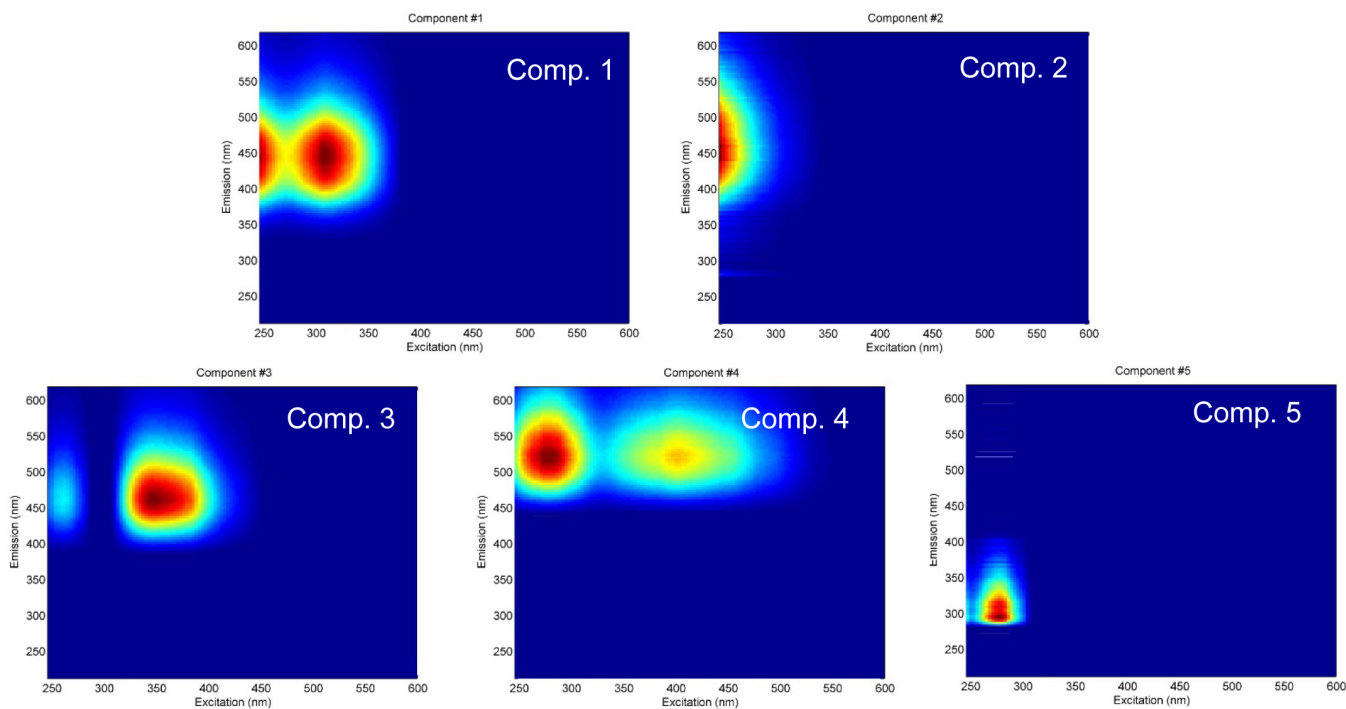


Fig. 3. Parallel factor analysis (PARAFAC)-resolved fluorescence components.

a large range in the relative aromaticity of DOC, as indicated by specific ultraviolet absorption at 254 nm ($SUVA_{254}$; 2.89–4.72 L mg-C⁻¹ cm⁻¹) and other spectrophotometric indexes (Table 1). Concentrations of the organic acids formate, acetate, propionate, and oxalate (known ILE matrix interferences) were below detection limits (0.02 mg L⁻¹); these data were thus not included in further analyses. A PARAFAC model described > 99% of fluorescence in all EEMs and was able to identify five component classes (Fig. 3, Table 2). Careful examination of residual fluorescence in EEM landscapes did not reveal any other component classes. It is worth noting also that manual integration of C_a/C_c was closely correlated with modeled Ca/C_c by PARAFAC ($R^2 = 0.95$, $p < 0.001$), which is in agreement with similar validation by Kothawala et al. (2012).

Principal component analysis (Fig. 4, Table 3) found that samples sorted along a redox and C source gradient. Positive loadings along PC1 corresponded to an increasing redox index (more reduced samples), and an increasing proportion of red-shifted fluorophores (%C2 and %C3). Positive loadings along PC2 corresponded to variation in indices of humification

Table 2. Excitation-Emission maxima and putative identities of Parallel factor analysis (PARAFAC)-derived fluorescence components.

Component	Excitation/emission	Putative classification
1	240, 325/440	Fulvic-like†
2	250/450	Terrestrial humic or fulvic (photo-refractory)‡
3	255, 375/450	Less-condensed humic or fulvic†
4	240, 325/525	Condensed aromatic; high MW humic †‡
5	275/328	Low MW, phenolic, microbial †§

† Kothawala et al. (2014).

‡ Olefeldt et al. (2013).

§ Cory and McKnight (2005).

(increasing H_p and $E4$; decreasing FI) and differences in the DOC content in the samples. The time course of the incubation experiment sorts along PC1, indicating that DOM in the samples became both more reduced and humified throughout the course of the experiment (Fig. 4).

An orthogonalized PLS model with the y -block loadings constrained to o -P Method total Fe recovery (versus ICP), (Q residual = 5.22%; Hotelling's $T^2 = 94.78\%$; Fig. 5a), and another with the y -block loadings constrained to o -P Fe^{2+} : o -P Total Fe (Q residual = 4.24%; Hotelling's $T^2 = 95.76\%$; Fig. 5b) were constructed, each consisting of eight latent variables. In these models, X-block loadings (sample chemical parameters from Table 1) with significant predictive power are projected further away from the origin along the first latent variable, as opposed to chemical variables with less predictive power which project along the orthogonal latent variable (Fig. 5). The loadings which are located most opposite to the vector describing method performance and which project the furthest Euclidean distance from the origin comprise the most probable matrix interferences for the o -P Method in this dataset (Table 4). Increasing H_p and increasing humic-like fluorescence (%C3) interfere the most with total Fe recovery (Fig. 5), while the spectral indices $E2$ and $SUVA_{254}$ provided little prediction of total Fe recovery in these samples. The loadings that corresponded to the aberrant $Fe^{2+}/Total\ Fe$ (ratio > 1.0) were %C5, C_a/C_c , and %C2 (Fig. 5a). Significant linear regressions of o -phenanthroline $Fe^{2+}/Total\ Fe$ vs. C_a/C_c (Fig. 6a; $R^2 = 0.34$; $p < 0.01$), %C5 ($R^2 = 0.26$; $p = 0.02$) and %C2 ($R^2 = 0.34$; $p < 0.01$) across all samples confirm that these factors represent chemical characteristics of DOM that are predictive of

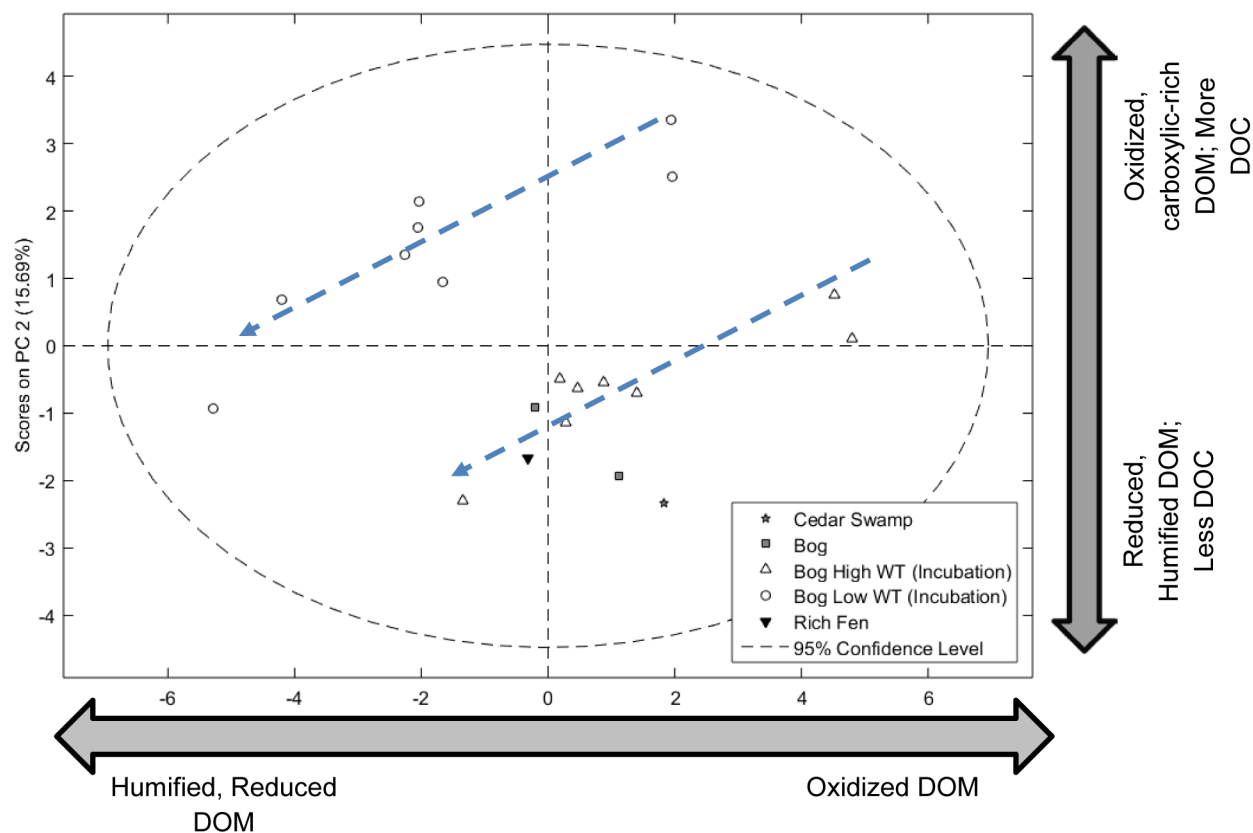


Fig. 4. Loadings along the first two principal components corresponding to principal component analysis (PCA) of sample pore water chemistry. Labeled axis arrows indicate general trends derived from loadings. Dashed arrows within the plot indicate the time course of the incubation experiment.

aberrant Fe^{2+} recovery using *o*-P. A linear regression of H_p versus *o*-P % total Fe recovery (Fig. 6b; $R^2 = 0.28$, $F = 6.81$, $p = 0.018$) confirms that this particular index of humification is predictive of *o*-P performance with regard to total Fe recovery.

Samples from the rich sedge fen and rich cedar fen did not represent the bulk of the dataset, but appeared to share chemical similarity with samples harvested from high water table, reducing environments (Fig. 4). Total Fe recovery using *o*-P was still below ICP–OES totals and the relationship between *o*-P performance and H_p was still significant with the rich fen and cedar swamp data excluded ($R^2 = 0.30$, $F = 6.88$, $p = 0.02$). The regression between %C3 and *o*-P total Fe recovery was not significant for all samples ($R^2 = 0.14$, $p = 0.10$), but was significant for the samples harvested from the bog site incubation ($R^2 = 0.37$, $p = 0.01$).

DISCUSSION

Dissolved Organic Matter Interference on Spectrophotometric Iron Methods

To our knowledge, this study is the first appraisal of ILE for Fe quantitation in peatlands. We found ILE to be a more accurate method than commonly used spectrophotometric methods (*o*-P and ferrozine) for Fe quantitation and speciation in pore water from very different bog and fen peatlands. We suggest ILE is better suited for Fe quantitation in highly humified, DOM-rich sample matrices than spectrophotometric approaches for three reasons: lower reaction pH, independence from reducing agents, and better discrimination between Fe^{3+} and Fe^{2+} .

Iron is more easily released from DOM at low pH and therefore complexing agents that bind at low pH are most desirable. The *o*-P assay operates at a lower pH that is also more representative of peatland ecosystems and is thus better suited

Table 3. Loadings along the first two principal components corresponding to principal component analysis (PCA) of sample porewater chemistry.†

	PC 1 (37.90%)	PC 2 (15.69%)
% Total Fe (<i>o</i> -P vs. ICP)	0.20	-0.13
SUVA ₂₅₄	-0.01	-0.09
E ₂	0.06	0.31
E ₄	-0.08	0.51
DOC	-0.20	0.41
TDN	-0.20	0.33
DOC/TDN	-0.02	0.20
Ca:Cc	0.36	0.14
H _p	-0.36	0.11
RI	-0.22	-0.11
FI	0.21	-0.28
%C ₁ ‡	-0.10	0.05
%C ₂ ‡	0.34	0.22
%C ₃ ‡	-0.35	-0.19
%C ₄ ‡	-0.26	-0.15
%C ₅ ‡	0.28	-0.12
C ₂ /C ₃ (Ca/Cc)	0.34	0.24

† *o*-P, *o*-Phenanthroline; ICP, inductively coupled plasma; SUVA, spectral ultraviolet absorbance at 254 nm; DOC, dissolved organic C; TDN, total dissolved N; H_p , index of humification; RI, Redox index; FI, fluorescence index.

‡ Parallel factor analysis data.

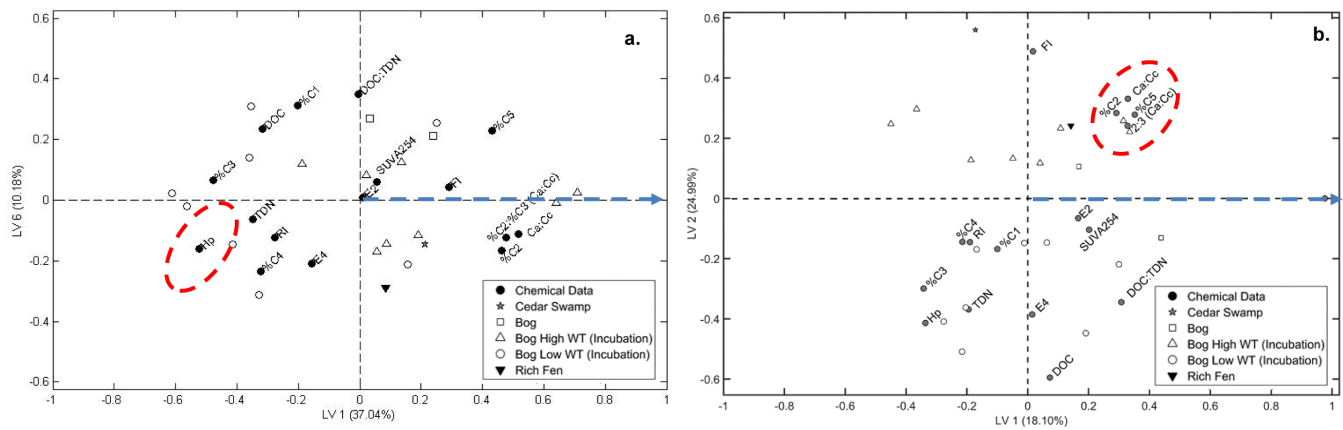


Fig. 5. (a) An orthogonalized partial least squares (PLS) model found that an index of dissolved organic matter (DOM) humification (H_p), depicted in the ellipse, correlated with decreased *o*-phenanthroline (*o*-P) total Fe recovery (as indicated by inductively coupled plasma spectrometry [ICP]). %C3 was not found to be indicative of matrix interference across the full dataset. The dashed arrow along the primary axis depicts a vector composed of a combination of matrix characteristics positively correlated with % Total Fe recovery in *o*-P; matrix interferences in our dataset project opposite this vector. (b) An orthogonalized PLS model found that an index of humic/fulvic redox (C_a/C_c) driven mostly by %C2, and one fluorescence component (C5) depicted in the ellipse, corresponded to recovery of $Fe^{2+} > \text{Total Fe}$ (using *o*-P) for some samples. The dashed arrow depicts a vector along which a combination of chemical variables positively correlated with $Fe^{2+} > \text{Total Fe}$.

to strip DOM-complexed Fe than the ferrozine assay. The *o*-P complexation reaction proceeds optimally at pH 3 to 5 while the ferrozine assay occurs at pH 9.5 (Komadel and Stucki, 1988; Amonette and Templeton, 1998; Viollier et al., 2000). Thus, reduced ferrozine performance in the presence of peat humic acids may be explained by the effect of reaction pH on humic/fulvic Fe binding, and the resulting complexation equilibrium of ferrozine with Fe in the sample matrix. In model humic substances, high pH ($pH > 6$) leads to tighter binding in metal-humic complexes as the predominant complexation sites shift away from carboxyl groups toward phenolics (Catrouillet et al., 2014). This shift in

type of binding site is accompanied by a greater capacity for Fe complexation overall (Fujii et al., 2014). Because the ferrozine assay is performed at pH 9.5, the dominant (ca. 60%, Catrouillet et al., 2014) binding sites in the sample matrix will shift toward phenolic groups. Because the Fe at these sites is more tightly bound, it is perhaps not quantifiable using ferrozine (Guo et al., 2007; Hu 2011). The unpredictable endpoint of the *o*-P-Fe reaction in systems rich in humic acids (Skogerboe and Wilson, 1981; Fujisawa et al., 2011) and the apparent inability of ferrozine to compete with plant polyphenols at circumneutral pH (Guo et al., 2007) suggest that humic substances in DOM

Table 4. Loadings of chemistry data along the two latent variables most explanatory of *o*-phenanthroline method performance. Euclidean distance is calculated from the origin.†

Variable	%Total Fe recovery			$Fe^{2+}/\text{Total Fe}$		
	LV 1 (37.04%)	LV 6 (10.18%)	Euclidean distance	LV 1 (18.10%)	LV 3 (24.99%)	Euclidean distance
SUVA ₂₅₄	0.041	0.081	0.091	0.21	-0.09	0.05
E2	0.007	0.011	0.013	0.17	-0.06	0.03
E4	-0.112	-0.286	0.307	0.01	-0.34	0.11
DOC	-0.226	0.319	0.391	0.07	-0.52	0.27
TDN	-0.249	-0.087	0.264	-0.20	-0.32	0.14
DOC/TDN	-0.003	0.478	0.478	0.32	-0.30	0.19
C_a/C_c	0.370	-0.151	0.400	0.34	0.29	0.20
H_p	-0.374	-0.218	0.433	-0.34	-0.36	0.25
RI	-0.197	-0.168	0.259	-0.19	-0.13	0.05
FI	0.208	0.059	0.216	0.02	0.43	0.18
%C1‡	-0.144	0.428	0.451	-0.10	-0.15	0.03
%C2‡	0.331	-0.228	0.402	0.30	0.25	0.15
%C3‡	-0.342	0.091	0.354	-0.35	-0.26	0.19
%C4‡	-0.232	-0.321	0.396	-0.22	-0.13	0.06
%C5‡	0.309	0.313	0.439	0.36	0.24	0.19
%C2/%C3	0.342	-0.168	0.381	0.34	0.21	0.16

† *o*-P, *o*-Phenanthroline; ICP, inductively coupled plasma; SUVA, spectral ultraviolet absorbance at 254 nm; DOC, dissolved organic C; TDN, total dissolved N; H_p , index of humification; RI, Redox index; FI, fluorescence index.

‡ Parallel factor analysis data.

pose challenges for either optical method. Based only on the extraction pH for each method ILE should be the most effective because it leverages an acid extraction at very low pH that fully displaces chelated Fe ions.

Both *o*-P and ferrozine depend on the formation of a chromophoric complex with Fe^{2+} . Thus, to measure total Fe these methods presume that first, all Fe^{3+} in a sample is reduced to Fe^{2+} and, second, that the entire pool of Fe^{2+} is subsequently available to the chelating agent. This complexation mechanism is inhibited in anoxic humic- and fulvic-rich systems wherein the DOM matrix itself is believed to be redox active and the predominant metal-DOM complexes are thought to exist as bulky bidentate or tridentate conformations of large molecules (Catrouillet et al., 2014). In our dataset, the samples with the most humified DOM appeared to pose the greatest challenge to *o*-P. It is likely that the efficacy of the reducing agent used in the *o*-P Method declined as the degree of complexity of humified DOM increased across samples. For example, complex aromatic polymers like tannins generally have a more condensed structure with low

H/C (Hedges, 1990), which have also been shown to correlate strongly with the electron accepting capacity of humic substances (Aeschbacher et al., 2010).

Spectrophotometric methods are inherently less selective than ILE extraction in that multiple metal species may produce similarly colored chromophores with overlapping absorption bands. The ILE Method effectively filters any signal from co-extracted ions by the ICP analysis, offering superior precision and accuracy over spectrophotometric methods. Copper (II) forms a stable chromophore with *o*-P that absorbs at $\lambda = 510$ nm and systems with appreciable copper content have long been known to interfere with *o*-P (Fortune and Mellon, 1938). This mechanism could likely explain the occurrences where *o*-P reported $\text{Fe}^{2+} >$ Total Fe (Fig. 1, Fig. 6a). To explore possible Cu^{2+} interference, we scaled up the ILE to process 500 mL of pore water taken from the bog site, with repeated harvests of the aqueous phase (under atmosphere) over the course of 288 h. The aim here was to ensure that all Fe was oxidized to Fe^{3+} and extracted into the organic phase, leaving other metal species behind. These samples were found to contain 0.2 mg L^{-1} Cu (determined using ICP-OES). After stripping out 97% of the Fe (determined by ICP), *o*-P still displayed color development that would indicate as much as 62% of the original Fe was still present. While this exploration is certainly not exhaustive, it suggests that peat Cu concentrations should be considered when evaluating *o*-P method performance for determining iron concentrations in peat.

Dissolved Organic Matter Character Effects on Detection of Iron Speciation

Multivariate modeling of matrix effects on *o*-P in this work suggests that successful quantitation and redox speciation of Fe in peatlands depends less on DOM concentration than it does on DOM character. The mechanism explaining *o*-P's overestimation of Fe^{2+} is not completely evident from the data collected in this

study, although DOM composition, humic redox status and Cu (which was only analyzed on a small subset of samples and not included in modeling exercises) appear to all play a role. Humic and fulvic substances have been shown to chelate Fe^{3+} from fluorescence spectroscopy quenching assays (Antunes et al., 2007), and peat-derived humics and fulvics are among the most tenacious humic substances in regard to Fe binding (Fujii et al., 2014). It is not surprising, then, that humic and fulvic-like fluorophores present in our sample matrices (H_p , %C2, and %C3) corresponded to reduced total Fe recovery with *o*-P. The H_p index presented the loading with the largest Euclidean distance away from the origin, in the direction most antagonistic to *o*-P performance within our dataset (Fig. 5a), suggesting that H_p may be a good way to determine if *o*-P is an appropriate method for Fe quantitation. Our data suggest that in the absence of %C2 and %C5 interferences, such as in open water systems, it is likely that results from *o*-P are accurate. However, in humic-rich waters from a variety of peatland ecosystems the C2 and C3 components can be substantial (e.g., ~20–60% of fluorescence across the different peatlands in this study; Table 1), suggesting that DOM matrix interference effects are likely to be high in peat pore water.

Ultraviolet-visible indices like SUVA_{254} , E2, and E4 are frequently utilized to deduce DOM character, particularly aromaticity (Worrall et al., 2002; Weishaar et al., 2003). In our dataset, E4 was a better indicator of differences in DOM between samples than either E2 or SUVA_{254} but none provided as strong predictive capability for *o*-P total Fe recovery as %C2, %C3 or H_p . In a study encompassing 15 standard humic- and fulvic acid mixtures, Fujii et al. (2014) reported that aromaticity was a strong predictive factor of iron binding capacity and coordination complex stability. We have found that the UV-Vis index of oxygen content (E4) was a stronger predictor of *o*-P method performance than aromaticity (proxied by SUVA_{254}),

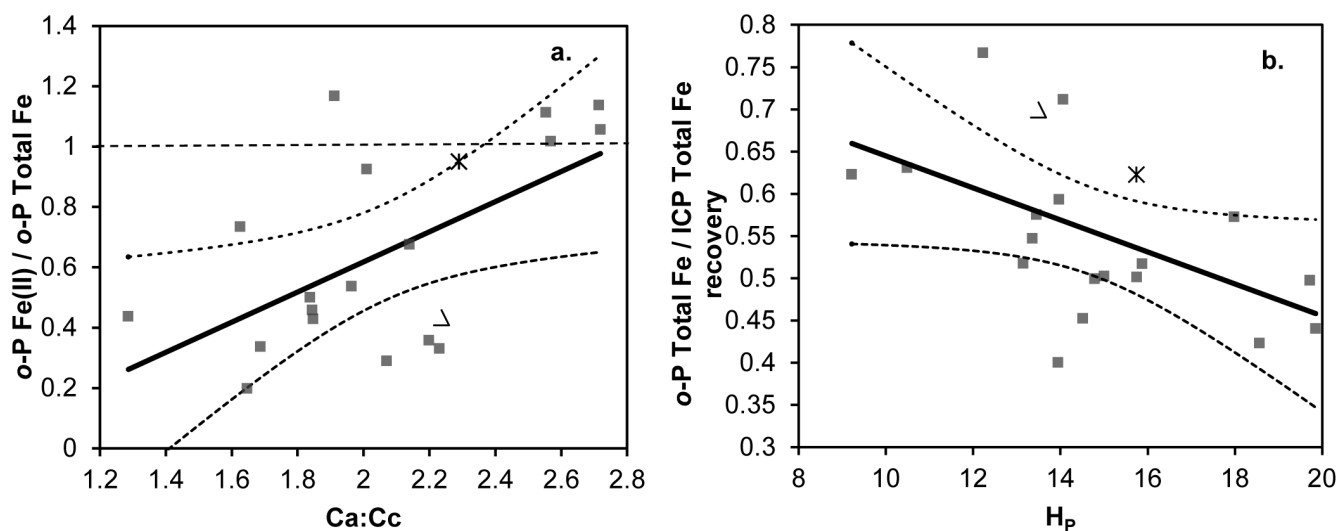


Fig. 6. (a.) Increasing Ca/Cc, an index of humic redox status, explained aberrant Fe^{2+} : Total Fe recovered by *o*-phenanthroline (*o*-P; $R^2 = 0.22$, $F = 5.11$, $p = 0.04$). (b.) The recovery of total Fe by the *o*-P method declined as the humification of dissolved organic matter, as indicated by humification index (H_p), increased ($R^2 = 0.28$, $F = 6.81$, $p = 0.018$). The Alaskan sedge (asterisk) and Michigan cedar (open triangle) rich fens were identified as outliers but are still included in this regression.

which agrees in principle with the concept that humic and fulvic carboxylic groups participate in Fe binding (Catrouillet et al., 2014). To fully understand DOM-Fe interactions in peat porewater, investigations coupling ILE and spectrophotometric Fe assays to NMR and high-field mass spectrometry in addition to fluorescence and UV-Vis spectroscopy (Tfaily et al., 2013) could provide greater mechanistic insight into site-dependent matrix effects.

Recommendations

While the *o*-P Method did not quantitatively total Fe across the different peat pore waters investigated in this study, total concentrations were linearly correlated with ILE Methods (Fig. 1). As such, we suggest that in this class of samples optical measurements of total Fe can be corrected for matrix interference effects using empirical relationships between *o*-P and ILE:

$$y_{corr} = 1.60 (\pm 0.23)x + 0.83 (\pm 0.82) \quad [1]$$

where y_{corr} is the corrected total Fe concentration and x is the total Fe concentration determined using *o*-P. Standard error for regression terms are given in parentheses ($R^2 = 0.72$; $F = 46.93$; $p < 0.001$). We note however that use of a general correction factor in this way has to consider DOM properties to assess the potential for matrix interference effects (e.g., Fig. 5), which likely vary widely in different soils and sediments.

Method selection is obviously not limited to concerns regarding quantitative yield, but also with regards to analytical time and cost. *o*-P and other colorimetric methods are considerably higher throughput, more facile, and less costly by far than ILE, which requires specialized equipment and generates considerable amounts of hazardous chemical waste. The optical methods we investigated may be suitable for detecting seasonal trends in Fe in natural systems or treatment effects in experimental systems, and correlate linearly with ILE methods. However we caution that the *o*-P method is not suitable if quantitative pool sizes of total Fe, or Fe species, are needed (e.g., in constructing energetic budgets for experimental or natural systems).

CONCLUSIONS

An underestimation of Fe using the spectrophotometric *o*-P method in peat pore water matrices has the potential to lead to an underestimate of Fe^{3+} by up to 100%. Matrix interferences in these systems were found to be the result of peat-derived humic and fulvic acids, DOM character, and possibly the presence of copper. Use of an ILE Method to quantitate total Fe, Fe^{2+} , and Fe^{3+} was shown to be reliable in spite of these potential interferences. The *o*-P Method was correlated to ILE, suggesting that empirical correction factors specific to the DOM character of a given ecosystem could be developed from ionic extraction methods when accurate pool sizes of total Fe and Fe species are required. These factors should be considered in interpreting quantitative pool data for total Fe and Fe^{3+} in complicated peat pore water matrices.

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