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IONIC LIQUID EXTRACTION UNVEILS PREVIOUSLY OCCLUDED HUMICBOUND IRON IN PEAT POREWATER

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IONIC LIQUID EXTRACTION UNVEILS PREVIOUSLY OCCLUDED HUMIC-
BOUND IRON IN PEAT POREWATER

By

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Preface

This Thesis is submitted in partial fulfillment of the degree of Master of Science in Applied Ecology at Michigan Technological University. The research described herein was conducted under the supervision of Dr. Evan Kane through the School of Forest Resources and Environmental Science and the USDA Forest Service Northern Research Station with additional supervision and assistance from Dr. Amy Marcarelli (Michigan Technological University School of Biological Sciences) and Dr. Sarah Green (Michigan Technological University Department of Chemistry). This is original work written by the lead author and is in preparation for submission for publication. The experimental procedures described herein were performed by the lead author with assistance from Dr. Kane; data analysis was performed by the lead author, Dr. Kane, and Dr. Amy Marcarelli. Dr. Sarah Green assisted with the design and optimization of the extraction scheme and provided great insight into the kinetics of the reactions described herein. Drs. Kane, Marcarelli, and Green provided substantial editorial comments which have helped shape this document.

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Abstract[1]

Extracellular iron reduction has been suggested as a candidate metabolic pathway that may explain a large proportion of carbon respiration in temperate peatlands. However, the *o*-phenanthroline colorimetric method commonly employed to quantitate iron and partition between redox species is known to be unreliable in the presence of humic and fulvic acids, both of which represent a considerable proportion of peatland dissolved organic matter. We propose ionic liquid extraction as a more accurate iron quantitation and redox speciation method in humic-rich peat porewater. We evaluated both *o*-phenanthroline and ionic liquid extraction in four distinct peatland systems spanning a gradient of physico-chemical conditions to compare total iron recovery and $Fe^{2+}:Fe^{3+}$ ratios determined by each method. Ionic liquid extraction was found to provide more accurate iron quantitation and speciation in the presence of dissolved organic matter. A multivariate approach utilizing fluorescence- and UV-Vis spectroscopy was used to identify dissolved organic matter characteristics in peat porewater that lead to poor performance of the *o*-phenanthroline method. Where these interferences are present, we offer an empirical correction factor for total iron quantitation by *o*-phenanthroline, as verified by ionic liquid extraction.

1. Introduction

Peatlands represent critical terrestrial carbon stores that are protected by a combination of hypoxic and anoxic soil environments. These chemical 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: nitrate (NO_3^-), manganese (Mn^{4+}), ferric iron (Fe^{3+}), sulfate (SO_4^{2-}), and ultimately CO_2. Although there is wide agreement that alternative TEA reduction is important for anaerobic decomposition, wide variation in the magnitudes of anaerobic CO_2 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), particularly in the expected sequential reduction of TEAs (Vile et al., 2003; Dettling et al., 2006; Keller & Bridgham, 2007; Knorr & Blodau, 2009; Deppe et al., 2010; Kane et al., 2013). A growing body of literature implicates a missing pool of electron acceptors responsible for relatively high carbon mineralization rates in saturated peatland soils (Keller and Bridgham, 2007; Blodau et al., 2007; Gupta et al., 2013). For example, Estop-Aragones et al. (2012) found that only ca. 55% of dissolved inorganic C production could be explained despite monitoring a suite of organic and inorganic dissolved TEAs. Because of this inability to account for all TEAs, comprehensive energy budgets for peatlands have remained elusive.

Due to the chemical characteristics of peat porewater and methodological

limitations, it is possible that underestimation of Fe^{3+} could account for the "missing"

TEAs in peatlands. Peat porewater is rich in dissolved organic matter (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 ferric iron (Antunes et al., 2007; Fuji et al., 2014).

These complexes are effective extracellular electron acceptors in anaerobic environments,

including peat (Benz et al., 1998; Dodla et al., 2009; Bauer et al., 2007; Lipson et al.,

2010; Keller and Takagi, 2013). Therefore, it is likely that complexed iron and humic

substances are important electron acceptors in peatlands, but unfortunately common

methods for estimating ferric iron (cf. EPA method 8008) 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-phenanthroline-Fe^{2+} complex when fulvic acids were present.

More recently, Fujisawa et al. (2011) demonstrated that humic acids prevent development

of the colored o-phenanthroline complex because complexation between iron and humic

acids is more kinetically favorable than formation of the Fe^{2+}-phenanthroline complex

and thus precludes color formation. Incomplete reaction with o-phenanthroline may lead

to skewed estimates of iron reduction in these systems and could explain why

quantification of the sequential chain of observed electron acceptors in peatlands often

does not explain much variability in anaerobic metabolism. This is an important

consideration because a large body of wetland research has depended on

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spectrophotometric assays to quantitate total and ferrous iron, and ferric iron by mass balance (cf. Delaune et al., 2014).

General caveats pertaining to matrix interferences in spectrophotometric determination of total and ferrous iron have been explored in the past (Skogerboe and Wilson, 1981; Anastacio et al., 2008), but environmental samples can vary widely in their composition (e.g, Tfaily et al. 2013) and there is no consensus as to which methods are most appropriate for samples containing highly humified, complex DOM. For example, DeLaune et al. (2014) recommend that *o*-phenanthroline is applicable for Fe quantitation in wetland sediments and soil porewater, based on the perceived quantitative recoveries of iron. Anástacio et al. (2008) compared the performance of *o*-phenanthroline with that of another popular reagent for iron, ferrozine, and also recommended *o*-phenanthroline across a wide range of ecosystems, including those with clay-sorbed amorphous iron oxides. Lipson et al. (2010) reported total Fe recoveries in Arctic peat soil using *o*-phenanthroline that were not significantly different from direct quantitation using inductively-coupled plasma methods. Other work has used the ferrozine assay to estimate iron redox in natural systems (Lovely and Philips, 1986; Pepper et al., 2010; Keller and Takagi, 2013). Despite reports of reliable results, both assays have been shown to experience interference in the presence of both model compounds and environmental DOM, including humic acids (Guo et al., 2008; Yamamoto et al., 2010; Fujisawa et al., 2011). Because iron reduction is energetically sufficient to completely oxidize organic matter under anoxic and hypoxic conditions (Lovely and Phillips, 1986;

Lipson et al., 2010), incomplete iron quantitation could potentially explain a large proportion of peatland anaerobic metabolism, especially if DOM-Fe^{3+} complexes are utilized as TEAs, as Estop-Aragones et al. (2012) suggest (see also Lipson et al., 2010; Keller and Takagi, 2013). Therefore, to accurately appreciate the role iron plays in anaerobic metabolism in bogs and fens it is important to critically evaluate spectrophotometric determination of iron in the challenging matrix of peat porewater and to identify conditions where spectrophotometric approaches are not suitable.

To offer an alternative means of iron extraction and subsequent direct quantitation in humic-rich matrices, we adapted an ionic liquid extraction (ILE) method that utilizes an acid organophosphorous compound, following methods described by Pepper et al. (2010). This method has long been used in industrial effluent cleanup and metal reclamation (e.g., Belkouche and Didi, 2005; Guezzen and Didi, 2012; and references therein), and provides quantitative extraction of ferric iron independent of DOC concentration (Pepper et al. 2010). The extraction portion of the ILE method proceeds via three-step mechanism. At low pH, H^+ ions donated to humic-Fe complexes cause the release 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 by 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)

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complex into the organic phase, leaving Fe^{2+} behind in the aqueous phase. The extracted ferric iron is back-extracted into aqueous media with strong acid for analysis and both aqueous ferric and ferrous iron are directly quantitated using inductively-coupled plasma optical emission spectrometry (ICP-OES). Given the evidence for reliable quantitative performance in highly chelating matrices, ILE may present a novel way of tracking iron redox in the challenging peat porewater matrix. Here, we evaluated interference effects on the quantitation of ferrous and total iron in peat porewater from a variety of peatland environments, from rich fen to bog. Performance of the *o*-phenanthroline method was compared to the ionic liquid extraction method in complex environmental matrices and in deionized water. In this study, we address three questions.

1. Is the *o*-phenanthroline method suitable for iron quantitation and oxidation state speciation in northern peatlands?

2. Can a novel ionic liquid extraction method provide improved quantitation and iron speciation in these systems?

3. Can UV-Vis and fluorescence spectroscopy be used to identify DOM character corresponding to reduced *o*-phenanthroline method performance in peat porewater?

2. Materials and Methods

2.1 Study Sites and Sampling Protocol

Study sites consisted of four locations encompassing a wide gradient of porewater chemistry and dissolved organic matter content and character (Table 1), including a rich

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fen from interior Alaska, USA (see Kane et al., 2010), a rich cedar fen peatland, and a water table manipulation in a bog in northern Michigan, USA (see Potvin et al. 2014). Sampling at the bog site was divided equally into plots with unaltered and lowered water table positions.

We collected porewater samples from a depth of 20 cm below the peat surface through established piezometers into airtight, headspace-free containers. All piezometers were purged prior to collection, and were outfitted with 120 μm Nitex mesh to avoid sampling coarse particulate matter. In the rich fen peatland, porewater samples were harvested with a syringe, injected into pre-evacuated vials, and shipped overnight on ice to the USDA Forest Service Northern Research Station in Houghton, MI for analysis. Samples from all other sites were harvested in the same manner, but were analyzed within 2 hours of collection.

To evaluate discrete interference effects of increased humification and reduction of DOM over time, porewater samples from the bog site (two from the high water table treatments, two from the low) were harvested as above and transferred under N_2 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). Destructive harvests (n=4) from these samples occurred at 0, 7, 21, and 48 d for iron 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 (TDI International model 60245DG, Tucson AZ, USA), and the atmosphere was exchanged five times or more with zero-grade N_2. As the samples equilibrated to laboratory

temperature, extraction reagents were degassed and prepared under N_2 to avoid the introduction of extraneous dissolved oxygen during the extraction process. After reaching ambient temperature, all samples were uncapped and filtered through 0.45 μm sterile nylon membrane filters (AQ Brand Microsolv) and divided into two aliquots each, which were transferred to glass vials fitted with Teflon-faced septa and analyzed using the two different iron quantitation methods

2.2 Iron quantitation

2.2.1   Quality Control

To compare the performances of *o*-phenanthroline and ILE in the absence of DOM and to capture any inadvertent oxidation during sample handling, duplicate quality control standards consisting of distilled-deionized water (DI $H_2O \geq 18.2$ MΩ cm^{-2}) at 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 extraction, and identical aliquots were analyzed simultaneously by *o*-phenanthroline.

2.2.2 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 N HCl (ACS grade; Fisher Scientific) was added,

followed by the addition of 20 mL 0.1 M *bis*-2-(ethylhexyl) phosphoric acid (hereafter

HDEHP; 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 iron found in

HCl. Laboratory trials in DI H_2O and environmental matrices alike confirmed that

extraction vessel geometry and resulting liquid turbulence greatly influenced the rate at

which a mass transfer-limited extraction such as this proceeds, and that mixing is

essential for results to be reliable. In our case, optimal mixing was accomplished by

laying the vials on their side during shaking. The forward extraction of Fe^{3+} 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 two hours.

Following forward extraction of Fe^{3+}, resulting emulsions were allowed to clear, and a 10

mL aliquot of the aqueous phase containing the Fe^{2+} fraction was then 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 N HCl,

and was shaken for 20 minutes at 500 rpm to back-extract Fe^{3+} into the aqueous phase.

The resulting aqueous phase in the back-extraction tube was similarly transferred to a

high-density polyethylene centrifuge tube for storage and analysis. Quantitation of

extracted Fe^{2+} and Fe^{3+} and un-extracted total iron were carried out on a Perkin-Elmer

Optima 7000DV ICP-OES 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 $\pm$ 10% of the mean.

2.2.3 o-*phenanthroline*

For comparison, total Fe and Fe^{2+} were also measured using the *o*-phenanthroline 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, for example, Sinsabaugh et al., 2000). Samples and corresponding matrix spikes were diluted 1:10 with DI H_2O and were reacted with 0.16% *o*-phenanthroline (for ferrous iron, Hach Method 8008), 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 (total iron, Hach method 8146), corresponding with the beginning of ionic liquid extraction. 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 (510 nm) of standards and samples at 15 min, 45 min, 60 min, 3 hrs, and 24 hrs. Laboratory trials indicated that the 3 minute reaction time recommended by the manufacturer was insufficient for environmental samples from peatland matrices to reach completion in either colometric assay, though 15 minutes was sufficient to allow absorbances of matrix-free standards to stabilize. DOM-rich samples required 3 hrs for stable color formation, and all results from *o*-phenanthroline methods reported in this study are from the 3 hr measurement. During the time between readings, plates were kept wrapped in foil to prevent photochemical reactions (DeLaune et al., 2013). We analyzed reagent-free samples simultaneously with reacted samples to correct for any background absorbance

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that would otherwise skew results. 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 *o*-phenanthroline calibration curves were built from these actual concentrations.

2.2.4   Ferrozine assay

For further intercomparison, Fe^{2+} and total Fe quantitation were performed on a subset of bog samples (n = 4) following the methods outlined in Viollier et al (2000).

2.3 Porewater Matrix Characterization

All matrix characterizations occurred simultaneously with iron quantitation. Filtered aliquots of sample were analyzed for organic acid and anion content on a Dionex ICS 2000 ion chromatograph (Dionex Corporation, Bannockburn, IL, USA). Ultraviolet-visible (UV-Vis) absorbance and fluorescence spectra (Excitation-Emission Matrices; EEMs) were both measured simultaneously with a Horiba-Jobin Yvon Aqualog C (Horiba Corporation, Edison, NJ, USA). Run parameters were Excitation: 240-600 nm by 3 nm increments; Emission: 212-608 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 H_2O, such that they were $0.20 < A_{254(sample)} < 0.6$ in order 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

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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 following the scheme presented in Lawaetz and

Stedmon (2009) and Singh et al. (2013) were employed. The resulting EEM fluorescence

intensities were normalized to the integral of the water Raman peak (Ex 350/Em 371-

428) of the sample (akin to normalization to an internal standard). 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. EEMs were then analyzed for fluorescence index (Johnson et al. 2011),

an index of humification (H_P; He et al. 2013) and for an index of DOM redox state

derived from Miller et al. 2006 using the area in a 10 nm x 10 nm window under the

component peak maxima (as a % total EEM fluorescence).

2.4 Statistical Analysis

Linear regressions of ILE and *o*-phenanthroline total iron recovery versus total

iron determined directly by ICP-OES were performed using SAS (SAS Institute Inc.,

Cary, NC, USA) 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 t-tests were used to identify significant (at $\alpha = 0.05$) departure from y = x 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 Kothwala et al (2013). Principal Component Analysis (PCA) was used as a means of arranging sample chemical matrices for qualitative exploration (Kothawala et al., 2013; Bro and Smilde, 2014) incorporating variables from Table 1 and including % total iron recovered by *o*-phenanthroline. Partial Least Squares analysis (PLS) was performed to identify correlative factors that best explain *o*-phenanthroline method performance using the same variables as for the PCA model, but constraining the y-block to be % total iron recovered by *o*-phenanthroline. Agglomerative Hierarchical Cluster Analysis (HCA) was performed on the PCA model to identify clusters of chemically similar samples using the K Nearest Neighbor approach. PARAFAC, PCA, and PLS, and HCA were performed using PLS_Toolbox (Eigenvector Research, Inc., Wenatchee, WA) in Matlab (R2014a).

3. Results

The *o*-phenanthroline method routinely underquantified total iron in samples with DOM matrix (Fig 1; significantly different from ICP-OES; paired t-test $p < 0.001$) but remained quantititative in DI water (data not shown—not significantly different from

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ICP-OES; paired t-test p = 0.199). ILE was not significantly different from ICP-OES in either DI water (paired t-test p = 0.453) or in environmental matrices (Fig 1; paired t-test p = 0.21). Because o-phenanthroline uses total iron to estimate Fe^{3+} by mass balance, this method estimated approximately 87% less Fe^{3+} than ILE (range = 53-100% less; Fig 2.), despite the tendency for Fe^{3+} to be slightly over-quantitated relative to Fe^{2+} in quality control standards (data not shown; average difference = 8.3%, paired t-test p = 0.05). ILE did not show any inadvertent oxidation in the QC standards (average difference = 0.6%, paired t-test p = 0.50), indicating that anaerobic conditions were persistent throughout each extraction. Ferrozine recovered ~30% less total Fe than ILE (68.9-69.7% recovery) and was in fact out-performed by o-phenanthroline in this regard (o-phenanthroline recovered 81.5-88.5% total Fe in the subset of samples).

Samples represented a range of DOC concentrations (32.6-172.3 mg L^{-1}) and $SUVA_{254}$ (2.89-4.72 L mg-C^{-1} cm^{-1}). Concentrations of the organic acids formate, acetate, prorpionate and oxalate 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 6 component classes (Fig 3, Table 2). Careful examination of residual fluorescence in EEM landscapes did not reveal any other component classes.

Principal component analysis (Fig 4, Table 3.) found that samples sorted along a redox and carbon source gradient. Positive loadings along PC 1 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 (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.

A PLS model with the y-block loadings constrained to *o*-phenanthroline method performance (as % total iron recovery vs ICP) was constructed. X-block loadings (sample chemical parameters from Table 1) with significant predictive power are projected further away from the origin, as opposed to orthogonal loadings which are nearest the origin (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 (Table 4) comprise the most probable matrix interferences for the *o*-phenanthroline method in this dataset. These loadings were found to be H_P, %C3 RI, and %C2 (Figure 5). A simple linear regression of H_P versus *o*-phenanthroline (Fig. 6) confirms that this particular index of humification is predictive of *o*-phenanthroline performance. Regressions of total Fe recovery by *o*-phenanthroline against RI, %C2, and %C3 within the context of the full breadth of samples were not significant at $\alpha = 0.05$ (RI: $p = 0.4150$; %C2: $p = 0.8575$; %C3: $p = 0.937$). Samples from the rich fen and cedar swamp were consistent outliers, with higher than predicted total Fe recovery by *o*-phenanthroline in spite of the presence of these interferences (though total Fe recovery was still not quantitative). The HCA output confirms that these samples share chemical similarity with each other, but do not

share similarity with the bulk of the dataset (not shown). The relationship between o-phenanthroline performance and Hp was still significant with the rich fen and cedar swamp data excluded ($R^2 = 0.301$, $F = 6.88$, $p = 0.018$).

4. Discussion

4.1. Appraisal of ILE for Use in Peatlands

ILE was found to be a more accurate method than o-phenanthroline for iron quantitation and speciation in peat porewater across a gradient of physico-chemical conditions. To our knowledge, this study is the first appraisal of this extraction scheme in peatlands. The greater accuracy provided by ILE may be useful in tracking iron biogeochemistry in these systems that has otherwise remained elusive. For example, ILE detected Fe^{3+} in all environmental samples assayed, and throughout the course of an incubation where o-phenanthroline indicated that all iron was Fe^{2+}. Further studies using this method instead of o-phenanthroline may indicate the persistence of humic-bound Fe^{3+} as an energetic reservoir following, e.g., water table drawdown and subsequent rewetting (Estop-Aragones et al., 2013). Mechanistic insight into humic-iron redox interactions could perhaps also be gained through use of ILE rather than o-phenanthroline. Specifically, this approach could prove useful for close examination of the reaction scheme proposed by Page et al. (2012).

4.2 DOM Interference on Spectrophotometric Iron Methods—Advantages of ILE

ILE appears to be an intrinsically better method for quantitation of iron in highly humified, DOM-rich sample matrices than spectrophotometric approaches for three reasons: lower reaction pH, smaller chelating agent size, and a more sensitive detection method. The *o*-phenanthroline complexation reaction proceeds optimally at pH 3-5 (Komadel and Stucki, 1988; Amonette and Templeton, 1998) while the ferrozine assay, a similar spectrophotometric method that has been utilized for iron quantitation and redox partitioning in some peatland systems (Viollier et al., 2000; Keller and Takagi, 2013) occurs at pH ~9.5. There is some debate as to the overall reliability of this method as well (e.g., Anástacio et al., 2008; Guo et al., 2008; Hu 2011; DeLaune et al., 2014). In our samples total Fe assayed by ferrozine was in fact out-performed by *o*-phenanthroline. The reason for 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 thus the complexation equilibrium that ferrozine might reach with iron in the sample matrix. In model humic substances, higher pH (pH > 6) has been shown to lead to tighter binding in metal-humic complexes as the predominant binding sites shift away from carboxyl groups toward phenolics (Catrouillet et al., 2014). This shift in binding site species is accompanied by a reduction in the overall number of sites in humic acids (Fuji et al. 2014), likely corresponding to the relatively smaller number of suitable phenolic sites vs. carboxylic sites overall (Catrouillet et al., 2014, Fuji et al., 2014). Because the ferrozine assay is carried out at a reaction pH of 9.5, the majority (ca. 60%,

Catrouillet et al., 2014) of dominant binding sites in the sample matrix will shift

toward phenolic groups, while some previously-complexed iron will enter the

solution where it is readily quantitated. A fraction of humic-complexed Fe will

remain unquantifiable by ferrozine because it is either too tightly bound or else

stearically inaccessible (Guo et al., 2007; Hu 2011). Complementary research within

the bog system investigated in this study suggests that H_P is strongly correlated with

Folin-Coicetreau assayed phenolics (r = 0.66; p < 0.001; n = 92), suggesting that

further matrix exploration may be necessary. The *o*-phenanthroline assay is carried

out at a more optimal pH to strip DOM-complexed Fe. Based on this information

alone *o*-phenanthroline should experience greater total Fe recoveries than ferrozine

but would theoretically still be less effective in this regard than ILE, which proceeds

at pH ~1.

Both *o*-phenanthroline and ferrozine depend on the ability of a colorimetric

complex to form with Fe. This mechanism is limited in humic- and fulvic-rich systems

wherein the predominant metal-DOM complexes are thought to exist as bidentate or

tridentate conformations of large molecules with bulky functional groups that serve as

binding sites (Catrouillet et al., 2014). These conformations exhibit considerable steric

hindrance from the standpoint of either spectrophotometric assay, effectively limiting

quantitation to free Fe (e.g., Fujisawa et al., 2011) and an unpredictable portion which is

accessible to the chelating agent. bis-2-ethylhexyl phosphoric acid is an aliphatic

molecule and so exhibits less steric hindrance, which likely further improves extraction efficiency.

Both *o*-phenanthroline and ferrozine rely upon spectrophotometric determination by chomophoric absorbance (Viollier et al., 2001; Delaune et al., 2013). Either of these methods could feasibly experience interference from inner filter effects in especially DOM-rich matrices (Kothwala et al., 2012), though DOM typically does not absorb strongly in either the 562 nm (ferronzine) or 510 nm (*o*-phenanthroline) regions, and a heuristic correction for these effects is achieved by simply subtracting the absorbance of reagent-free samples alongside samples undergoing iron quantitation. More important than the potential for slight inner filter effects is the fact that analytical equipment for these methods is inherently less sensitive than ICP-OES, and especially less sensitive than ICP-MS methods. Because ILE utilizes these quantitation techniques, it offers superior precision over spectrophotometric methods.

The unpredictable endpoint of the *o*-phenanthroline-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) suggests that humic substances in DOM pose challenges for either optical method. ILE offers an alternative approach for exploring iron biogeochemistry for applications where quantitative iron recoveries are needed, in systems where highly-chelating matrices may pose analytical challenges for spectrophotometric methods.

4.3 DOM Character—Matrix Effects

Multivariate modeling of matrix effects on o-phenanthroline in this work suggests that successful quantitation and redox speciation of iron in peatlands depends less on DOM concentration than it does on the character of DOM. Humic and fulvic substances have been shown to chelate Fe^{3+} using fluorescence spectroscopy quenching assays (Antunes et al. 2007), and peat-derived humics and fulvics are among the most tenacious humic substances in regard to iron binding (Fuji 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 o-phenanthroline method performance. The H_P index presented the loading with the largest Euclidean distance away from the origin, in the direction most antagonistic to o-phenanthroline performance within our dataset, suggesting that H_P may be a good way to determine if o-phenanthroline is an appropriate method for iron quantitation. Our data suggest that in the absence of %C2 and %C3 interferences, it is entirely likely that results from o-phenanthroline are accurate, though these components combined comprised 22-63% of fluorescence described by PARAFAC in these samples, suggesting that they are perhaps ubiquitous in peat porewater.

UV-Vis indices like $SUVA_{254}$ 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 $SUVA_{254}$ but neither provided predictive capability with regards to o-phenanthroline performance as strong as %C2, %C3 or H_P. In a study encompassing 15 standard humic- and fulvic

acid mixtures, Fuji et al. (2014) reported that aromaticity was a strong predictive factor of iron binding capacity and coordination complex stability. We have found that a UV-Vis index of oxygen content (E4) is a stronger predictor of *o*-phenanthroline method performance than aromaticity (proxied by SUVA$_{254}$), which agrees in principle with the concept that humic and fulvic carboxylic groups participate in Fe binding (Catrououillet et al., 2014). In order to fully understand DOM-Fe interactions in peat porewater, investigations coupling ILE and spectrophotometric iron 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.

In our dataset, it appears that *o*-phenanthroline is not well-suited for use in peatlands due in part to the preponderance of highly humified DOM. In other systems possessing relatively less humified or more aliphatic DOM (see, e.g., Kothwala et al., 2013), this method may be appropriate for quantitative studies of iron biogeochemistry.

4.4 Future recommendations

Providing that the presence of the peat-derived matrix interferences we found in these samples can be confirmed, we propose a simple linear correction factor for the *o*-phenanthroline method:

$$(\text{Eq. 1}) \qquad y_{corr} = 1.6 \, (\pm 0.23)x + 0.83 \, (\pm 0.82)$$

Where y_{corr} is the corrected total iron in peatlands, when *o*-phenanthroline is used

and x is the total iron previously quantitated using o-phenanthroline. Standard error for regression terms are given in parentheses. $R^2 = 0.72$; F = 46.93; p <0.001.

Method selection is obviously not limited to concerns regarding quantitative yield, but also with regards to analytical time and cost. o-phenanthroline 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 iron in natural systems or treatment effects in experimental systems, but we caution against using the o-phenanthroline method in studies investigating pools of electron acceptors in anaerobic metabolism.

5. Conclusions

An underestimation of total iron using the o-phenathroline method in peat porewater matrices has the potential to lead to an underestimate Fe^{3+} by up to 100%. Matrix interferences in these systems were found to be the result of peat-derived humic and fulvic acids. Use of an ionic liquid extraction method to quantitate iron and elucidate the redox fraction of the iron was shown to be reliable in spite of these potential interferences and is recommended in these systems. An empirical correction factor between ICP-OES total Fe and o-phenanthroline total Fe in this work may inform studies of heterotrophic metabolism in humic-rich environments like peat porewater.

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7. Tables and Figures

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

Sample	$SUVA_{254}$[a]	E2[b]	E4[c]	DOC[d]	DOC:TDN[e]	H_P[f]	RI[g]	FI[h]	%C1[i]	%C2[i]	%C3[i]	%C4[i]	%C5[i]	%C6[i]	%C$_a$:%C$_c$[j]
Rich Fen	4.07	4.45	14.88	58.28	23.31	15.74	0.77	1.16	0.30	0.30	0.30	0.08	0.00	0.02	20.47
Cedar Swamp	2.89	4.61	3.41	32.55	18.82	13.49	0.68	1.17	0.34	0.26	0.27	0.09	0.01	0.03	7.51
Bog Sedge	4.82	3.71	4.88	98.60	34.96	12.22	0.64	1.14	0.27	0.19	0.16	0.15	0.19	0.04	0.80
Bog Ericoid	4.66	4.17	7.62	116.00	40.42	14.07	0.65	1.12	0.28	0.21	0.17	0.12	0.19	0.04	0.63
Bog High WT	3.93	4.21	8.10	81.98	30.41	13.07	0.63	1.15	0.28	0.16	0.14	0.19	0.18	0.05	1.08
Bog Low WT	3.75	4.50	19.82	146.75	35.78	16.89	0.67	1.11	0.27	0.21	0.20	0.09	0.21	0.02	0.45

[a] $SUVA_{254}$ L mg-C^{-1} cm^{-1} Specific Ultraviolet absorbance at 254 nm. Weishaar et al., 2003.
[b] E2 Unitless. $A_{\lambda254}$:$A_{\lambda365}$ Index of aromaticity (UV-Vis). Worrall et al., 2002
[c] E4 Unitless. $A_{\lambda465}$:$A_{\lambda665}$. Index of oxygen content (UV-Vis). Worrall et al., 2002; Osborne et al., 2007.
[d] DOC mg L^{-1}
[e] DOC:TDN Unitless.
[f] H_P Unitless. Index of humification (fluorescence). He et al., 2013.
[g] RI Unitless. Redox index (fluorescence). Miller et al., 2006.
[h] FI Unitless. Fluorescence index (Johnson et al., 2012).
[i] %C1-6 Parallel Factor Analysis scores; % of total modeled fluorescence; this work.
[j] %C$_a$:%C$_c$ Ratio of %C4:%C5 (Kothwala et al., 2012).

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Table 2. Excitation-Emission maxima and putative identities of PARAFAC-derived fluorescence components.

1	240, 325/420	Fulvic-like[c]
2	240, 325/525	Condensed aromatic; high MW humic [b,c]
3	255, 375/450	Less-condensed humic or fulvic[c]
4	250/450	Terrestrial humic or fulvic (photo-refractory)[b]
5	335/460	Terrestrial humic or fulvic[b]
6	275/328	Low MW, phenolic, microbial [a,b,c]

[a] Cory and McKnight (2005)
[b] Olefeldt et al. (2013)
[c] Kothawla et al. (2013)

Table 3. Loadings along the first two principal components corresponding to PCA of sample porewater chemistry.

PCA Loadings	PC 1 (29.73%)	PC 2 (25.50 %)
% Recovery *o*-P	-0.039	-0.251
SUVA254	-0.091	0.028
E2	-0.036	-0.013
E4	-0.046	0.241
DOC	-0.103	0.438
DOC:TDN	-0.186	0.226
Hp	0.256	0.356
RI	0.319	0.149
FI	0.000	-0.386
%C1	0.185	-0.320
%C2	0.429	0.033
%C3	0.416	0.038
%C4	-0.362	-0.155
%C5	-0.259	0.330
%C6	-0.324	-0.226
Ca:Cc	0.283	-0.221

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	LV 1 (24.10%)	LV 3 (24.97%)	Euclidean Distance
$SUVA_{254}$	0.093	0.088	0.128
E2	-0.030	0.200	0.202
E4	-0.227	0.215	0.313
DOC	-0.408	0.233	0.470
DOC:TDN	-0.129	0.268	0.297
Hp	-0.44	-0.171	0.472
RI	-0.197	-0.348	0.400
FI	-0.358	-0.083	0.367
%C1	0.224	-0.164	0.278
%C2	-0.115	-0.376	0.393
%C3	-0.114	-0.390	0.406
%C4	0.245	0.315	0.399
%C5	-0.299	0.271	0.404
%C6	0.350	0.222	0.414
$C_a:C_c$	0.219	-0.283	0.358
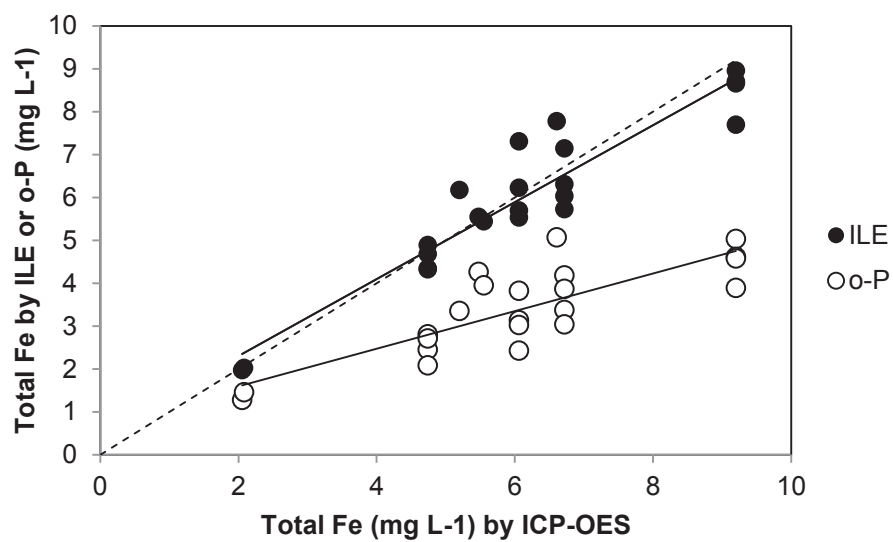
Figure 1: ILE yielded quantitative results (vs ICP-OES derived total Fe) in DOM-rich matrices ($y = 0.90 \, x + 0.5036$, $R^2 = 0.89$, $F = 169.76$) whereas *o*-phenanthroline experienced reduced accuracy ($y = 0.44x + 0.71$, $R^2 = 0.67$, $F = 46.93$).
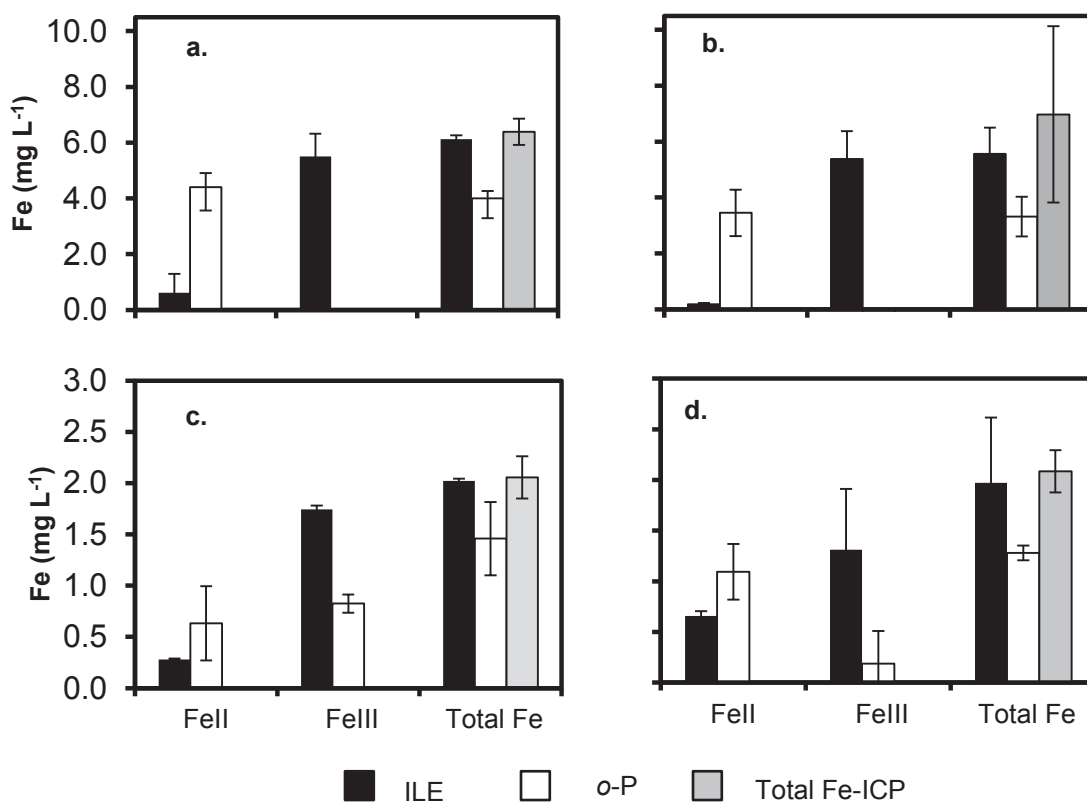
Figure 2: o-phenanthroline underestimated total Fe, leading to estimates of Fe^{3+} 53-100% less than ILE in four distinct peatlands a. *Sphagnum* bog high water table; b. *Sphagnum* bog Ericoid community low water table; c. rich cedar fen; d. rich *Sphagnum* peatland). Error bars depict 1 standard deviation of replicate measurements for ILE (n = 10), o-P (n = 6) and Total Fe-ICP (n = 10).
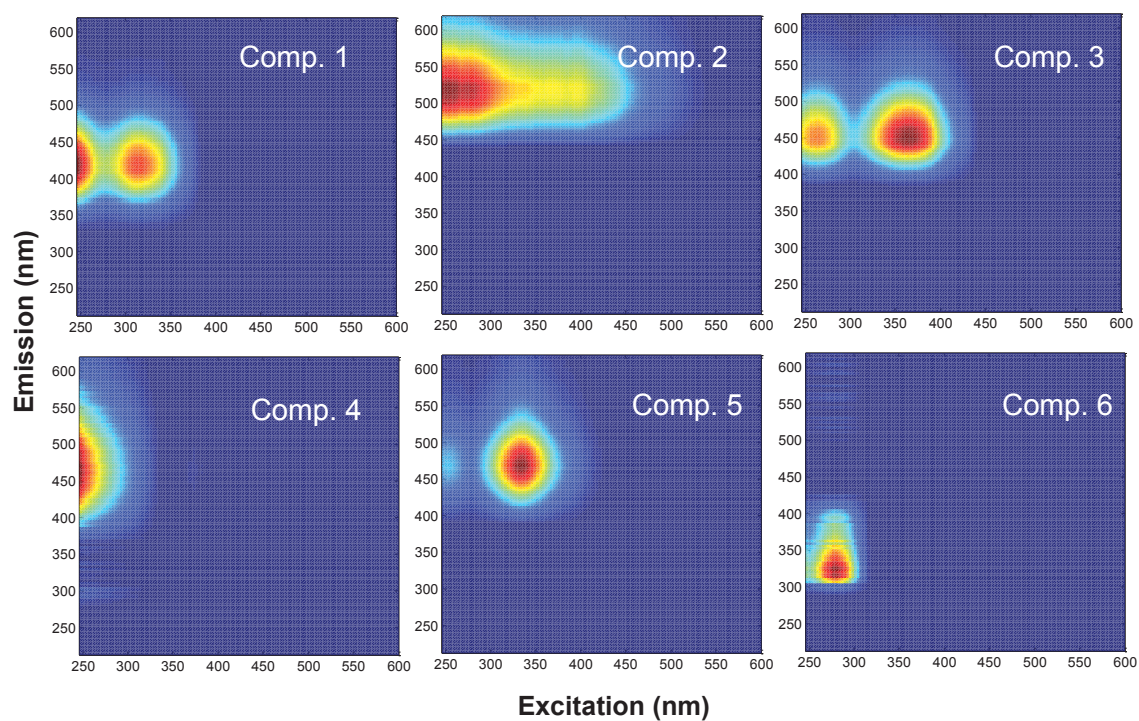
Figure 3: PARAFAC analysis revealed six fluorescence components, explaining >99% of fluorescence in sample EEMs.
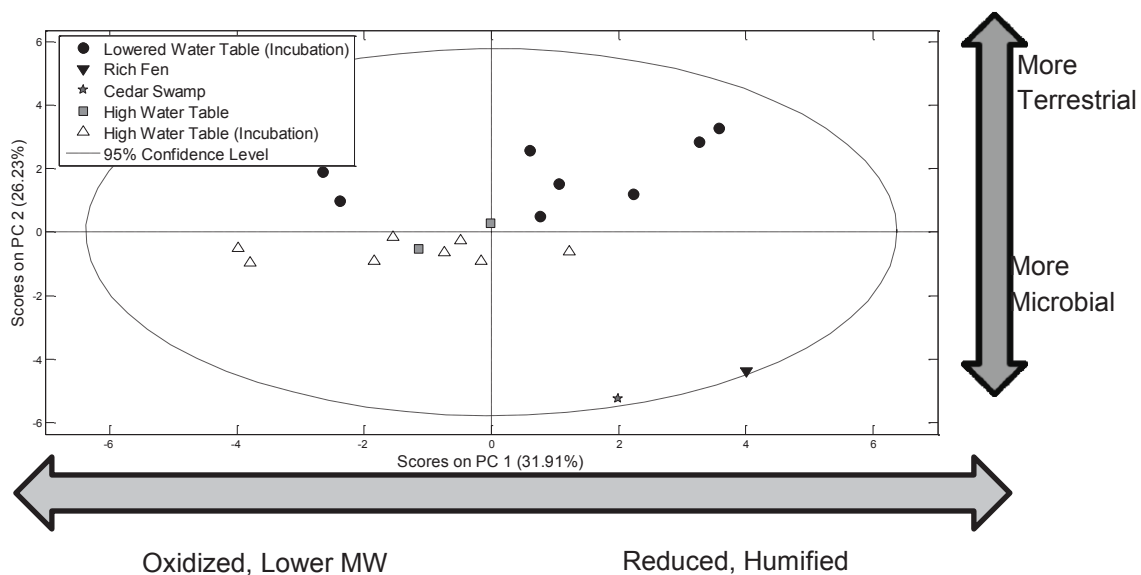
Fig 4. Loadings along the first two principal components corresponding to PCA of sample porewater chemistry. Labeled arrows indicate general trends derived from loadings.
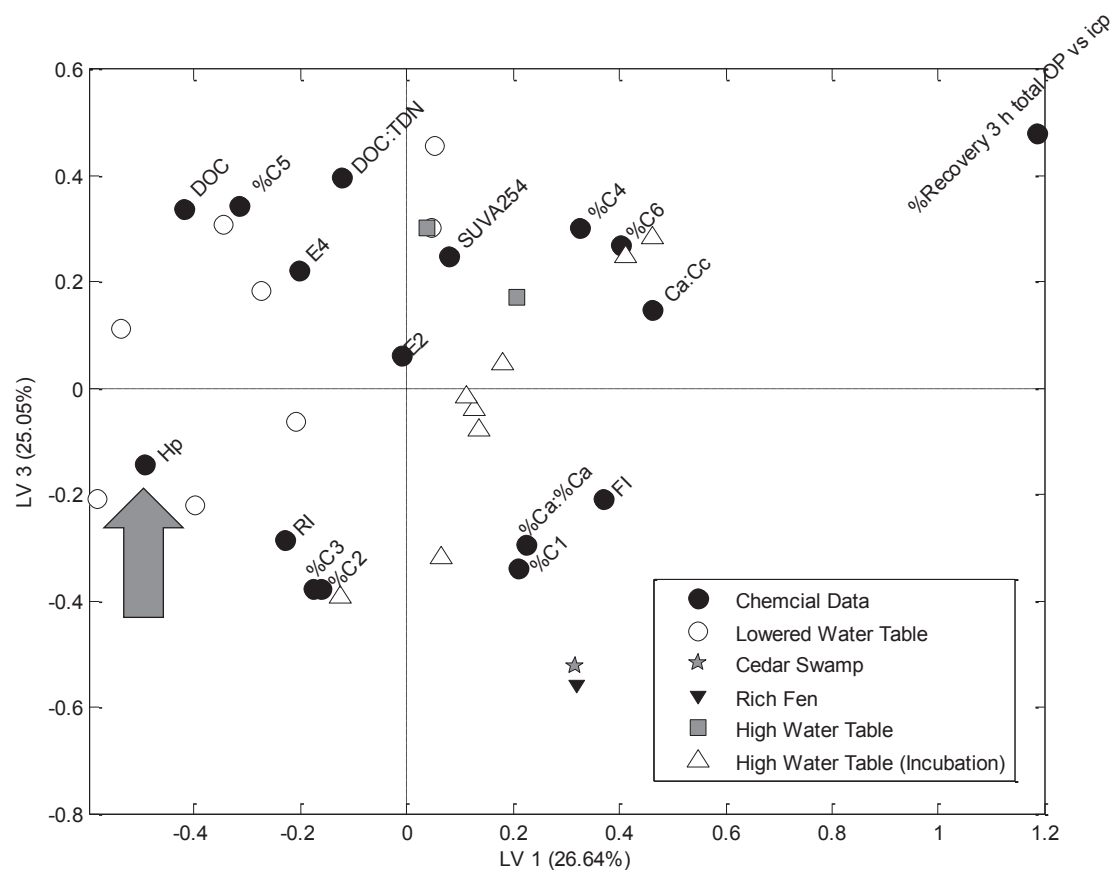
Figure 5. A PLS model found that H_P (indicated by the shaded arrow) is the most significant chemical predictor antagonistic to *o*-phenanthroline method performance.
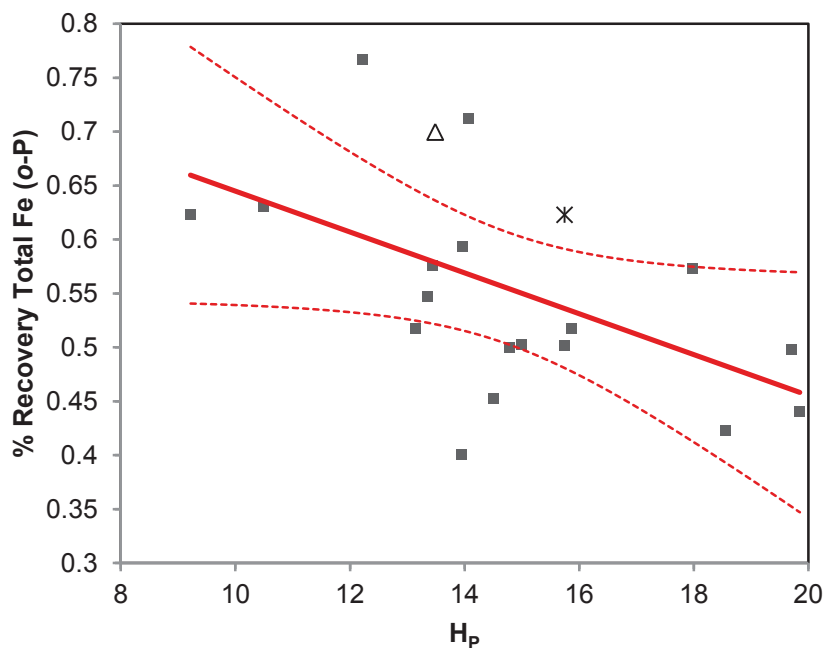
Figure 6. The recovery of total Fe by the *o*-phenanthroline method declined as the humification of dissolved organic matter, as indicated by humification index Hp, increased ($R^2 = 0.28$, F = 13.49, p = 0.018). The Alaskan (asterisk) and Michigan cedar (open triangle) rich fens were identified as outliers but are still included in this regression. Bog samples outside of the 95% confidence interval projected near the origin in the PLS analysis, indicating that fluorescence and UV-Vis spectroscopy alone may not provide a full appraisal of DOM-Fe interactions.

Appendix A

Table 5: Raw data collected during this experiment

Sample	FeII ILE	FeIII ILE	Total ILE	II:III ILE	FeII OP 3h	FeIII OP 3h	Total OP	3o-p vs ILE	o-P vs ILE	%Recover	% Recove	Total Fe IC	SUVA254	A254	A365	A465	A665	E2	E4	DOC	TDN	DOC:TDN	Ca:Cc	Hp	RI	FI
Rich Fen	1.092826	1.312	1.97	0.832946	1.092826	0.187258901	1.280084	0.649789	1.660829	0.622912	0.958637	2.055	0.040679	2.37075	0.5326	0.0967	0.0065	4.451277	14.87692	58.28	2.5	23.312	2.162161	15.74266	0.771799	1.155732
Cedar Swamp	0.28	1.742459	2.022459	0.160692	0.632786	0.825432268	1.458218	0.721013	2.259951	0.699721	0.97047	2.084	0.028902	0.94076	0.204	0.04184	0.01228	4.611569	3.407166	32.55	1.73	18.81503	2.235588	13.48746	0.679084	1.168254
Sedge Community 2014	2.45592	5.322269	7.778189	0.461442	3.809945	1.258055	5.068	0.651566	0.691977	0.766833	1.176909	6.609	0.048159	4.7485	1.2809	0.3354	0.0687	3.707159	4.882096	98.6	2.82	34.96454	2.070793	12.22465	0.64422	1.142081
Ericoid Community 2014	1.584	3.858866	5.442866	0.410483	6.855785	-2.904285	3.9515	0.725996	3.346261	0.711726	0.980343	5.552	0.046581	5.4034	1.2949	0.2856	0.0375	4.172832	7.616	116	2.87	40.41812	1.911903	14.06577	0.647057	1.116261
d01	1.096	4.936	6.032	0.222042	4.762065	-0.5762075	4.185858	0.693942	4.34495	0.622895	0.897619	6.72	0.036604	3.371	0.79165	0.17185	0.0166	4.258195	10.35241	92.095	3.38	27.24704	2.714563	9.217334	0.624448	1.102436
d02	0.144	6.08	6.224	0.023684	4.03716	-0.214889	3.822271	0.614118	28.03583	0.630738	1.027063	6.06	0.038829	3.26785	0.73875	0.16315	0.02195	4.423486	7.432802	84.16	1.9	44.29474	2.718904	10.48928	0.61775	1.155063
d05	0.216	4.672	4.888	0.046233	2.86074	-0.048431	2.812309	0.57535	13.24416	0.593314	1.031224	4.74	0.036808	4.5327	0.9272	0.13515	0.00433	4.888589	31.21247	123.145	3.93	31.33461	2.567649	13.96819	0.621796	1.12187
d06	0.652	8.056	8.708	0.080933	5.60217	-0.5679435	5.034226	0.578115	8.592285	0.547198	0.946522	9.2	0.037598	6.33895	1.37875	0.2505	0.0131	4.597607	19.12214	168.6	3.24	52.03704	2.553496	13.3629	0.631683	1.147837
d71	0.538	6.608	7.146	0.081416	2.074575	1.791475	3.86605	0.541009	3.856087	0.575305	1.063393	6.72	0.045234	4.4574	1.15812	0.25968	0.03264	3.848824	7.955882	98.54	4.14	23.80193	1.963778	13.45202	0.63676	1.157021
d72	0.436	6.876	7.312	0.063409	2.90035	0.23589	3.13624	0.428917	6.652179	0.517531	1.206601	6.06	0.033792	2.68445	0.52565	0.1217	0.0233	5.106915	5.223176	79.44	2.26	35.15044	2.009872	13.14721	0.629286	1.179977
d75	0.304	4.376	4.68	0.06947	1.225055	1.225375	2.45043	0.523596	4.029786	0.516968	0.987342	4.74	0.036486	4.918318	1.064489	0.153003	0.010559	4.620354	14.48987	134.8	5.54	24.33213	1.837274	15.87896	0.647522	1.136508
d76	0.492	7.208	7.7	0.068257	3.38717	1.22527	4.61244	0.599018	6.884492	0.501352	0.836957	9.2	0.036486	6.345796	1.29955	0.248649	0.0131	4.883073	18.98081	172.32	3.94	43.73604	1.626181	15.75389	0.654415	1.114459
d211	0.258	6.048	6.306	0.042659	2.280216	1.094974	3.37519	0.535235	8.838047	0.50226	0.938393	6.72	0.047277	3.789752	0.977079	0.24169	0.022921	3.878654	10.5445	80.16	4.08	19.64706	2.139815	15.00411	0.634683	1.125928
d212	0.23	5.468	5.698	0.042063	1.001224	2.02464	3.025864	0.53104	4.353148	0.499317	0.940264	6.06	0.043828	3.398434	0.83753	0.199458	0.01747	4.057685	11.41724	77.54	2.4	32.30833	2.230428	14.78631	0.634178	1.141912
d215	0.198	4.128	4.326	0.047965	1.16292	1.552432	2.715352	0.627682	5.873333	0.572859	0.912658	4.74	0.041789	5.248735	1.158313	0.206928	0.005964	4.531361	34.69697	125.6	4.6	27.30435	1.846764	17.98312	0.652316	1.104786
d216	0.274	8.388	8.662	0.032666	2.00004	2.578384	4.578424	0.528564	7.299416	0.497655	0.941522	9.2	0.043312	6.910904	1.734699	0.323373	0.031627	3.983921	10.22476	159.56	3.68	43.3587	1.286111	19.72177	0.688605	1.083563
d481	0.36	5.364	5.724	0.067114	1.09003	1.95152	3.04155	0.531368	3.027861	0.452612	0.851786	6.72	0.036072	2.6758	0.668	0.15825	0.019	4.005689	8.328947	74.18	4.24	17.49528	2.198391	14.51588	0.638445	1.150146
d482	0.284	5.252	5.536	0.054075	0.47965	1.9452125	2.424863	0.438017	1.688908	0.400142	0.913531	6.06	0.032788	2.2873	0.5547	0.0932	0.0262	4.12349	3.557252	69.76	2.68	26.02985	1.647316	13.95013	0.662225	1.157294
d485	0.18	4.168	4.348	0.043186	0.954995	1.133066	2.088061	0.480235	5.305528	0.440519	0.9173	4.74	0.034097	4.33845	0.98225	0.17025	0.012385	4.416849	13.74647	127.24	5.2	24.46923	1.844158	19.85315	0.651612	1.111221
d486	0.238	8.72	8.958	0.027294	1.31266	2.579442	3.892102	0.434483	5.515378	0.423055	0.973696	9.2	0.033016	5.3737	1.30585	0.27785	0.0173	4.115097	16.06069	162.76	4.1	39.69756	1.687702	18.57002	0.829958	1.087596

Appendix B

Table 6: Key to Table 5

Sample............Name of Sample
FeII ILE............Fe^{2+} recovered by ILE (mg L^{-1})
FeIII ILE............Fe^{3+} recovered by ILE (mg L^{-1})
Total ILE............Sum of Fe^{2+} Fe^{3+} recovered by ILE (mg L^{-1})
II:III ILE............Ratio of Fe^{2+} : Fe^{3+} recovered by ILE
FeII OP 3h............Fe^{2+} recovered by *o*-phenanthroline (mg L^{-1})
FeIII OP 3h............ Fe^{3+} estimated by *o*-phenanthroline (mg L^{-1})
Total OP 3h............Total Fe recovered by *o*-phenanthroline (mg L^{-1})
o-p vs ILE Tot Fe............Ratio of Total Fe (*o*-P) to Total Fe (ILE)
o-P vs ILE FeII............Ratio of Fe^{2+} (*o*-phenanthroline) tot Fe^{2+} (ILE)
%Recovery 3 h total OP vs icp............Total Fe by *o*-phenanthroline
divided by total Fe by ICP-OES
% Recovery ILE..........Total Fe by ILE divided by Total Fe by ICP-OES
Total Fe ICP............Total Fe determined directly by ICP-OES (mg L^{-1})
SUVA254............
A254............UV Absorbance at 254 nm
A365............UV Absorbance at 365 nm
A465............UV Absorbance at 465 nm
A665............UV Absorbance at 665 nm
E2............A254/A365
E4..........A465/A665
DOC............Dissolved organic carbon (mg L^{-1})
TDN............Total dissolved nitrogen (mg L^{-1})
DOC:TDN............Ration of DOC to TDN
Ca:Cc............Ratio of PARAFAC Component 4: Component 5
Hp............Ratio of EEM region (III+V)/(I+II+IV)
RI............Ratio of reduced / oxidized component peak maxima
FI............Fluorescence index calculated from EEMs.