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# *Interactive comment on* "Molecular characterization of dissolved organic matter from subtropical wetlands: a comparative study through the analysis of optical properties, NMR and FTICR/MS" *by* N. Hertkorn et al.

**N. Hertkorn et al.**

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We greatly appreciate the constructive comments from both reviewers and have done our best to address all comments (see below). Our replies to reviewers' comments are shown after each suggestion starting as 'R'.

Anonymous Referee #1

This paper by Hertkorn et al. uses analyses of optical properties (absorbance, EEM-PARAFAC, etc), NMR, and FTICR/MS to characterize and compare dissolved organic



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matter from three subtropical wetlands. The results of these analyses are very comprehensive and reported in detail throughout the manuscript, and on the whole these data are an important contribution to the characterization of wetland DOM. The novelty of combining detailed NMR and FTICR/MS analyses to environmental DOM samples was previously demonstrated in Hertkorn et al., 2013, but the application to a different system (wetland DOM) plus the addition of optical property analyses and comparison between sites makes this a valuable contribution to the field. The NMR results in particular are extremely detailed, especially in comparison to the other two analyses, and as such I recommend some reorganization and editing to the manuscript. My specific comments are detailed below. If these moderate revisions are addressed, I would recommend publication in Biogeosciences.

R: We appreciate the constructive comments from this reviewer.

Major comments:

The abstract is too long and needs to be shortened - in particular the amount/length of the results presented in the abstract needs to be reduced and/or summarized more efficiently.

R: Done

Primarily because the results of the NMR analyses are much more extensive than the other two analytical approaches, the results and discussion section seems disjointed and would benefit from reorganization. As sections 3.2 to 3.6 are exclusively about NMR, the headings of these sections should reflect that explicitly - I would suggest three parts to the results and discussion section: optical properties, FTICR/MS, and NMR. Then sections 3.2-3.6 should be subsections under the NMR section (though there are also some issues with a few of these specific subsections which are mentioned below).

R: Done. The removal of original sections 3c and 3f, which exclusively refer to NMR



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data, also restores better balance between NMR and FTMS section of this contribution. We now have 4 NMR and 4 FTMS figures in the main text as well as similar word count for both NMR and FTMS description.

Section 3.3 seems out of place within this manuscript. A test of the extraction selectivity is not mentioned in the abstract, introduction, or conclusions as part of this study and is not addressed in the methods section. If the authors wish to include this test in this paper, it needs to be evaluated specifically with regards to wetland DOM. Also the extraction selectivity is only tested for the NMR analyses - what are the implications for FTICR/MS analysis, which also was performed on PPL extracts? Another option would be to move this discussion to the supplemental material along with Figure 3.

R: Done. We have removed the original section 3c entirely: Extraction selectivity of DOM. This section had in fact been introduced at a late stage of the manuscript to address the issue of the origin of methoxy groups in SPE-DOM. As it does not directly contribute to the main story of wetland SPE-DOM characterization, this omission has no other consequences on the residual text.

The comparison to an open ocean sample (section 3.6) is useful given the biogeochemical links between wetland and marine systems, but this section again only includes the NMR analyses. A few references to this open ocean sample are included in the FTICR/MS section, but the comparison should ideally include all three analytical approaches and be combined into one separate section along with any biogeochemical implications from the data (possible DOM degradation signatures, terrestrial vs. marine components, etc). If this comprehensive comparison is not available, this section should become a sub-section under the NMR results and be clearly identified as such.

R: Done. We have moved the section 3f (Comparison of wetland SPE-DOM FCL-L with a South Atlantic open ocean SPE-DOM) entirely to the Supporting Online section, where it resides along with Fig. S5. We rate this comparison still important, because no other 2D NMR spectra of sp2-hybridized carbon in any SPE-DOM are available in



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the current literature. Nevertheless, this comparison can be clearly separated from the main text without loss of coherence. We have inserted a small text section which leads to the respective SI text.

Is it possible to quantify the differences in NMR analyses between sites? The cluster analysis (Figure 9, panel A) from the FTICR/MS data is a nice visualization of the quantitative differences between sampling sites, and something similar that included all the results of all the analyses (if possible) would really strengthen the authors' conclusion that DOM composition varied among wetlands. The quantitative extent of differences among samples can be difficult for readers less well-versed in NMR to grasp from simple comparisons (i.e., FCE-L > FCE-S > PAN > OKA). The data presented in Table 2 helps with this.

We have added a 1H NMR derived hierarchical cluster analysis (HCA) and a principal component analysis (PCA) of the six wetlands SPE-DOM in the new Figure S2.

A summary or conclusions section is missing though section 3.8 gives a broader overview-type perspective to the results. This should be clearly titled as conclusions or a separate conclusions section added with the most pertinent results and their implications (or both; i.e., section 4 'Conclusions and biogeochemical significance').

R: Done.

Minor comments:

- The entire manuscript should be thoroughly checked for consistent use (or non-use) of Oxford commas. Did best we can.

- Please check that all abbreviations are defined (for example, SUVA254 is not defined in the text, and EEM-PARAFAC is only defined in the abstract). R: Done.

- pg 13716, lines 5-6: this sentence is repetitive (South/southern Florida mentioned twice). R: Done.



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- pg 13718, line 4: should this reference be Dittmar et al., 2008 in LO Methods? R: Done.

- pg 13720, line 25: a word is missing here, perhaps for? (‘. . . was acquired for an acquisition time of 1 s, . . .’) R: Done.

- pg 13725, line 19: should this word be ‘where’ rather than ‘were’? R: Done.

- pg 13732, line 26: Yuan et al., 2011 does not appear in the reference list. R: Done.

- pg 13744, line 7: move ‘that’ to before ‘many’ (‘. . . suggest, in agreement with previous reports . . . , that many of the bulk . . .’). R: Done.

- pg 13752, line 12: Yang et al., 2011 appears in the reference list but is not referenced in the manuscript (perhaps this should be Yuan from pg 13732 or vice versa?). R: Done.

- pg 13759, Figure 4: panels are out of order (A should be upper left) and the first part of the caption is confusing and should be reworded for clarity (panel B is SPE-DOM from which site?) R: Corrected. Figure 3 (TOCSY NMR spectra of SPE-DOM) has been rearranged accordingly.

- pg 13764, Figure 9 caption: extra word in (b) description, remove either ‘in’ or ‘to’ from ‘molecular compositions common in to all six wetlands SPE-DOM’. R: Done.

Anonymous Referee #2

In general, the paper is hard to follow with so much information describing some data and on the hand lacking a clear conclusion and explanation of the observations and linking it to the geochemical and the environmental conditions in each ecosystem. I think the authors lost an opportunity to compare and link between the NMR and the ICR results. Each section appeared to be separate from the section before it or after it.

The paper needs to be re-organized and focused on the main conclusions. I personally don’t think the optical priorities data are needed, . . ...



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R: The optical properties data are directly connected to critical ecological functions of wetland organic matter. Absorption of electromagnetic radiation refers to ïĄř- and non-bonding electrons which also govern chemical reactivity of critical functional groups in natural organic matter. In addition, due to availability and ease of operation, many more publications refer to UV/VIS and EEM data than to meaningful FTMS and NMR descriptions of DOM. Hence, this contribution which offers a DOM characterization by all three methods will provide useful leads for future use of optical methods in conjunction with a deeper molecular assessment in DOM research. Besides, the comprehensive optical data in the manuscript indicate a well discussed and reasonable variance in-between DOM, even if intrinsic averaging characteristic of any optical spectroscopy of NOM will inevitably result in a restricted variance of these "bulk" parameters. In addition, studies combining optical techniques with molecular characterizations are becoming more commonplace in the literature, clearly reflecting the needs for detailed comparative studies. This is now highlighted in the Introduction with several references added.

. . ...as well as the comparison between the terrestrial DOM and the marine DOM samples. It might be worth mentioning how different these samples are from each other (which is not surprising), however I would only spend few sentences on that and not 2-3 paragraphs.

R: We have moved the section 3f (Comparison of wetland SPE-DOM FCL-L with a South Atlantic open ocean SPE-DOM) entirely to the Supporting Online section, where it resides along with Fig. S5. We rate this comparison still important, because no other 2D NMR spectra of sp2-hybridized carbon in any SPE-DOM are available in the current literature. Nevertheless, this comparison can be clearly separated from the main text without loss of coherence. We have inserted a small text section which leads to the respective SI text.

This paper needs major changes. The data sets on the other hand are extensive and would be a great addition to the DOM society and a substantial contribution to scientific progress.



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R: We appreciate this constructive comment and fully agree about the importance of this contribution. While the DOM community fully agrees that most of the DOM remains molecularly uncharacterized, this paper provides a wealth of such information, which should help in moving this field forward. As to changes in the manuscript, we are doing our best in accommodating the requests by the reviewers the best we can, without giving up important data.

Abstract: I don't think this sentence add anything to the abstract and a result I suggest deleting it. "Analogies were such that an established excitation emission matrix fluorescence parallel 15 factor analysis (EEM-PARAFAC) model for the Everglades was perfectly applicable to the other two wetlands").

R: We believe this sentence contains a very important message, particularly to those working on optical properties of DOM. We have re-phrased it to clarify.

The abstract contains a lot of information. I suggest rewriting it to summarize the main points of the study only.

R: The Abstract was shortened.

Introduction: The authors of the paper fail to include references of main groups that are known for using FT ICR MS, NMR in their organic matter characterization, rather, it appears that the authors are mainly referencing their papers.

R: We have included a large number of important references from other groups working on FT ICR/MS and NMR of DOM, but obviously can't include them all. We have added some additional references that may have fallen short in the previous version of the manuscript.

Experimental: What time of the year did the sampling take place in the everglades? It was confusing to me which FCE samples were collected in 2011 and which was collected over the years. "Sampling was performed during 2011 for the Florida coastal Everglades (FCE; for SPE-DOM) and dur- 20 ing the summer 2010 for the Pantanal



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(PAN) and the Okavango Delta (OKA) as part of on-going research programs. For EEM-PARAFAC comparisons, multiple samples collected monthly over several years (Chen et al., 2013) for the FCE, 38 samples collected along a trans-Okavango gradient (Cawley et al., 2012), and 22 samples collected in different sub-environments of the Pantanal wetland (rivers, lagoons, marshes; unpublished) were used to assess differences and similarities." How representative are these 6 samples to their ecosystems? The number of samples chosen for this study is too low for such a detailed comparison. Are these the samples that were collected for the ICR? Or these are the summary of the 38 samples? I am still confused on which samples are included in this study. "For the Florida Coastal Everglades (FCE), samples were collected from the freshwater marsh, peat-soil dominated Shark River Slough (FCE-L) and the marl-soil dominated Taylor Slough (FCE-S), from the Okavango Delta (OKA) seasonal floodplain (OKA-L) and occasional floodplain (OKA-S) along the Boro River (Cawley et al., 2012), and the Paraguay River (PAN-L) and a wetland channel in Pantanal National Park (PAN-S; Chacra de Solange) for the Pantanal (PAN)."

R: The sampling, site selection and number of samples analyzed for optical properties and for SPE-DOM have been clarified.

How long were the extracts stored in the freezer? From my experience, even if the samples are stored in MeOH, you will see changes in OM composition with time; "the isolated SPE-DOM extracts (referred from here on as DOM for the NMR and FT-ICRMS data) were stored in pre-combusted glass vials and kept in a freezer until analyzed."

R: Storage periods ranged from a few weeks to a few months; on reacquisition of 1H NMR spectra of SPE-DOM dissolved in CD3OD we could not observe visible alterations upon direct overlay even in 800 MHz 1H NMR spectra acquired with cryogenic detection on storage time intervals exceeding several months.

How was the concentration of the extracts in an organic solvent measured? "Diluted SPEDOM (5 $\mu$gmL$-$1 15 in methanol) were injected into the electrospray source using



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a microliter pump at a flow rate of 120 $\mu$L h$-$1 with a nebulizer gas pressure of 138 kPa and a drying gas pressure of 103 kPa".

R: Upon preparation of NMR samples, defined aliquots of methanolic SPE-DOM solution are dried and weighed on the analytical balance; this provided the dilution ratio employed in methanol solutions used for FTICR mass spectrometry. In addition, this dried sample in standard methanol is exchanged 3-4 times with CD3OD (for several times); the integration of residual CD2HOH and its 13C satellites and SPE-DOM 1H NMR resonances provides an alternative lead to the DOM content.

What was the number of unassigned peaks? "Final formulae were generated and categorized into groups containing CHO, CHNO, CHOS or CHNOS molecular compositions which were used to reconstruct the group-selective mass spectra (Schmitt-Kopplin et al., 2010)."

R: Because we use rather strict and conservative exclusion criteria, about 50% of mass peaks were assigned to CHO, CHNO, CHOS and CHNOS molecular compositions, respectively. We have abstained from providing the count of total mass peaks; nevertheless, we have added a respective note in the experimental section.

Results section: I don't think the optical properties added anything to this study. I think the problem is with the low number of samples used for comparison. Considering that the authors spend more time and space explaining the NMR and the ICR data, I would suggest removing the optical properties data to the supporting information.

R: See above the explanation why we consider this information should stay in the manuscript.

Why was the NMR done on the SPE samples? Why not the original samples? Freeze drying the samples then dissolving them in D2O would be less biased than using SPE.

R: For two reasons: (a) Field logistics – for both the Pantanal and the Okavango, due to the remoteness of the sampling locations, and logistical problems of air travel



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with large water samples, the samples were filtered and extracted (PPL) in Brazil and Botswana respectively. (b) While a certain extraction selectivity of PPL solid phase extraction is acknowledged, SPE nevertheless shows appreciable carbon yield and produces materials with excellent properties to be used in FTICR mass spectrometry and NMR spectroscopy. Freeze-drying of natural organic matter solutions on the other hand provides rather variable results depending on the extent of organo-mineral interactions and hydrophobic collapse which may impede complete re-dissolving of organic matter. Residual salt interferes with both NMR spectroscopy (metal coordination of organic ligands affects relaxation time and causes line broadening or disappearance of 2D NMR cross peaks) and FTICR mass spectroscopy (metal salt clusters commonly ionize much better than organic compounds, thereby suppressing mass peaks from original organic matter). Therefore, in the interest of comparability of all wetland samples, SPE was selected as the most appropriate method of DOM isolation in this study.

The IHSS extraction protocol utilizes strong acids and bases which could (or is) altering the composition of organic matter. I personally don't recommend this protocol.

R: This section has been removed.

Can you elaborate on what you mean by "identical source materials". A sample description would be useful here.

R: This section has been removed.

The whole section "Extraction selectivity of dissolved organic matter" appears out of place. Maybe move to supporting information and summarize main conclusion in the manuscript? Maybe the methods section?

R: We have removed this section entirely: Extraction selectivity of DOM. This section had in fact been introduced at a late stage of the manuscript to address the issue of the origin of methoxy groups in SPE-DOM. As it does not directly contribute to the main story of wetland SPE-DOM characterization, this omission has no other consequences



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on the residual text.

The NMR section is very detailed. It is very easy to get lost. Why not focus on the main results and conclusions only.

R: NMR and FTMS record and express SPE-DOM molecular characteristics from complementary perspectives. (Non-destructive) NMR spectroscopy samples atom-specific data of intact molecules which are reconstructed into SPE-DOM substructures; and several distinct NMR spectra have to be acquired to enable these conclusions. In this contribution, 5 different types of complementary 1D and 2D NMR spectra of wetland were discussed (some more had been acquired and were not even mentioned or discussed at all). In contrast to the very information-rich NMR spectra, in which even subtle molecular differences are manifest from primary data, FTICR mass spectra suffer from extensive projection of structural diversity onto single mass peaks (which represent entire molecular ions composed of many atoms). Initially, ionization selectivity within very complex mixtures causes non-appearance of mass peaks altogether; otherwise, projection of all isomeric molecules, which in case of SPE-DOM likely range in the hundreds to thousands per any single recorded mass peak, represents a strong case of intrinsic averaging which has a tendency to make all FTICR mass spectra of (very complex) SPE-DOM look rather similar. This intrinsic disparity of SPE-DOM molecular complexity and FTMS resolution makes elaborate mathematical data treatment indispensable, to ensure an adequate compositional distinction of wetland SPE-DOM. This has been done in this contribution and has revealed remarkable distinction of OKA, PAN and FCE wetland SPE-DOM not otherwise available. With the removal of the NMR based section “Extraction selectivity of SPE-DOM” altogether and transfer of the section “Comparison of wetland and marine SPE-DOM. . .” into the Supporting Online Material we have restored the balance between NMR and FTMS sections, both with respect to numbers of figures (4 each) and word count (about equal).

What do you attribute the reason of the high number of unassigned formulas? (table 3) DID you compare the samples using Kendrick plots that utilize all the observed



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masses?

R: See reply above

A PCA or NMDS or both the NMR and the ICR data could be useful.

R: We have added a 1H NMR derived hierarchical cluster analysis (HCA) and a principal component analysis (PCA) of the six wetlands SPE-DOM in the new Figure S2.

Biogeochemical significance: The biological significant paragraph fails to explain the main factors behind the differences or lack of differences observed between the samples. A summary of the main conclusions is lacking. Each section in this manuscript appears to be separate from the other and rarely linked to one another. A paragraph that links all the observations from different techniques is lacking.

R: While we do not agree with this statement, we have modified this section hoping it will be more comprehensive now.

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Interactive comment on Biogeosciences Discuss., 12, 13711, 2015.