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Interactive comment on "Fast-freezing with liquid nitrogen preserves bulk dissolved organic matter concentrations, but not its composition" *by* Lisa Thieme et al.

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We thank referee #3 for the constructive comments. Please find below our response.

Introduction: The introduction focuses a great deal on fluorescence, while not mentioning the experimental approach of freezing until much later in the discussion. The extensive literature review on fluorescence isn't necessary given the common nature of the technique and the focus of the paper. I suggest reducing the discussion of fluorescence and spending more time summarizing current research on freezing and identifying knowledge gaps in this area. Specifically, I think it would be important to see if any experiments have been conducted on freezing soil solution. Highlighting the novelty of the approach is critical for the impact of this study. In addition, keep the

information of freezing organic matter in general. Some time could also be dedicated to discussing what might be different between stream samples and soil samples after freezing. Finally, a clear justification and rationale for the study needs to be part of the introduction.

We will reduce the discussion of fluorescence and review the scientific literature on the effect of freezing on fluorescence characteristics in water samples. We will include a clearer justification and rationale for the study. We will rephrase the introduction as follows: "In addition to dissolved organic carbon (DOC) concentrations, properties of dissolved organic matter (DOM) are crucial for its role in biogeochemical cycles of carbon and nutrients as well as for its effect on pollutant dynamics (Bolan et al., 2011). Spectroscopic methods like UV-vis absorption and fluorescence spectroscopy used as single excitation/emission scans, synchronous scans and excitation-emission matrices (EEMs) in combination with different indices and/or parallel factor analysis (PARAFAC) are increasingly applied to characterize chromophoric dissolved organic matter (cDOM) in various environments (e.g., Murphy et al., 2008; Yamashita et al., 2010; Stedmon and Markager, 2005; Graeber et al., 2012; Otero et al., 2007, Traversa et al., 2014, Kalbitz et al., 1999). The applicability of optical methods for characterizing DOM and the comparability of results in multidisciplinary studies relies on the preservation of samples prior to their analysis. DOM properties depend on many physicochemical and biological boundary conditions, so that artefacts caused by sample storage or sample pre-treatment may be produced easily. For these reasons it is recommended to directly filter samples after collection and store them in the cold and dark prior to measurement as soon as possible (Santos et al. 2010; Spencer and Coble, 2014;). However, immediate measurement is often not possible for practical reasons such as a large number of samples, remote or separated sampling sites, so that freezing of filtered DOM samples is often the selected storage method (Murphy et al., 2008; Yamashita et al., 2010; Graeber et al., 2012). Freezing can affect the physicochemical composition of samples (Edward and Cresser, 1992) so that improved conservation techniques, which avoid or minimize potential artifacts of freezing, are required. During

the freezing process, DOM is preferentially excluded from the ice phase and enriched in the remaining liquid phase (Belzile et al., 2002; Xue et al., 2015). The increasing solute concentrations and changing physical conditions in the remaining liquid phase during the freezing process could promote conformational and configurational changes of DOM molecules as well as particle and complex formation depending on DOM composition and sample type (Zaritzky, 2006; Edward and Cresser 1992). One potential technique for minimizing these effects could be fast freezing with liquid N2, by radically reducing the freezing time. Whereas studies on sample preservation of marine waters (Del Castillo and Coble 2000, Yamashita et al. 2010a, Conmy et al. 2009) showed only a small freezing effects on DOM fluorescence characteristics, research with a variety of freshwater samples produced inconsistent results. Fellman et al. 2008 measured DOC concentrations and UV absorption in fresh and frozen/thawed Alaska stream water samples and reported a significant decrease of DOC concentration and specific ultraviolet absorption at 254nm (SUVA254). They recommended freezing as an acceptable storage method for freshwater samples with low DOC concentration and/or low SUVA254 values. In contrast, Yamashita et al. 2010 observed only minor changes in absorption based indices after freezing and thawing of Venezuela river water but significant alterations (decrease and increase) for PARAFAC component intensities. A freeze/thaw experiment with water samples from a large number of UK locations conducted by Spencer et al. 2009 showed large and variable changes (decreasing and increasing) in DOM fluorescence intensity and absorbance after freezing and thawing. Likewise Peakock et al. (2015) found strong and inconsistent effects of freezing and thawing on absorbance properties of cDOM in water from bog pools, fen ditches and lakes. In a study of sample preservation on rainwater cDOM fluorescence, Santos et al. (2010) found a decrease of protein-like fluorescence intensity due to freezing. While many studies investigated the influence of different soil sample pre treatments on DOC concentrations and DOM composition (e.g. Christ and David 1994; Sun et al. 2015) only few studies focused on the influence on these properties when using different preservation methods for the extracted soil solutions. Otero et al. (2007) con-

ducted freeze/thaw experiments on salt marsh pore water and found no changes in characteristics of synchronous fluorescence scans. The impact of sample preservation like freezing seems highly variable depending on sample and DOM characteristics. While most studies focused on samples from marine or freshwater ecosystems, there is a lack of information on sample pre-treatment effects on cDOM properties of water samples from terrestrial ecosystems, especially soil solution. Due to different sources of DOM in land and water environments (Bolan et al. 2011) and therefore different chemical characteristic, it is unlikely that insight regarding the alterations of samples during storage can be transferred from one sample type to another. To help closing this gap, we investigate in this study the influence of freezing and thawing on DOC concentration, spectral absorption and fluorescence properties for a wide range of water samples (throughfall, litter leachate and soil solution) from different terrestrial ecosystems (grasslands and forests). We tested in how far fast-freezing with liquid nitrogen might prevent concentration and partitioning effects and minimize structural changes of DOM. We hypothesized i) that sample type affects freeze/thaw effects on DOC concentrations and DOM properties, because of different physical and chemical DOM characteristics and therefore different response to changing conditions during freezing and ii) that fast-freezing with liquid nitrogen reduces these freeze/thaw effects, because it minimizes the freezing time and thus prevents partitioning effects and their physical consequences.

Sampling and sample preparation: The approach for sampling, replication, and defining the subject for the analysis needs to be clarified. The existing description is hard to follow. It might help to provide a diagram for where the samples originate and their fate, with a clear identification of what is composited and analyzed. This will clearly highlight the mixing of the grassland and forest samples.

We did not mix sample solutions from forest and grassland plots for obtaining composite samples for chemical or spectroscopic analysis. All samples were analyzed separately in the laboratory. We used replicated sampling devices per sample type

(e.g. topsoil solution or throughfall) on the individual plots (forest: W1, W2, W3, W5 and W9; grassland: G3, G5 and G39) in order to gain composite samples with sufficient volume for the experiment. We will rearrange the description of sampling and sample preparation for clarification. While the forest and grassland samples were processed separately in the laboratory, the results were analyzed in one statistical analysis. This analysis did not reveal significant differences between grassland samples and forest samples (PERMANOVA, R^2 = 0.05184, p = 0.2401). The freezing procedures are somewhat tedious. Is this operational? What happens if a large quantity of water is stored? Is there a potential difference given the small amounts used as test subjects in the study? The procedure for freezing samples at -18°C corresponds to the routine procedure in the above mentioned BECYCLES project. We commonly keep the sample volume that is stored frozen as small as possible because of space limitations in deep freezers. We think that increasing the volume of samples that are subjected to freezing also increases the risk of artifacts, because of increasing concentration effects due to extended freezing time. We will included a short discussion of this in the revised manuscript.

Results: The overall average change of 6% (1.6 mg L-1) seems small given the high DOC concentrations in the samples. Is the lower average a result of the composite?

This comment may be a misunderstanding of the methods applied in the study. We did not produce composite samples across different sample types, as we tried to explain with the answer of your comment to Sampling and sample preparation (above). A good point is to test the influence of the initial DOC concentrations on the changes of DOM properties due to different treatments. We found a significant correlation between initial DOC concentration of the fresh sample and the changes of DOC concentrations for the -18°C freezing treatment (Spearmans rank r = -0,447, p = 0,0194). This indicates a larger decrease of DOC concentration during freezing at -18°C for samples with higher initial DOC concentration. Additionally we run a new PERMANOVA extended with DOC concentrations (NPOC concentration in the following table) of the fresh samples as fac-

tor (see Fig.1:PERMANOVA_results) The interaction between the freezing treatment (either at -18°C or with liquid N2) and DOC concentration of the fresh sample explains a reasonable part of the variance of DOC concentrations (R2 = 0.14) and is highly significant. However, the fraction of the variation that is explained by the main treatment is as low as before (R2 = 0.05). It is important to note that the tested dependent variables of the PERMANOVA were the DOM composition variables without DOC concentration. Therefore, fast-freezing with liquid N2 still eliminates the significant effect of freezing on DOC concentrations. Altogether initial DOC concentration well explains the different strength of the effect of treatment on DOM composition. In other words, the higher the initial DOC concentration, the stronger the effect by freezing on DOM composition. But, (as before) there are more variables, which may add to the explanation. We will add the new statistic and their results to the results section.

L30: This doesn't make sense. SUVA values increase, so aromatic compounds or aromaticity increase. But, humification index decreases?

SUVA is an absorbance-based indicator, reflecting aromaticity, whereas HIX is a common indicator of humification based on low-Stokes shift fluorescence (protein-like), relative to high-Stokes shift fluorescence (humic-like). Therefore, both indicators allow different interpretations and can have opposite tendencies within a dataset. In fact, HIX is not necessarily linked to aromaticity but rather to a wavelength shift in the emission of so-called humified DOM (Fellman et al. (2010) Limnology and Oceanography 55:2452–2462). Theoretically (and in an extreme case), if a sample is only consistent of amino acids with aromatic groups (Tyrosine, Tryptophan, Phenylalanine), it could have a high SUVA but a very low HIX.

Conclusion: There needs to be some discussion of the results related to very high DOC concentrations in the sample. What are the implications for changes in the DOM character with freezing?

We will include a discussion of the results of the new statistic, showing that higher

initial DOC concentrations lead to higher losses of DOC during freezing at -18°C . This finding is consistent with results of Fellman et al. 2008 who suggested freezing as preservation method only for water samples with DOC concentrations < 5mgL-1. Our results for fast-freezing with liquid nitrogen show the opportunity of freezing as conservation method for samples with higher concentrations without altering the bulk DOC amount.

Also, is freezing with N2 practical?

Of course, in field freezing with liquid nitrogen would take some extra effort concerning material and costs and is probably only applicable for small sample volumes. The objective of our experiment was testing if the increased effort and cost of using liquid nitrogen in the field is justified by advantages regarding the minimization of freezing artifacts. We will add this consideration to the conclusion section of the revised manuscript.

Figure 1: Is cDOC an accepted convention? A label of DOC with the units usually implies a concentration. I suggest adding 'in' for Change in DOC concentration, Or DOC change

Graphs a and b in Figure 1 show the DOC concentrations of the samples before freezing them. We will change the label of the y-axis of these graphs into "DOC concentration (mg L-1)". The label of the y-axis of graphs c and d will be changed into "change in DOC conc. (mg L-1)". The label of graphs e and f will be changed into "change in DOC conc. (%)".

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	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
treatment	2	32.41	16.2066	2.3584	0.05065	1e-04 ***
treatment:npoc.factor	3	92.21	30.7358	4.4728	0.14407	1e-04 ***
Residuals	75	515.38	6.8717		0.80528	
Total	80	640.00			1.00000	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Fig. 1. PERMANOVA_results