Fast-freezing with liquid nitrogen preserves bulk dissolved organic matter concentrations, but not its composition

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Reply to RC1

We thank reviewer #1 for the constructive hints. Please find below our response.

P4L5ãAˇARevise "500 ml" to "500 mL". P4L26, 28ãˇAˇARevise "ml" to "mL". P6L25 Reviseˇ "mg L-1" to "mg C L-1". P7L5 Revise "Fellman et al., (2010)" to "Fellman et al. (2010)" P8L24 Revise "formerr" to "former"

In the revised manuscript we will correct the mentioned mistakes. We will change "ml" to "mL" when necessary, "mgL-1" to "mg C L-1" and "former" to "former". We will also correct the wrong comma setting in Fellman et al., (2010) to Fellman et al. (2010).

Reply to RC2

We thank referee #2 for the constructive comments. Please find below our response

1.

In section "Abstract", the last sentence highlight important findings "We recommend fast-freezing with liquid nitrogen for preservation of bulk DOC concentrations of samples from terrestrial sources, whereas immediate measuring is preferable to preserve spectroscopic properties of DOM." However, the last part of the sentence was also suggested by the study of Santos et al. (2010) for bulk deposition samples (rainwater samples), which show that such study should be used in the discussion of the present manuscript.

We will consider and cite Santos et al (2010) in the revised version of our manuscript (see point 3 and 7).

2.

In section "1 Introduction", page 2, reformulate the sentence "In addition to cDOM in samples from aqueous systems, water-extractable soil organic matter and cDOM in soil pore water samples (Otero et al., 2007; Hur et al., 2014; Traversa et al., 2014) were investigated using EEMs plus PARAFAC as well as isolated humic substances from soil and litter (Kalbitz et al., 1999; D'Orazio et al., 2014)." The study of Otero et al. (2007) did not used the EEMs plus PARAFAC as well as isolated humic substances from soil and litter.

Thank you for the hint. We will reorganized the introduction and rephrase the sentence into:

"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)."

(page 14, line 27-31)

3.

In section "1 Introduction", page 3, I suggest to add also the reference of Santos et al. (2010) to the following sentence "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 (Spencer and Coble, 2014)".

We strongly agree with the reviewer on the importance of immediate filtration, as well as cold and dark storage. In our experiment samples were immediately filtered and stored cold in the dark. The reference Santos et al. (2010) will be added in the respective sentence in front of "Spencer and Coble, 2014".

(page 15, line 2)

4.

In section "2 Material and methods", subsection "2.2 Sampling and sample preparation", page 4, the first and fourth sentences seems to be contradictory, because it is presented that samples were collected on 17 and 18 June 2014, and then is presented that bottles were biweekly used. Please, clarify.

The samples for the cDOM storage experiment described in this manuscript were taken within a biweekly sampling routine of above and belowground water samples. It takes two days to collect samples from all research sites. Therefore we state in the Materials and Methods section that samples were taken on the 17th and 18th of June. For the in-field sample collection we use the same PE bottles for the same sample every 14 days.

We will rephrase the respective paragraph into:

"For the experiments, we collected solution samples from five forest and three grassland plots on 17 and 18 June 2014 within a bi-weekly 2 day sampling routine of above and below-ground water samples in the DFG priority programm "Biodiversity Exploratories". Together we collected 27 samples for the freezing experiment including six throughfall (TF), five stemflow (SF), five forest litter leachate (LL) as well as six top- and five subsoil solution samples. Volume-weighted composite samples were produced from replicated samplers of the same type (e.g. throughfall collectors, shallow suction cups) of one plot for the experiment in "aged" 500 ml PE bottles. The bottles were bi-weekly used in the field for the same samples, after washing in the dishwasher and with deionised water."

(page 16, line 23-29)

5.

In section "2 Material and methods", subsection "2.2 Sampling and sample preparation", page 4: why were not used glass bottles and vials to store the samples? Glass material should be used to avoid contaminations. Blanks of procedure were performed?

Since we froze the samples, glass bottles could not be used because they could break when the water sample expands during the freezing process. For collecting the samples, we used aged HDPE bottles, which do not release detectable amounts of DOM according to our experience. We had blanks for all steps of the experiment. For all of them, no detectable DOC release (concentrations) and fluorescence was detectable. We will add this information to the Materials and Methods section of the revised manuscript and the data in table form in the supporting information.

(page 17, line 23; supporting information S2)

6.

In section "3 Results", subsection "3.2 PARAFAC fluorescence components", reformulate the sentence "The maximum increase was +10% (-18◦C) and +12% (N2)". Remove the plus sign and extend the sentence with the types of freeze.

Thank you for the indication, we will rephrase the sentence accordingly.

(page 20, line 29-30)

7.

In section "4 Discussion", the reference of Santos et al. (2010) should be used together with the reference to Spencer et al. (2007) to the following sentence "This is in contrast to the results of Spencer et al. (2007), which could be related to similar fluorescence characteristics, but different chemical composition of proteinaceous fluorescence material from aquatic sources and the solutions from terrestrial ecosystems tested in this study."

The reference Santos et al. (2010) will be added. We will rephrase the sentence into:

"This is in contrast to the results of Spencer et al. (2007) and Santos et al. (2010), which could be related to similar fluorescence characteristics, but different chemical composition of proteinaceous fluorescence material from aquatic sources, rainwater and the solutions from terrestrial ecosystems tested in this study."

(page 21, line 31)

Reply to RC3

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

1.

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 N_2, 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 ($SUVA_{254}$). They recommended freezing as an acceptable storage method for freshwater samples with low DOC concentration and/or low $SUVA_{254}$ 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) conducted 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 insights 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.

(page 13, line 25 to page 16, line 8)

2.

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, $R^2 = 0.05184$, $p = 0.2401$).

(page 16, line 23-29)

3.

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 include a short discussion of this in the revised manuscript.

(page 22, line 12-15)

4.

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 absolute 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 factor:

Als Fig.1 eingefügt

	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*

The interaction between the freezing treatment (either at -18°C or with liquid N_2) 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 N_2 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.

(page 19 line 26-29 + page 20, line 17-20)

5.

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.

6.

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.

(page 21, line11-12 + line 17-18)

7.

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.

(page 13, line 12-15)

8.

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. (%)".

(page 30)

Fast-freezing with liquid nitrogen preserves bulk dissolved organic matter concentrations, but not its composition

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Abstract. Freezing can affect concentrations and spectroscopic properties of dissolved organic matter (DOM) in water samples. Nevertheless, water samples are regularly frozen for sample preservation. In this study we tested the effect of different freezing methods (standard freezing at -18°C and fast-freezing with liquid nitrogen) on DOM concentrations measured as organic carbon (DOC) concentrations and on spectroscopic properties of DOM from different terrestrial ecosystems (forest and grassland). Fresh and differently frozen throughfall, stemflow, litter leachate and soil solution samples were analyzed for DOC concentrations, UV-vis absorption and fluorescence excitation-emission matrices combined with parallel factor analysis (PARAFAC). Fast-freezing with liquid nitrogen prevented a significant decrease of DOC concentrations observed after freezing at -18°C. Nonetheless, the share of PARAFAC components 1 (EXmax <250 nm (340 nm), EMmax: 480 nm) and 2 (EXmax: 335 nm, EMmax: 408 nm) to total fluorescence and the humification index (HIX) decreased after both freezing treatments, while the shares of component 3 (EXmax: <250 nm (305 nm), EMmax: 438 nm) as well as $SUVA_{254}$ increased. The contribution of PARAFAC component 4 (EXmax: 280 nm, EMmax: 328 nm) to total fluorescence was not affected by freezing. We recommend fast-freezing with liquid nitrogen for preservation of bulk DOC concentrations of samples from terrestrial sources, whereas immediate measuring is preferable to preserve spectroscopic properties of DOM**.**

Keywords

freezing, dissolved organic matter, fluorescence, absorption

1 Introduction

~~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. Optical methods have been used to assess origin, dynamics, biogeochemical cycling and fate of cDOM in a wide range of marine and freshwater systems (e.g., Murphy et al., 2008; Yamashita et al., 2010; Stedmon and Markager, 2005; Stedmon et al., 2007; Graeber et al., 2012). In addition to cDOM in samples from aqueous systems, water extractable soil organic matter and cDOM in soil pore water samples (Otero et al., 2007; Hur et al., 2014; Traversa et al., 2014) were investigated using EEMs plus PARAFAC as well as isolated humic substances from soil and litter (Kalbitz et al., 1999; D'Orazio et al., 2014). 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~~

~~physico-chemical and biological boundary conditions, so that artefacts caused by sample storage or sample pre-treatment may easily be produced. 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 (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). Studies on marine waters (Del Castillo and Coble, 2000; Yamashita et al., 2010; Conmy et al., 2009) showed only a small freezing effect on DOM fluorescence characteristics, but tests with a variety of freshwater samples produced significant changes in DOM composition, however with inconsistent results in terms of direction of the changes (e.g., Fellman et al., 2008; Yamashita et al., 2010; Spencer et al., 2007).~~

~~Apparently the impact of sample preservation like freezing is 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. Here we investigate 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). 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 conditions like viscosity (Zaritzky, 2006) and pH (Shafique et al., 2011) 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. We tested in how far fast freezing with liquid nitrogen might prevent concentration and partitioning effects and minimize the time for conformational 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.~~

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 artifacts 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 N_2, 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 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 254 nm ($SUVA_{254}$). They recommended freezing as an acceptable storage method for freshwater samples with low DOC concentration and/or low $SUVA_{254}$ 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) conducted 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 insights 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.

2 Material and methods

2.1 Study sites

The study was conducted on experimental plots in the Schorfheide Chorin Exploratory of the German "Biodiversity Exploratories", which were established as platform for large-scale and long-term functional biodiversity research (Fischer et al., 2010). The experimental plots are located in a young glacial landscape in NE Germany with an annual mean temperature of 8 to 8.5°C and an annual mean precipitation of 500 to 600 mm. The forest plots are dominated either by pine (*Pinus sylvestris* L.) or beech (*Fagus sylvatica* L.) on Cambisols (IUSS working group WRB, 2014). The grassland plots are meadows, pastures and mown pastures on Histosols, Gleysols and Cambisols.

2.2 Sampling and sample preparation

~~For the experiments, five composite samples from grassland sites and 22 composite samples from forest sites were collected on 17 and 18 June 2014. Together we had 27 samples including six throughfall (TF), five stemflow (SF), five forest litter leachate (LL) as well as six top- and five subsoil solution samples. Volume-weighted composite samples were produced in "aged" 500 ml PE bottles by merging samples of the same sample type per site. The bottles were biweekly used for the same samples on a routine sampling campaign of above- and below ground water at the biodiversity exploratory sites, after washing in the dishwasher and with deionised water.~~ For the experiments, we collected solution samples from five forest and three grassland plots on 17 and 18 June 2014 within a bi-weekly 2 day sampling routine of above and below-ground water samples in the DFG priority programm "Biodiversity Exploratories". Together we collected 27 samples for the freezing experiment including six throughfall (TF), five stemflow (SF), five forest litter leachate (LL) as well as six top- and five subsoil solution samples. Volume-weighted composite samples for the experiment were produced from replicated samplers of the same type (e.g. throughfall collectors, shallow suction cups) of one plot in "aged" 500 mL PE bottles. The bottles were bi-weekly used in the field for the same samples, after washing in the dishwasher and with deionised water. -TF was sampled with funnel-type collectors (diameter 0.12 m, polyethylene) 0.3 m above soil surface. We pooled five replicates at grassland and 20 replicates arranged in two lines of 10 samplers in a cross shaped form at forest sites. To minimize

alterations of the sample and contamination such as evaporation, photo chemical reactions and algae growth, the sampling bottles were wrapped with aluminium foil and closed with a 1.6 mm polyester mesh and a table-tennis ball. SF was sampled with sliced polyurethane hoses (diameter: 0.04 m) as a collar sealed with a polyurethane-based glue to the bark of three trees per site at approximately 1.5 m height and connected with a polypropylene (PP) or polyethylene (PE) barrel via a PE tube. LL was collected with three zero-tension lysimeters per site (280 cm^2 sampling area) consisting of polyvinyl chloride plates covered with a PE net (mesh width 0.5 mm) connected with PE hoses to 2 L PE bottles stored in a box below ground. We sampled soil solution with nylon membrane (0.45 µm) suction cups (ecoTech, Germany). Three samplers were installed beneath the A horizon (Top) at approximately 10 cm depth. Another three were installed in the B horizon (Sub) in approximately 50 cm depth in the forest plots and 60 or 70 cm depth in the grassland sites. Suction cups were connected to 2 L PE bottles in an insulated aluminium box placed into a soil pit. Soil water was extracted by applying a vacuum of 50 kPa to the PE bottles with an electric pump after each sampling.

After mixing, the samples were transported on ice to the laboratory and stored overnight at 5°C. We measured pH (Knick, Germany) and electrical conductivity (WTW, Germany) in all samples prior to filtration through ~ 0.7 µm glass microfiber filters (Whatman GF/F). The filters were washed with 100 mL deionised water and 10 mL of sample before sample filtration. The filtered sample was split in three aliquots for different preservation treatments: i) no preservation (fresh) for which samples were stored at 5°C in the dark and DOC concentrations were measured 24 h after sampling while fluorescence as well as absorbance were measured within 48 h; ii) preservation by freezing for which the samples were stored at -18°C for four weeks, and iii) fast-freezing with liquid nitrogen (N_2), for which 1 mL sample aliquots were filled in pre-rinsed 15 mL (5 mL sample) PP falcon tubes, dipped in liquid nitrogen for 30 s and then stored at -18 °C for 42 days. Fresh samples and samples frozen at -18°C were stored in 20 ml PE scintillation vials (NeoLab) that were pre-rinsed with 5 ml sample before filling. Fluorescence, absorbance and DOC concentration from all frozen samples were measured after defrosting over night at 5 °C in the dark. For all preparation steps and treatments control samples of ultrapure water (EVOQUA, Germany) were analyzed, showing no release of DOM (DOC concentration and DOM fluorescence) from laboratory equipment.

2.3 Laboratory analysis

We measured the concentration of DOC as non-purgeable organic carbon on a Shimadzu TOC-5050A (Duisburg, Germany) with a limit of quantification of 2 mg C L^{-1}. Absorption spectra of DOM were scanned at wavelength from 400 to 600 nm using a Lambda 20 UV-vis spectrometer (Perkin Elmer, USA) and a 1 cm quartz cuvette. Absorbance measurements were baseline corrected using ultrapure water. All fluorescence EEMs were measured on a Hitachi F-4500 fluorescence spectrometer (Hitachi, Japan) directly after absorption measurement in the same cuvette. We measured excitation from 240 to 450 nm (5 nm steps) and emission from 300 to 600 nm (2 nm steps) with a slit width of 5 nm and scan speed 12000 nm min^{-1}. We corrected our EEMs according to the protocol from Murphy (2010) with the fdomcorrect function in the drEEM toolbox (version 2.0) of Murphy et al. (2013) using Matlab (Version Matlab2011b, The MathWorks Inc.). We used the

supplies provided by the manufacturer for the excitation and emission correction factors. We measured ultrapure water fluorescence spectra for blank correction and to convert EEMs to Raman units by normalizing them to the area under the Raman peak at 350 nm excitation wavelength (Lawaetz and Stedmon, 2009). In order to apply the inner-filter correction of Lakowicz (2006) integrated in the drEEM toolbox, all aliquots were diluted with ultrapure water to achieve an absorption of <0.3 at 254 nm (Ohno, 2002). For this reason, not all treatments of one sample were diluted with the same dilution factor. To test the possible influence of different dilutions on the pH-related changes in fluorescence (Patel-Sorrentino et al., 2002; Baker et al., 2007), dilution series with samples (n = 14) from the same plots and same sample types but with different sampling dates where measured for pH, absorption and fluorescence according to the protocol described above. We compared the differences of 31 dilutions and calculated the mean absolute deviation (MAD). These were compared to the MAD of measurement precision, determined by analysing 11 samples in three replications. For the PARAFAC components %C1, %C2 and %C3 and $SUVA_{254}$ the MAD caused by dilution were less or equal than the precision MAD, so that there was no influence of dilution on the three humic-like components and the specific UV absorbance at 254 nm. For %C4 and HIX the effect of dilution could exceed the precision of fluorescence measurements. For detailed information see supporting information.

2.4 Spectroscopic indices and PARAFAC modelling

Based on the absorbance spectra, we calculated specific ultraviolet absorbance ($SUVA_{254}$) as the absorbance at 254 nm divided by the DOC concentration. The $SUVA_{254}$ is reported in $L\,mg^{-1}\,m^{-1}$, and is associated with bulk aromaticity (Weishaar et al., 2003). Moreover, we calculated the humification index (HIX) from fluorescence EEMs (Ohno, 2002). The HIX ranges from 0 to 1 and allows characterizing samples based on their degree of DOM humification.

In addition to the calculation of indices, we used parallel factor analysis (PARAFAC) to mathematically decompose the trilinear data of the EEMs into fluorescence components of DOM (Stedmon et al., 2003). Further pre-processing steps of EEMs (smoothing of Rayleigh and Raman scatter and sample normalization), as well as the PARAFAC analysis were conducted with the drEEM toolbox (version 2.0, Murphy et al., 2013). We chose a four component PARAFAC model (components referred as C1-C4), visually checked the randomness of residuals and the component spectral loadings, split-half validated the model and generated the best fit by random initialization. For comparison in statistical analysis we used the relative percentage distribution of the four PARAFAC components (% of the sum of total peak fluorescence of all PARAFAC components), so that percentage values for the components will be given as %C1 to %C4.

2.5 Statistical analysis

The DOM composition variables used for statistical analysis were the PARAFAC components %C1 to %C4, the spectroscopic indices HIX and $SUVA_{254}$, as well as the DOC concentration. For all statistical analysis the variables were scaled and centred. We conducted a pair-wise (samples as strata) permutational multivariate analysis of variance

(PERMANOVA) with DOC concentrations of the fresh samples as factor based on Euclidean distances in R (Oksanen et al., 2015; R core team, 2015). The adonis function was used to assess the influence of sample preparation (fresh, frozen, fast-freezing) and of the initial DOC concentration on DOM variables. To investigate preservation effects on single variables we conducted linear mixed-effect models (sometimes called multi-level models, lme function, Linear and Nonlinear Mixed Effects Models package for R, Pinheiro et al., 2015) with samples as random intercept on each of the DOM composition variables. These were used instead of simple linear models or ANOVAs, since we could not expect the same intercept for all samples due to different sample concentrations. To test the influence of the initial DOC concentration on single preservation treatments we performed Spearman Rank Order Correlation. To assess the influence of sample type (TF, SF, LL, Top or Sub) on the relative change of DOM composition due to fast-freezing with liquid nitrogen or freezing at -18°C in relation to the measurement of fresh, cooled samples, we used an ANOVA with the sample type as fixed factor (aov function in R). To remove sample concentration-related effects and to calculate relative changes, the differences between the two preservations (either fast-freezing or freezing at -18°C) relative to the measurements of fresh samples were calculated for each sample before the ANOVA. This was only done for variables, for which we found strong, significant effects with the linear mixed-effect models.

3 Results

3.1 DOM concentrations

The samples covered a wide range of DOC concentrations (Fig. 1a, b). Fresh TF samples showed the lowest concentrations ranging from 5 to 17 mgC L^{-1}, SF samples had the highest DOC concentrations ranging from 12 to 138 mgC L^{-1} (Fig. 1b). High concentrations up to 75 mgC L^{-1} were also found for LL samples, but average values were smaller than for SF (Fig. 1b). In the mineral soil, concentrations decreased from 13 to 124 mgC L^{-1} in topsoil samples to 9 to 47 mgC L^{-1} in subsoil samples.

We found a significant treatment effect (linear mixed-effect models (lme), $p<0.05$) on DOC concentration when comparing the fresh and frozen samples (Fig. 1c). In 24 of 27 samples DOC concentrations decreased after freezing at -18°C and subsequent thawing, with an average change of - 1.6 mgC L^{-1} or - 6% respectively. The maximum decrease that was found equalled - 6 mgC L^{-1} and - 25%, respectively. In contrast to freezing at -18°C, fast-freezing with liquid nitrogen did not result in significant changes (lme, $p>0.05$) of DOC concentrations (Fig. 1c). This different behaviour between normal freezing and fast-freezing was also found for the influence of the initial DOC concentration on changes of DOM properties. Only the -18°C treatment showed a significant correlation (Spearmans rank r = -0.447, p = 0.0194), indicating a larger decrease of DOC concentrations due to freezing for samples with higher initial DOC concentrations.

3.2 PARAFAC fluorescence components

The analysis of fluorescence spectra using PARAFAC resulted in four components that were characterized according to the review of Fellman et al.~~,~~ (2010) (Table 1). C1 exhibited its main excitation maximum at < 250 nm, a secondary maximum at 340 nm and an emission maximum at 480 nm and was described as UVA humic-like fluorophore with a terrestrial source and a high molecular weight (Murphy et al., 2006; Stedmon et al., 2003; Shutova et al., 2014; Fellman et al., 2010). C2 had a maximum excitation at 335 nm and an emission maximum at 408 nm and was named also UVA humic-like, but associated with low molecular weight (Murphy et al., 2006; Fellman et al., 2010; Stedmon et al., 2003). C3 was defined by an excitation maximum at < 250 nm, a secondary maximum at 305 nm and an emission maximum at 438 nm. This component dominated fulvic acid fractions of humic substances (Santín et al., 2009; He et al., 2006). Finally, C4 was characterized by its excitation maximum at 280 nm and an emission maximum at 328 nm and was classified as tryptophan-like, as its fluorescence resembles free tryptophan. Therefore, this component was associated with free or bound proteins (Fellman et al., 2010).

We found different distributions of PARAFAC components for different sample types (Fig. 2). The contribution of %C1 to the total fluorescence increased from TF over SF to LL and then decreased again from LL to Sub (Fig. 2), while %C2 showed just the opposite trend. In contrast, %C3 tended to increase from TF to Sub, whereas %C4 showed a decreasing trend (Fig. 2).

The conducted PERMANOVA was highly significant ($p < 0.001$), indicating that the preservation significantly affects the DOM composition. The interaction between treatment and initial DOC concentration of the fresh treatment explains a reasonable part of the variance ($R^2 = 0.14$) and is highly significant ($p < 0.001$). Therefore the original DOC concentration of the fresh sample well explains the variable strength of the treatment effect.

Similar changes in component distribution were found as a consequence of freezing at -18°C and fast-freezing with liquid nitrogen (Fig. 3). We observed a significant (lme, $p<0.05$) decrease in all samples for the relative fraction of the humic-like components %C1 and %C2 after freezing at -18°C and fast-freezing compared to the fresh control samples (Fig. 3a, b). The contribution of %C1 to the total fluorescence decreased on average by -3% with maximum changes of -5% for freezing at -18°C and -6% for fast-freezing with liquid nitrogen. The average decrease of %C2 was -3% and the maximum -8% for both treatments.

In contrast to %C1 and %C2, the share of %C3 to the total fluorescence intensity increased upon freezing (Fig. 3e, f). All samples frozen at -18°C showed an increase in the relative intensity of the %C3 signal, with an average increase of +6% for both treatments. ~~The maximum increase was +10% (-18°C) and +12% (N_2).~~ The maximum increase was 10% (freezing at -18°C) and 12% (freezing with liquid N_2). No significant effects of sample preservation (lme, $p>0.05$) were found for %C4, the protein-like-component (Fig. 3g, h).

3.3 Aromaticity and humification index

We found $SUVA_{254}$-values ranging from 1.1 L mg^{-1} m^{-1} up to 4.5 L mg^{-1} m^{-1} for fresh samples (Fig. 4a, b). Samples frozen at -18°C and fast-frozen samples showed a significant increase (lme, $p<0.05$) of their $SUVA_{254}$ (Fig. 4c). The average change was +0.4 L mg^{-1} m^{-1} equivalent to +20% for samples frozen at -18°C and +0.5 L mg^{-1} m^{-1} equivalent to +24% for samples that were fast-frozen with liquid nitrogen.

The humification index of the freshly measured samples ranged from 0.806 to 0.931 in TF and SF samples and from 0.849 to 0.975 for Sub, Top and LL samples (Fig. 5a, b). We found a significant decrease (lme, $p<0.05$) of the HIX when comparing the freshly measured samples with the frozen and the fast-frozen samples (Fig. 5c). The average change was -0.016 or -2% for samples frozen at -18°C and -0.020 or -2% for samples fast-frozen with liquid nitrogen. The maximum decrease was -0.128 or -15% for -18°C samples and -0.076 or -8% for liquid nitrogen samples (Fig. 5 c, d, e, f).

4 Discussion

We found that freezing at -18°C significantly reduced DOC concentrations across all sample types and that the effect is higher with higher initial DOC concentrations. This is in line with results of Fellman et al. (2008) investigating the effect of freezing and thawing on Alaskan stream water samples. This loss of DOC concentration might be due to aggregation and irreversible particle formation (Giesy and Briese, 1978) induced by partitioning and concentration effects during the freezing process (Belzile et al., 2002; Xue et al., 2015). Indeed, our results indicated that fast-freezing with liquid nitrogen can prevent significant reductions of bulk DOC ~~concentrations, mainly because topsoil and subsoil solution DOC concentrations were less affected than during freezing at -18°C.~~ for samples with a large range of DOM concentrations.

In contrast to effects on DOC concentrations, we found similar significant effects of fast-freezing as well as freezing at -18°C on the chromophoric humic fraction of DOM (PARAFAC components, HIX and $SUVA_{254}$). The increase of aromaticity as indicated by higher $SUVA_{254}$ values indicates a stronger removal of non-aromatic DOM during freezing and thawing. On the other hand, the decrease in the HIX suggests a preferential removal of humified cDOM. One potential explanation for the fact that fast-freezing in liquid nitrogen resulted in significant changes of DOM fluorescence properties, but only small changes of bulk DOC concentrations, is that cDOM reacted stronger to freezing and thawing than the remaining DOM so that spectroscopic properties were affected, but bulk DOC concentrations were not. Fast freezing may have failed to prevent changes of cDOM composition because i) cDOM changes occurred not only during the freezing process (-18°C or -196°C in liquid nitrogen), but also in frozen state at -18°C in the freezer during storage or ii) cDOM was affected by the thawing process that was identical for both freezing treatments. The former might be supported by a recrystallisation of ice crystals in frozen state (Luyet, 1967; Meryman, 2007).

No significant changes of protein-like fluorescence (%C4) due to freezing and thawing were observed. This is in contrast to the results of Spencer et al. (2007) and Santos et al. (2010), which could be related to similar fluorescence characteristics, but

different chemical composition of proteinaceous fluorescence material from aquatic sources and the solutions from terrestrial ecosystems tested in this study.

In our experiment we used relative small sample volumes (fresh, -18°C: 20 mL, N_2: 12 mL) because we commonly keep the volume that is stored frozen as small as possible due to 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.

5 Conclusion

Freezing and thawing affected the DOC concentration, spectral absorption and fluorescence properties of water samples (throughfall, litter leachate and soil solution) from different terrestrial ecosystems (grasslands and forests). In contrast, fast-freezing with liquid nitrogen minimized the changes of bulk DOC concentrations but not the changes of spectroscopic cDOM properties. Different thawing protocols for minimizing sample storage effects on DOM should be tested in future studies. We suggest the use of fast-freezing for preservation of bulk DOC concentrations, especially for highly concentrated samples, when the increased effort and cost of using liquid nitrogen in the field is justified by advantages regarding the minimization of freezing artefacts. To preserve cDOM characteristics of samples from terrestrial sources normal freezing or fast-freezing should be avoided.~~of samples, but normal freezing or fast-freezing should be avoided to preserve cDOM characteristics of samples from terrestrial sources.~~ Instead, filtration, cooling and measurements soon after the sampling should be the method of choice, if possible.

Data availability

The data is available in the supplementary information

Author contribution

L.Th, M.K., and J.S. designed the experiment, L.T.h performed the experiments. All authors analyzed the data and wrote the manuscript.

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References

Baker, A., Elliott, S., and Lead, J. R.: Effects of filtration and pH perturbation on freshwater organic matter fluorescence, Chemosphere, 67, 2035–2043, doi:10.1016/j.chemosphere.2006.11.024, 2007.

Belzile, C., Gibson, J. A. E., and Vincent, W. F.: Colored dissolved organic matter and dissolved organic carbon exclusion from lake ice: Implications for irradiance transmission and carbon cycling, Limnol. Oceanogr., 47, 1283–1293, doi:10.4319/lo.2002.47.5.1283, 2002.

Bolan, N. S., Adriano, D. C., Kunhikrishnan, A., James, T., McDowell, R., and Senesi, N.: Dissolved Organic Matter: Biogeochemistry, Dynamics, and Environmental Significance in Soils, in: Adv. Agron., 1–75, 2011.

Christ, M. and David, M. B.: Fractionation of dissolved organic carbon in soil water: Effects of extraction and storage methods, Communications in Soil Science and Plant Analysis, 25, 3305–3319, doi:10.1080/00103629409369266, 1994.

Conmy, R. N., Coble, P. G., Cannizzaro, J. P., and Heil, C. A.: Influence of extreme storm events on West Florida Shelf CDOM distributions, J. Geophys. Res-Biogeo., 114, n/a-n/a, doi:10.1029/2009JG000981, 2009.

D'Orazio, V., Traversa, A., and Senesi, N.: Forest soil organic carbon dynamics as affected by plant species and their corresponding litters: a fluorescence spectroscopy approach, Plant Soil, 374, 473–484, doi:10.1007/s11104-013-1897-4, 2014.

Del Castillo, C. E. and Coble, P. G.: Seasonal variability of the colored dissolved organic matter during the 1994–95 5NE6 and 5SW6 Monsoons in the Arabian Sea, Deep-Sea Res. PT II, 47, 1563–1579, doi:10.1016/S0967-0645(99)00154-X, 2000.

Edwards, A. C. and Cresser, M. S.: Freezing and Its Effect on Chemical and Biological Properties of Soil: Advances in Soil Science, Stewart, B. A. (Ed.), Springer New York, New York, NY, 59–79, 1992.

Fellman, J. B., D'Amore, D. V., and Hood, E.: An evaluation of freezing as a preservation technique for analyzing dissolved organic C, N and P in surface water samples, Sci. Total Enciron., 392, 305–312, doi:10.1016/j.scitotenv.2007.11.027, 2008.

Fellman, J. B., Hood, E., and Spencer, R. G. M.: Fluorescence spectroscopy opens new windows into dissolved organic matter dynamics in freshwater ecosystems: A review, Limnol. Oceangr., 55, 2452–2462, doi:10.4319/lo.2010.55.6.2452, 2010.

Fischer, M., Bossdorf, O., Gockel, S., Hänsel, F., Hemp, A., Hessenmöller, D., Korte, G., Nieschulze, J., Pfeiffer, S., Prati, D., Renner, S., Schöning, I., Schumacher, U., Wells, K., Buscot, F., Kalko, E. K. V., Linsenmair, K. E., Schulze, E.-D., and Weisser, W. W.: Implementing large-scale and long-term functional biodiversity research: The Biodiversity Exploratories, Basic Appl. Ecol., 11, 473–485, doi:10.1016/j.baae.2010.07.009, 2010.

Giesy, J. P. and Briese, L. A.: Particulate formation due to freezing humic waters, Water Resources Res., 14, 542–544, doi:10.1029/WR014i003p00542, 1978.

Graeber, D., Gelbrecht, J., Pusch, M. T., Anlanger, C., and Schiller, D. von: Agriculture has changed the amount and composition of dissolved organic matter in Central European headwater streams, Sci. Total Environ., 438, 435–446, doi:10.1016/j.scitotenv.2012.08.087, 2012.

He, Z., Ohno, T., Cade-Menun, B. J., Erich, M. S., and Honeycutt, C. W.: Spectral and Chemical Characterization of Phosphates Associated with Humic Substances, Soil Sci. Soc. Am. J., 70, 1741, doi:10.2136/sssaj2006.0030, 2006.

Hur, J., Lee, B.-M., and Shin, K.-H.: Spectroscopic characterization of dissolved organic matter isolates from sediments and the association with phenanthrene binding affinity, Chemosphere, 111, 450–457, doi:10.1016/j.chemosphere.2014.04.018, 2014.

IUSS working group WRB: World reference base for soil resources 2014: International soil classification system for naming soils and creating legends for soil maps, World Soil Resources Reports No. 106, Rome, 2014.

Kalbitz, K., Geyer, W., and Geyer, S.: Spectroscopic properties of dissolved humic substances — a reflection of land use history in a fen area, Biogeochemistry, 47, 219–238, doi:10.1007/BF00994924, 1999.

Lakowicz, J. R.: Principles of Fluorescence Spectroscopy, 3rd ed., Springer Science+Business Media, 2006.

Lawaetz, A. J. and Stedmon C. A.: Fluorescence Intensity Calibration Using the Raman Scatter Peak of Water, Appl. Spectrosc., 63, 936–940, 2009.

Luyet, B.: Various Modes of Recrystallization of Ice, Physics of Snow and Ice proceedings = 雪氷の物理学 論文集, 1, 51–70, 1967.

Meryman, H. T.: Cryopreservation of living cells: principles and practice, Transfusion, 47, 935–945, doi:10.1111/j.1537-2995.2007.01212.x, 2007.

Murphy, K. R., Butler, K. D., Spencer, R. G. M., and Stedmon, C. A., et al.: Measurement of Dissolved Organic Matter Fluorescence in Aquatic Environments: An Interlaboratory Comparison, Environ. Sci. Technol., 44, 9405–9412, doi:10.1021/es102362t, 2010.

Murphy, K. R., Ruiz, G. M., Dunsmuir, William T. M., and Waite, T. D.: Optimized Parameters for Fluorescence-Based Verification of Ballast Water Exchange by Ships, Environ. Sci. Technol., 40, 2357–2362, doi:10.1021/es0519381, 2006.

Murphy, K. R., Stedmon, C. A., Graeber, D., and Bro, R.: Fluorescence spectroscopy and multi-way techniques. PARAFAC, Anal. Methods, 5, 6557, doi:10.1039/c3ay41160e, 2013.

Murphy, K. R., Stedmon, C. A., Waite, T. D., and Ruiz, G. M.: Distinguishing between terrestrial and autochthonous organic matter sources in marine environments using fluorescence spectroscopy, Mar. Chem., 108, 40–58, doi:10.1016/j.marchem.2007.10.003, 2008.

Ohno, T.: Fluorescence Inner-Filtering Correction for Determining the Humification Index of Dissolved Organic Matter, Environ. Sci. Technol., 36, 742–746, doi:10.1021/es0155276, 2002.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O ' Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., and Wagner, H.: vegan: Community Ecology Package, R package version 2.2-1}: http://CRAN.R-project.org/package=vegan, last access: 12 November 2015, 2015.

Otero, M., Mendonça, A., Válega, M., Santos, E. B.H., Pereira, E., Esteves, V., and Duarte, A.: Fluorescence and DOC contents of estuarine pore waters from colonized and non-colonized sediments: Effects of sampling preservation, Chemosphere, 67, 211–220, doi:10.1016/j.chemosphere.2006.10.044, 2007.

Patel-Sorrentino, N., Mounier, S., and Benaim, J. Y.: Excitation–emission fluorescence matrix to study pH influence on organic matter fluorescence in the Amazon basin rivers, Water Res., 36, 2571–2581, doi:10.1016/S0043-1354(01)00469-9, 2002.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R core team: {nlme}: Linear and Nonlinear Mixed Effects Models, R package version 3.1-120: http://CRAN.R-project.org/package=nlme, last access: 12 November 2015, 2015.

R core team: R: A language and environment for, R Foundation for Statistical Computing, Vienna, Austria, 2015.

Santín, C., Yamashita, Y., Otero, X. L., Álvarez, M., and Jaffé, R.: Characterizing humic substances from estuarine soils and sediments by excitation-emission matrix spectroscopy and parallel factor analysis, Biogeochemistry, 96, 131–147, doi:10.1007/s10533-009-9349-1, 2009.

Santos, P., Otero, M., Santos, E., and Duarte, A. C.: Molecular fluorescence analysis of rainwater: Effects of sample preservation, Talanta, 82, 1616–1621, doi:10.1016/j.talanta.2010.07.048, 2010.

Shafique, U., Anwar, J., Munawar, M. A., Zaman, W.-u., Rehman, R., Dar, A., Salman, M.Saleem, M., Shahid, N., Akram, M., Naseer, A.,and Jamil, N..: Chemistry of ice: Migration of ions and gases by directional freezing of water, Arabian Journal of Chemistry, -, doi:10.1016/j.arabjc.2011.02.019, 2011.

Shutova, Y., Baker, A., Bridgeman, J., and Henderson, R. K.: Spectroscopic characterisation of dissolved organic matter changes in drinking water treatment: From 5PARAFAC6 analysis to online monitoring wavelengths, Water Res., 54, 159–169, doi:10.1016/j.watres.2014.01.053, 2014.

Spencer, R. G., Bolton, L., and Baker, A.: Freeze/thaw and pH effects on freshwater dissolved organic matter fluorescence and absorbance properties from a number of UK locations, Water Res., 41, 2941–2950, doi:10.1016/j.watres.2007.04.012, 2007.

Spencer, R. G. and Coble, P. G.: Sampling Design for Organic Matter Fluorescence Analysis, in: Aquatic Organic Matter Fluorescence, 1st ed., Coble, P. G., Lead, J. R., Baker, A., Reynolds, D. M., and Spencer, R. G. (Eds.), Cambridge environmental chemistry series, Cambridge University Perss, 125–146, 2014.

Stedmon, C. A. and Markager, S.: Tracing the production and degradation of autochthonous fractions of dissolved organic matter using fluorescence analysis, Limnol. Oceangr., 50, 1415–1426, doi:10.4319/lo.2005.50.5.1415, 2005.

Stedmon, C. A., Markager, S., and Bro, R.: Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy, Mar. Chem., 82, 239–254, doi:10.1016/S0304-4203(03)00072-0, 2003.

Stedmon, C. A., Thomas, D. N., Granskog, M., Kaartokallio, H., Papadimitriou, S., and Kuosa, H.: Characteristics of Dissolved Organic Matter in Baltic Coastal Sea Ice: Allochthonous or Autochthonous Origins?, Environ. Sci. Technol., 41, 7273–7279, doi:10.1021/es071210f, 2007.

Sun, S.-Q., Cai, H.-Y., Chang, S. X., and Bhatti, J. S.: Sample storage-induced changes in the quantity and quality of soil labile organic carbon, Scientific Reports, 5, 17496 EP -, 2015.

Traversa, A., D'Orazio, V., Mezzapesa, G. N., Bonifacio, E., Farrag, K., Senesi, N., and Brunetti, G.: Chemical and spectroscopic characteristics of humic acids and dissolved organic matter along two Alfisol profiles, Chemosphere, 111, 184–194, doi:10.1016/j.chemosphere.2014.03.063, 2014.

Weishaar, J. L., Aiken, G. R., Bergamaschi, B. A., Fram, M. S., Fujii, R., and Mopper, K.: Evaluation of Specific Ultraviolet Absorbance as an Indicator of the Chemical Composition and Reactivity of Dissolved Organic Carbon, Environ. Sci. Technol, 37, 4702–4708, doi:10.1021/es030360x, 2003.

Xue, S., Wen, Y., Hui, X., Zhang, L., Zhang Z., Wang, J., and Zhang, Y..: The migration and transformation of dissolved organic matter during the freezing processes of water, J. Environ. Sci., 27, 168–178, doi:10.1016/j.jes.2014.05.035, 2015.

Yamashita, Y., Cory, R. M., Nishioka, J., Kuma, K., Tanoue, E., and Jaffé, R.: Fluorescence characteristics of dissolved organic matter in the deep waters of the Okhotsk Sea and the northwestern North Pacific Ocean Deep-Sea Res. PT II, 57, 1478–1485, doi:10.1016/j.dsr2.2010.02.016, 2010.

Zaritzky, N.: Physical-Chemical Principles in Freezing, in: Handbook of frozen food processing and packaging, Da-Wen Sun (Ed.), CRC Press, 3–32, 2006.

Tables

Table 1: Characteristics of PARAFAC components based on Fellman et al., 2010

Component	Maximum exitation wavelength(EX_{max}) (nm)	Maximum emission wavelength (EM_{max}) (nm)	Description
C1	<250 (340)	480	humic-like, terrestrial
C2	335	408	humic-like
C3	<250 (305)	438	fulvic-acid-type
C4	280	328	tryptophan-like

Figure captions

Figure 1: Absolute DOC concentrations (measured in fresh samples) and changes of DOC concentrations after freezing (-18°C) and fast-freezing with liquid nitrogen; a, c, e: all samples (n= 27); b, d, f: ordered by sample type (throughfall (TF) n=6, stemflow (SF) n=5, litter leachate (LL) n=5, top soilsolution (Top) n=6, sub-soilsolution (Sub) n=5); gray dashed line: analytical reproducibility; *** significant changes (linear mixed models (lme), $p<0.05$); Boxplots: solid line: median, dashed line: mean

Figure 2: Mean distribution of PARAFAC components %C1-%C4 for different sample types

Figure 3: Changes of relative distribution of PARAFAC components after freezing (-18°C) and fast-freezing with liquid nitrogen; a, c, e, g: all samples (n=27); b, d, f, h ordered by sample type (throughfall (TF) n=6, stemflow (SF) n=5, litter leachate (LL) n=5, top soilsolution (Top) n=6, sub-soilsolution (Sub) n=5); gray dashed line: analytical reproducibility; *** significant changes (linear mixed models (lme), $p<0.05$) ;Boxplots: solid line: median, dashed line: mean

Figure 4: Absolute values (measured in fresh samples) and changes of SUVA254 after freezing (-18°C) and fast-freezing with liquid nitrogen; a, c, e: all samples (n= 27); b, d, f: ordered by sample type (throughfall (TF) n=6, stemflow (SF) n=5, litter leachate (LL) n=5, top soilsolution (Top) n=6, sub-soilsolution (Sub) n=5); gray dashed line: analytical reproducibility; *** significant changes (linear mixed models (lme), $p<0.05$); Boxplots: solid line: median, dashed line: mean

Figure 5: Absolute values (measured in fresh samples) and changes of HIX after freezing (-18°C) and fast-freezing with liquid nitrogen; a, c, e: all samples (n= 27); b, d, f: ordered by sample type (throughfall (TF) n=6, stemflow (SF) n=5, litter leachate (LL) n=5, top soilsolution (Top) n=6, sub-soilsolution (Sub) n=5); gray dashed line: analytical reproducibility; *** significant changes (linear mixed models (lme), $p<0.05$); Boxplots: solid line: median, dashed line: mean

Figures

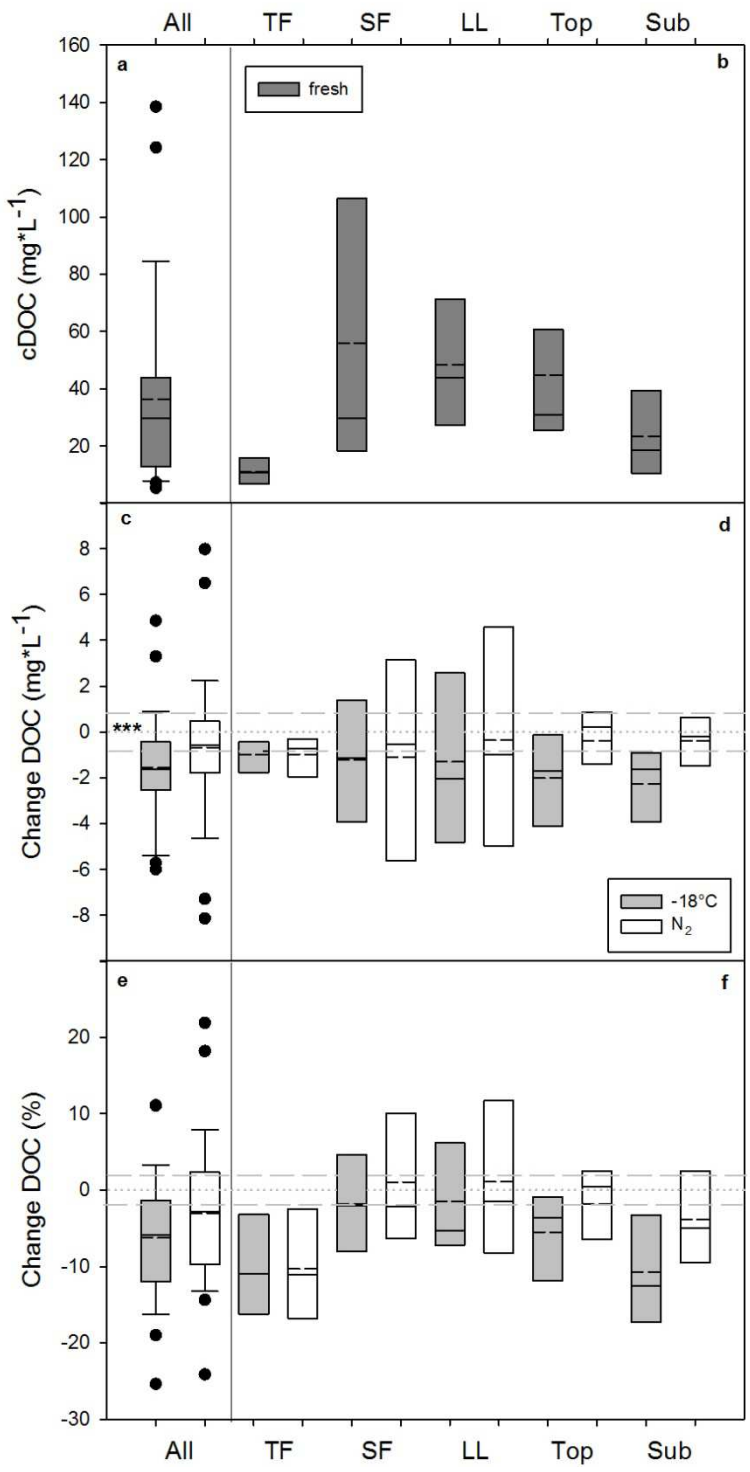
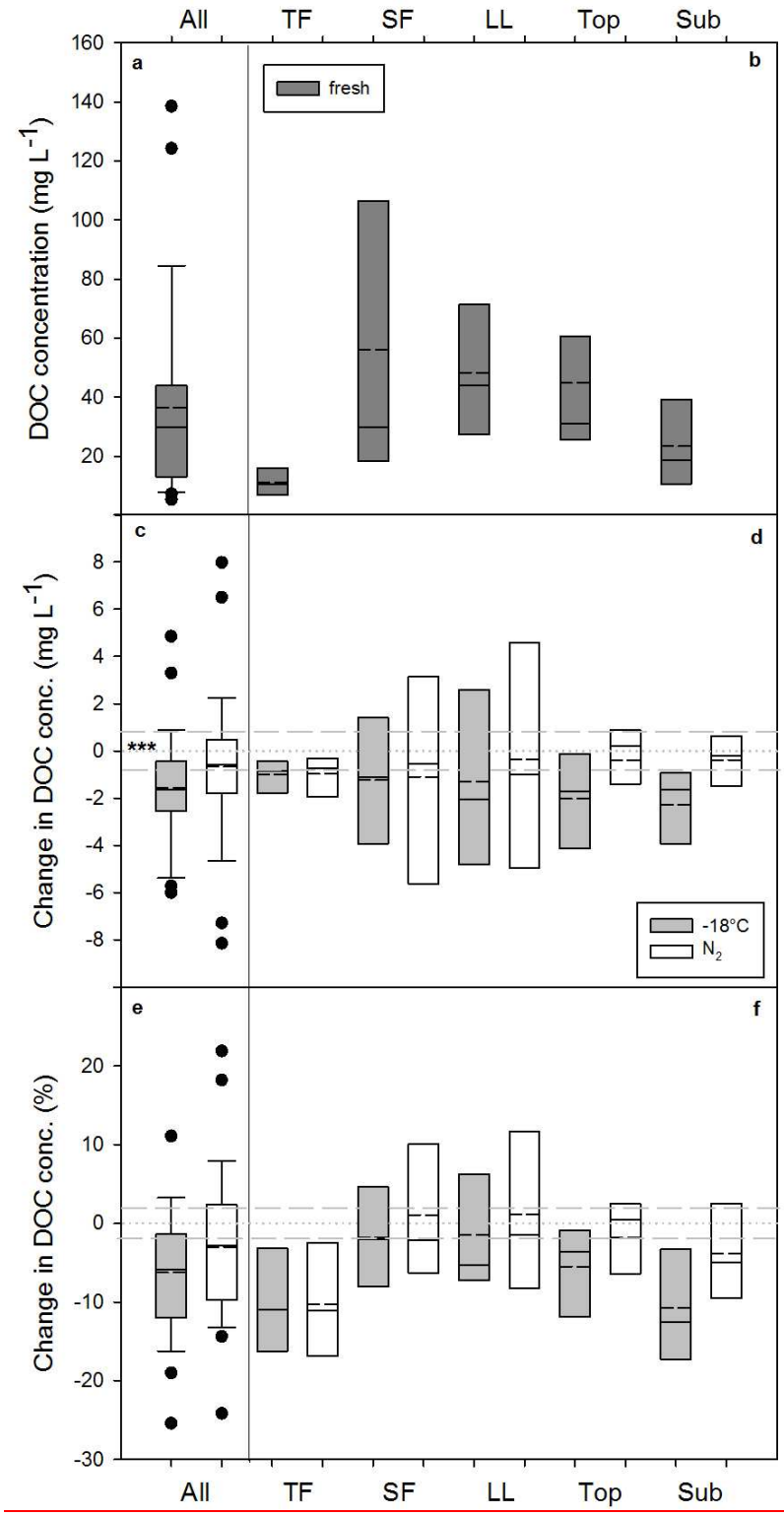


Figure 1


All
TF
SF
LL
Top
Sub
a
b
fresh
DOC concentration (mg L-1)
c
d
***
Change in DOC conc. (mg L-1)
-18°C
N2
e
f
Change in DOC conc. (%)

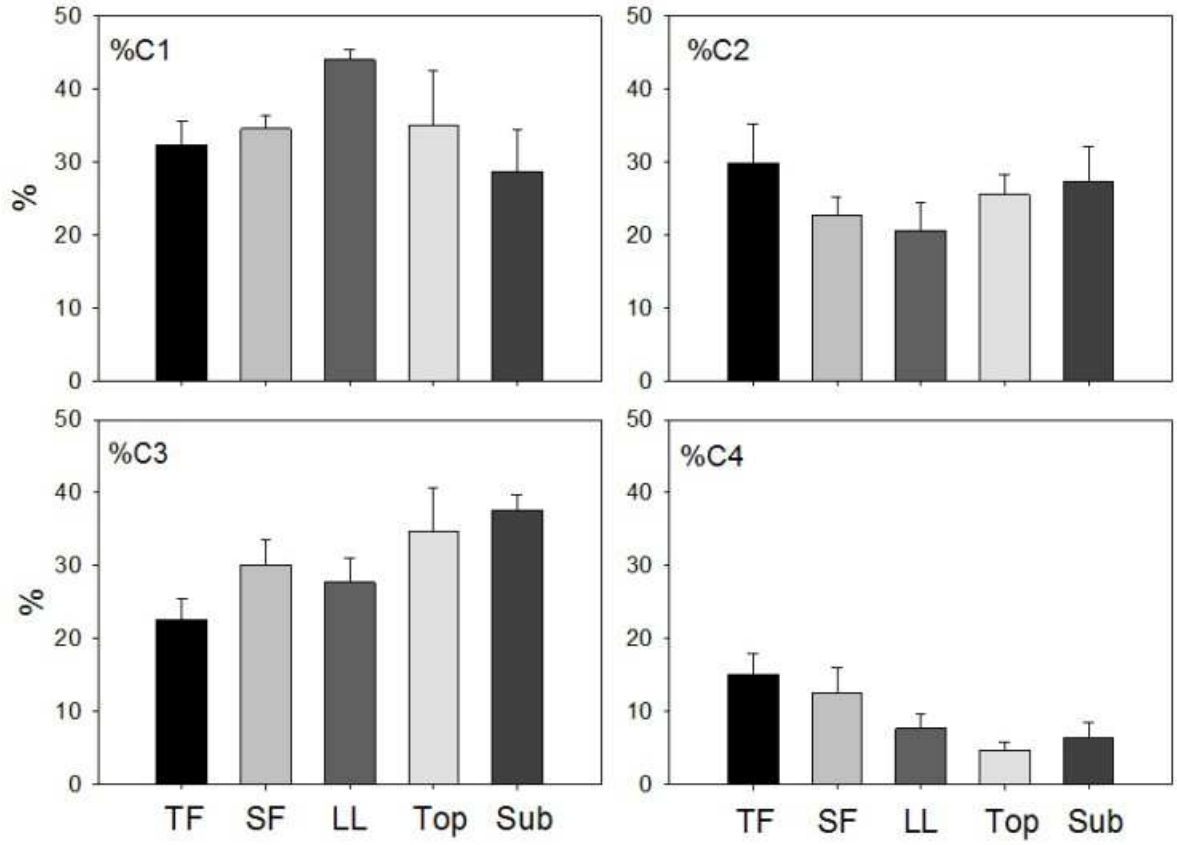


Figure 2

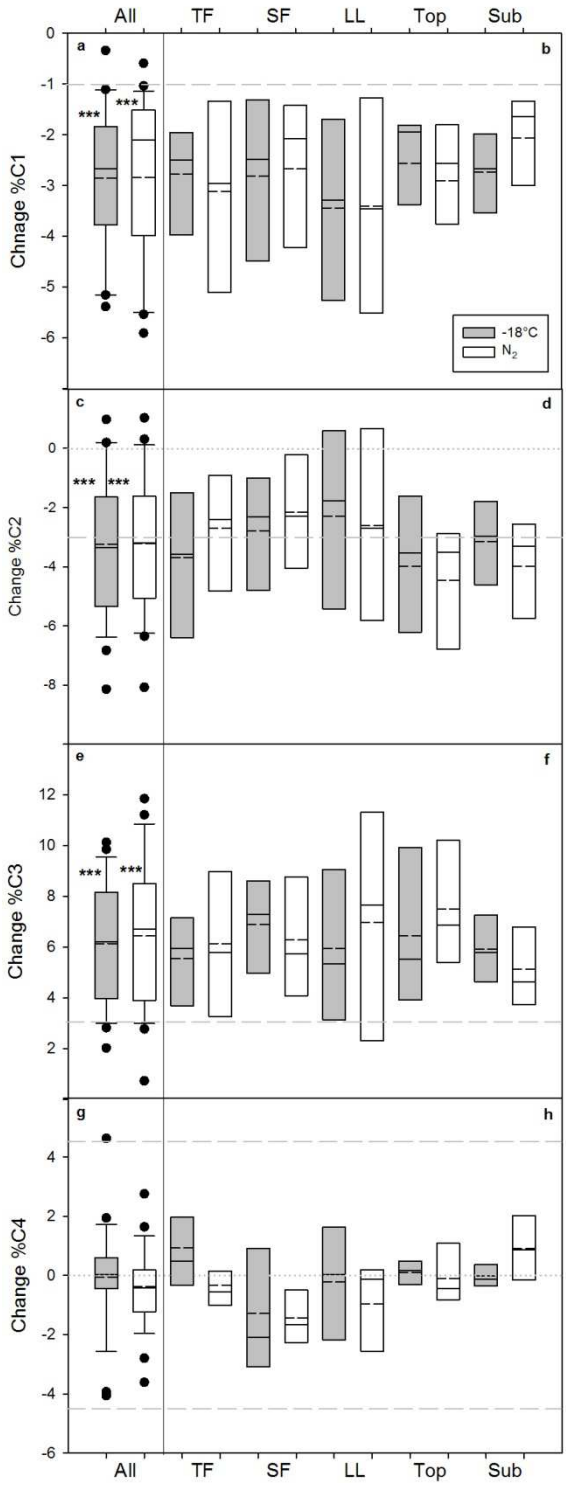

All
TF
SF
LL
Top
Sub
a
b
c
d
e
f
g
h
Chnage %C1
Change %C2
Change %C3
Change %C4
***
-18°C
N2

Figure 3

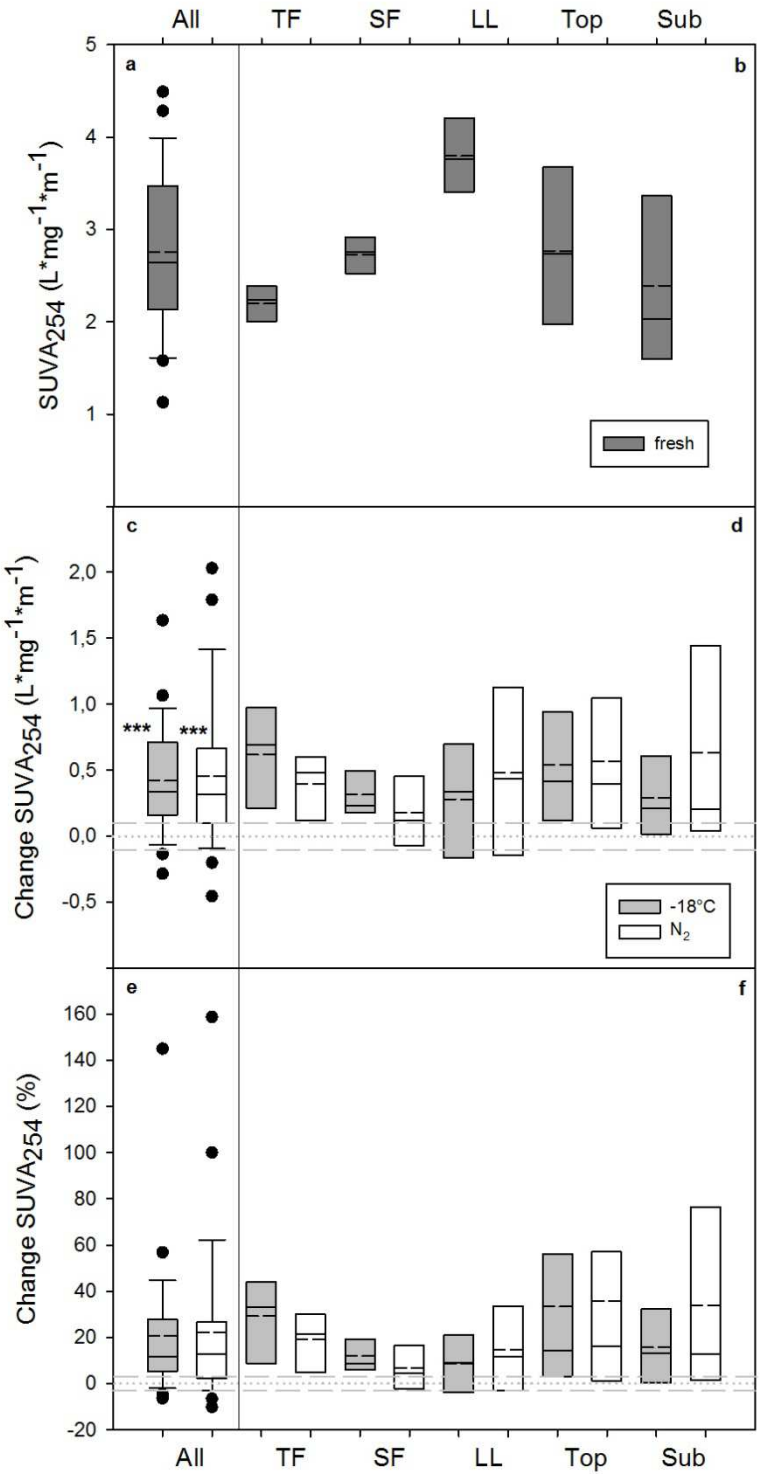

All
TF
SF
LL
Top
Sub
a
b
c
d
e
f
SUVA254 (L*mg-1*m-1)
Change SUVA254 (L*mg-1*m-1)
Change SUVA254 (%)
fresh
-18°C
N2
***
***

Figure 4

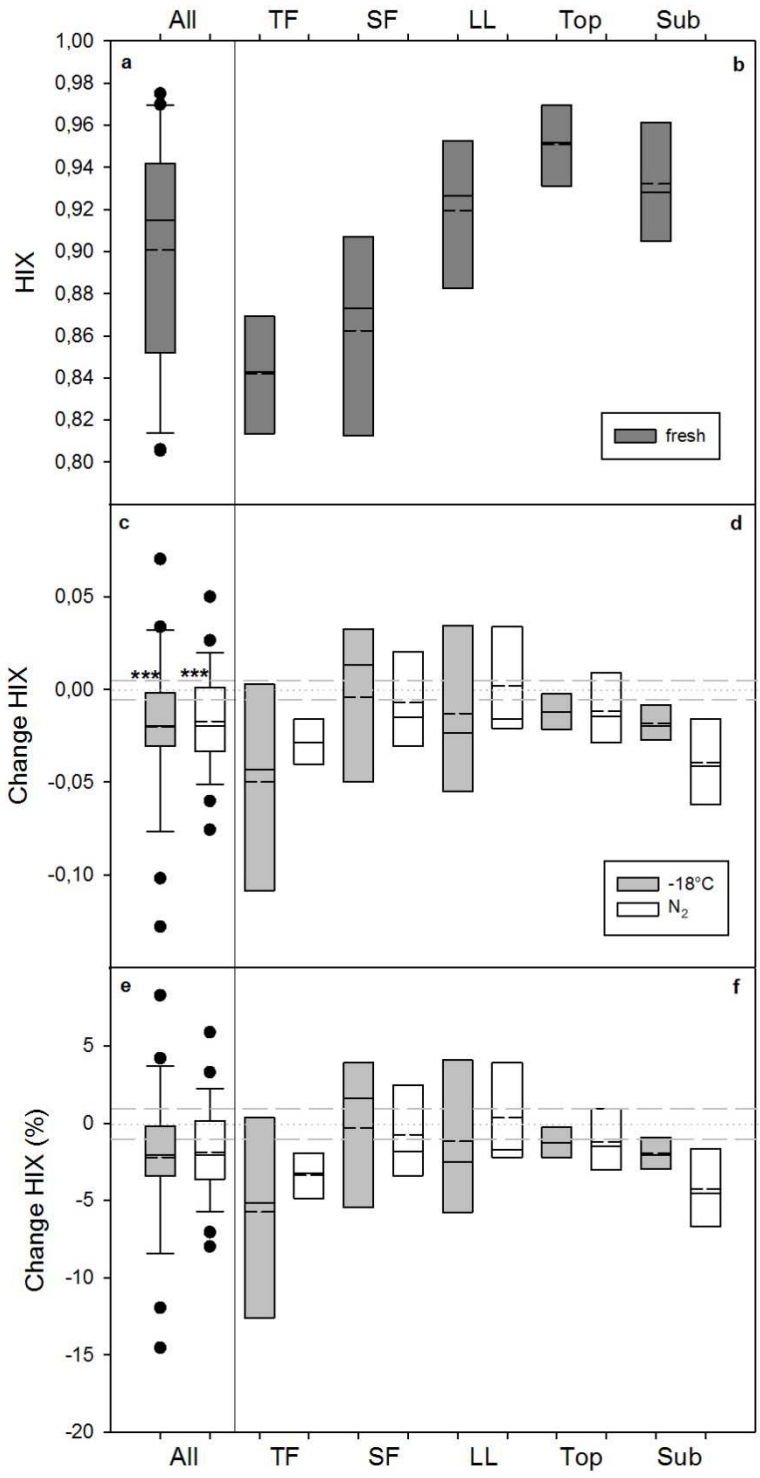

All
TF
SF
LL
Top
Sub
a
b
c
d
e
f
HIX
Change HIX
Change HIX (%)
fresh
-18°C
N2
***
***

Figure 5