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Storage effects on quantity and composition of dissolved organic carbon and nitrogen of lake water, leaf leachate and peat soil water

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Abstract

This study aimed to evaluate the effects of freezing and cold storage at 4°C on bulk dissolved organic carbon (DOC) and nitrogen (DON) concentration and SEC fractions determined with size exclusion chromatography (SEC), as well as on spectral properties of dissolved organic matter (DOM) analyzed with fluorescence spectroscopy. In order to account for differences in DOM composition and source we analyzed storage effects for three different sample types, including a lake water sample representing freshwater DOM, a leaf litter leachate of *Phragmites australis* representing a terrestrial, 'fresh' DOM source and peatland porewater samples. According to our findings one week of cold storage can bias DOC and DON determination. Overall, the determination of DOC and DON concentration with SEC analysis for all three sample types were little susceptible to alterations due to freezing. The findings derived for the sampling locations investigated here may not apply for other sampling locations and/or sample types. However,

DOC size fractions of formerly frozen samples should be interpreted with caution when sample concentrations are high. Alteration of some optical properties (HIX and $SUVA_{254}$) due to freezing were evident, and therefore we recommend immediate analysis of samples for spectral analysis.

Keywords: freezing, cold storage, size exclusion chromatograpy (SEC),dissolved organic nitrogen (DON), dissolved organic carbon (DOC)

1. Introduction

Dissolved organic matter (DOM) is a mixture of various soluble compounds differing in their molecular weight, structure and complexity (Leenheer and Croué, 2003). The composition of DOM can determine its bioavailibility (Parr et al. 2015, Petrone et al. 2009) and consequently strongly influences the fate and persistance of DOM in aquatic ecosystems. Changes of environmental conditions such as alterations of pH or ion density, as well as freezing and thawing can affect the structure of these compounds (Dryer et al., 2008; Giesy and Briese, 1978; Pace et al., 2012) and as a consequence thereof, also DOM concentration and the optical properties of chromophore DOM (Fellman et al., 2008; Gao et al., 2015; Peacock et al., 2015; Spencer et al., 2007; Thieme et al., 2016). Acidification can be applied to arrest biological activity during cold storage (Schneider-Zapp et al., 2013), and is a common sample preservation method for later analysis of bulk dissolved organic carbon (DOC). However, for subsequent fluorescence and absorbance analysis (Schneider-Zapp et al., 2013; Spencer et al., 2007) or analysis with size exclusion chromatography (SEC; Sandron et al., 2015) it is not recommended, since acidification may result in drastic alterations of the molecular structure and confirmation of DOM molecules (Dryer et al., 2008; Pace et al., 2012). When optical properties of DOM are to be addressed and immediate sample analysis is not possible, freezing samples may constitute an appropriate preservation method. For freezing various effects on chromophoric DOM composition, as well as on bulk DOC and dissolved organic nitrogen (DON) concentration have been observed so far (Fellman et al., 2008; Otero et al., 2007; Peacock et al., 2015; Spencer et al., 2007; Thieme et al., 2016). For instance, Fellman et al. (2008) reported that DOC and DON concentration decreased due to freezing, whereas Peacock et al. (2015) reported that DOC

concentration in peatland samples was mostly unaffected by freezing. Similarly, Otero et al. (2007) did not observe effects of freezing for sediment pore water samples in an estuary. Previous findings on fluorescence and absorbance properties of DOM were likewise inconsistent, reporting either no effects (Otero et al., 2007), variable responses (Spencer et al., 2007) or sometimes strong effects (Fellman et al., 2008; Peacock et al., 2015; Thieme et al., 2016) of freezing. Thereby factors like freezing and/or thawing temperature (Chen et al. 2016, Xue et al. 2015), ionic strength (Müller et al. 2011) and DOC concentration (Fellman et al. 2008, Thieme et al. 2016) and DOM composition influence the physico-chemical processes during freezing and thawing and thus may explain the various results observerd in literature. Size exclusion chromatography (SEC) can be used to determine bulk DOC and DON concentration and DOM composition, in particular the C:N ratio and the distribution of DOC and DON in different molecule size classes of DOM (Huber et al., 2011). SEC applied in parallel with analysis of optical properties enables to detect changes of DOM composition and concentration (Graeber et al. 2012a; Graeber et al., 2015; Heinz et al., 2015). While acidification affects size fractionation with SEC (Sandron et al. 2015) and is not a suitable preservation method for this analysis, the effects of freezing and cold storage at 4 °C on DOM size fractions determined with SEC have not been investigated yet at least in freshwater samples. For salt water from the Baltic sea no freezing effects were detected (Müller et al. 2011). However, the sometimes strong effects of freezing on spectral DOM properties (Fellman et al., 2008; Peacock et al., 2015; Thieme et al., 2016) indicate structural DOM alterations and suggest that SEC fractioning may be likewise vulnerable to freezing. Thieme et al. (2016) demonstrated that even if bulk

77 concentration is not affected by fast freezing with liquid nitrogen, alterations of DOM structure
78 and hence optical properties cannot be precluded.

79 In order to present a recommendation for storage and preservation of DOM samples for
80 later SEC analysis as well as fluorescence and absorbance analysis, this study aims to evaluate
81 the effects of freezing and cold storage on bulk DOC and DON concentration and SEC fractions.
82 To account for differences in DOM composition we analyzed storage effects for three different
83 sample types (freshwater DOM, leaf litter leachate, peatland pore water). To assess also the
84 effects of freezing on DOC and DON concentration and SEC fractions for a set of different
85 samples of the same sample type, but covering a range of DOM concentrations, we analyzed a
86 set of peatland pore water samples from two oligotrophic nutrient poor bogs in different
87 geographic regions.

88 We hypothesized that leaf leachates are more vulnerable to storage and freezing than lake
89 samples due to the more 'labile' nature of leachate samples. The other way around, we expect
90 that the peatland pore water samples constituting a less reactive humic sample type behave
91 more or less conservative, independent from the geographical region where they derive from
92 and independent of DOM concentration. In our study we aim to give a recommendation for
93 storage and sample preservation for three different types of natural samples.

94 ## 2. Methods

95 ### 2.1. Sampling and preparation of the leaf leachate

96 To test the effects of cold storage and freezing on different types of DOM samples we used
97 water from Lake Müggelsee (52.446°N, 13.640°E). For lake details see Recknagel et al., 2016)

representing a freshwater DOM source (hereafter referred to as lake sample). The leaf leachate from *Phragmites australis* grown in an inundated peatland 'Polder Stangenhagen' south of Berlin (52.199, 13.086) represents a purely terrestrial, but microbially unaltered, 'fresh' DOM source (hereafter referred to as leachate sample). The lake sample was taken at the lakeshore of Lake Müggelsee and filtered with a 0.45 µm cellulose acetate syringe filter (Sartorius). To prepare the leaf leachate the following leaching procedure was performed: About 50.0 g air-dried plant material of *Phragmites australis* was placed in 2 L polyethylene bottles. The plant material consisted mainly of leaves which were cut in 5-10 cm pieces to improve handling before leaching. 1.5 L of 1.5 mM NaCl solution was added to the bottle resulting in complete inundation of the plant material. The bottle was closed and stored at room temperature with occasional manual agitation over 24 hours. After leaching the resulting leachate was filtered with a 0.45µm cellulose acetate syringe filter (Sartorius). The filters were always rinsed with 100ml deionized water and preconditioned with 20 ml sample to minimize filter effects.

Lake and leachate samples had similar DOC and DON concentrations but differed in DOC and DON SEC-fractions and optical properties (Table 1). Leachate samples were characterized by higher contributions of low-molecular weight DOC (DOC_{LMWS}), higher aromaticity ($SUVA_{254}$) and less contribution of recently, microbial produced DOM and more terrestrial derived sources (FI, β:α) compared to the lake sample.

Additionally, pore water samples from oligotrophic acidic ombotrophic peatlands (bogs, hereafter referred to as peatland samples) located in two different geographical regions in Scotland (SCT; 3 sites: 58.397°N, -3.341°E; 58.373°N, -3.960°E; 58.376°N, -3.952°E; 5 dialysis samplers each) and Estonia (EST; 6 sites: 59.042°N, 25.520°E; 58.977°N, 25.649°E; 58.572°N,

25.183°E; 58.094°N, 25.044°E; 58.084°N, 25.132°E; 58.215°N, 27.360°E; 52.446°N, 13.641°E; 3 dialysis samplers each) were analyzed. Peatland samples were taken using the dialysis sampler technique (Hesslein, 1976). Dialysis samplers are thin Perspex plates covered by a 0.2 µm polysulfone membrane (HT-Tuffryn 200®, Pall®, Gelman Laboratory) containing 14 spaced chambers filled with de-ionised water. Prior to insertion into the peat, oxygen from the chamber water and the sampler material (Perspex) was displaced by degassing with nitrogen for 24 h. For that purpose samplers were stored in watertight polyvinyl chloride (PVC) vessels (diameter 25 cm and length 80 cm) filled completely with de-ionised water. After degassing, vessels were sealed with airtight cups for transportation to the sampling sites. Dialysis samplers were always inserted completely into the upper horizon of the peat (0–60 cm). The samplers were used to obtain integrated pore water samples by combining the 14 chambers to a composite sample for the DOM analysis. The exposure time of the samplers in the peat was at least 7 days so that the concentrations of dissolved substances in the pore water could equilibrate with the chamber water. After recovering and cleaning the samplers with deionized water, the chamber water of the dialysis sampler was taken rapidly within a few minutes with a multi-pipette (Eppendorf). Samples were transported to the lab at 4 °C and analyzed within 24 hours or frozen for further analysis. Samples of dialysis sampler were not 0.45 µm filtrated since the pore size of the membrane is about 0.2 µm so that bacteria are widely excluded from the samples in the chamber.

2.2. Experimental setup and laboratory analyses

To test the effects of cold storage and freezing, five replicate samples of lake and leachate samples were measured within 24 hours (original sample) or stored for one weeks at 4°C in the

142 dark (cold storage), or frozen at -20°C (freezing). Additionally 5 replicate blank samples
143 (deionized water), subject to the same storage treatments as lake and leachate samples were
144 analyzed. Samples from the two different geographical regions (EST, SCT) were analyzed before
145 (original sample) and after freezing at - 20°C, in order to test for the effects of freezing on the
146 DOC and DON concentration and SEC fractions of peatland pore water samples.

147 All samples were stored in 25 ml polypropylene (PP) vessels (washed with 10% HCl before
148 usage) during storage and analyzed at the same day after removing them from the refrigerator
149 or thawing at room temperature. The DOC and DON concentration and respective size fractions
150 we determined using size exclusion chromatography (SEC) combined with UV- and IR- organic
151 carbon detection and UV-organic nitrogen detection (Huber et al., 2011; Graeber et al., 2012a).
152 SEC enables to differentiate between DOC and DON in form of non-humic high molecular
153 weight substances of hydrophilic character (DOC_{HMWS}, DON_{HMWS}; e.g. polysaccharides and
154 proteins), humic-like substances (DOC_{HS}, DON_{HS}) and low molecular weight neutral, hydrophilic
155 to amphiphillic substances (DOC_{LWMS}; e.g. aldehydes, sugars, amino acids). The C:N ratio of bulk
156 DOM ($C:N_{DOM}$) was calculated as the molar ratio of DOC to DON. Absorbance and fluorescence
157 properties were measured using an Aqualog spectrophotometer (Horiba, USA). An excitation
158 wavelength range from 230 to 600 nm with a 5 nm increment was used. Emission spectra were
159 collected for the wavelength range 214.1 – 619.3 nm with a 1.6 nm increment, using 1 s
160 integration time, a pixel bin of 4 and medium detector gain. Absorbance spectra were collected
161 from 230 to 600 nm in 5 nm steps. Absorbance and fluorescence were measured at room
162 temperature. Spectral correction was performed using the automated algorithms provided

within the AQUALOG software (Horiba Scientific) and fluorescence intensity was normalized to Raman units using excitation wavelength of 350 nm (Lawaetz and Stedmon, 2009).

Following indices were calculated: From the absorbance data we calculated the $SUVA_{254}$ of DOM, which is the specific absorbance of the sample at 254 nm and a measure for aromaticity (Weishaar et al., 2003; Huber et al., 2011). For the peatland samples SEC was used to measure $SUVA_{254}$, since no absorbance data was available. The fluorescence data we used to calculate the humification index (HIX) (Ohno and Bro, 2006); the fluorescence index (FI), an indicator of DOM origin (more microbial (FI ~ 1.9) or terrestrial and higher plant (FI ~ 1.4) origins) (McKnight et al., 2001); as well as the β:α ratio, an indicator for the freshness of the material (0.6-0.8 more terrestrial input, > 1 freshly produced and released to water) (Parlanti et al., 2000).

2.3. Statistical analyses

All statistical analyses were performed using 'R' (2016, Version 3.3.1, The R Foundation for Statistical Computing) except for the Wilcoxon signed rank test which was performed using JMP Pro (Version 11.0.0, SAS Institute Inc. 2003). To test for the main and interaction effects of DOM source (lake, leachate) and storage treatment (cold storage at 4 °C, freezing) on changes of DOC and DON concentration, we applied a permutational 2-way ANOVA (factors: DOM source, storage treatment, 10000 iterations) with interactions (based on aov(), package 'stats', R). We used a permutational 2-way ANOVA, since for DOC and DON concentration the assumptions of variance homogeneity and normal distribution of residuals were not met. To test whether storage treatment and/or DOM source affect alterations of DOM composition a permutational MANOVA (PERMANOVA) testing for the factor DOM source (lake, leachate) and storage treatment (cold storage at 4 °C, freezing) was performed (adonis() function, package 'vegan',

185 Euclidean distance, 1000 iterations; Oksanen et al., 2015). The PERMANOVA was performed on
186 the changes of DOM composition, hence the differences between the values of the original
187 sample (measured immediately) and the samples after storage treatment and included the
188 following parameters: relative contribution of SEC fractions to bulk DOC and DON, $C:N_{DOM}$, HIX,
189 FI, β:α, and $SUVA_{254}$. To test whether differences of DOC and DON concentration and DOM
190 composition between original sample and after storage treatment in lake and leachate samples
191 were significant we used the Mann-Whitney U test (wilcox.test() function, package 'stats').
192 Individual parameters of DOM composition (SEC fractions, $C:N_{DOM}$, $SUVA_{254}$) and as well as DOC
193 and DON concentration of the peatland samples were tested for differences before and after
194 freezing applying Wilcoxon signed rank test ($p = 0.05$).

195 ## 3. Results

196 ### 3.1. DOC and DON concentration

197 Permutational ANOVA revealed neither effects of DOM source nor of storage treatment on
198 changes of DOC and DON concentration (perm. ANOVA, $p < 0.05$). Overall, changes of DOC and
199 DON concentration in the samples due to freezing were lower than changes due to cold
200 storage. This was in particular true for leachate samples where comparatively high changes of
201 DOC and DON concentration occurred after cold storage (Fig. 1 a, b). Cold storage resulted in
202 stronger changes of DOC and DON concentration in the leachate samples (-0.31 to 1.84 mg C *
203 L^{-1} ; -11 to 64 %) compared to lake samples (-0.23 to 0.14 mg C * L^{-1}; -7 to 4 %) (Fig. 1). Freezing
204 of lake and leachate samples resulted only in minor decreases of DOC (lake: 0.2 mg C L^{-1}, ≤ 5%;
205 leachate: ≤ 0.3 mg C L^{-1}, ≤ 10%) and DON (median lake: 0.01 mg N L^{-1}, leachate: 0.01 mg N L^{-1})

concentration and were within the standard deviation from the mean for the 5 replicate original samples (Fig. 1). However, individual replicates showed strong changes of DON concentration in lake and leachate samples lake (up to 31%, 0.06 mg N L^{-1}) and leachate (up to 60%, 0.07mgN L^{-1}), but the observed changes were not statistically significant (Mann-Whitney U, $p > 0.05$). In peatland samples changes of DOC concentration due to freezing were lower than 10% of the initial DOC concentration for most of the peatland samples (EST: 73%, SCT: 87%). Thereby DOC concentration increased in most of the EST samples (89%), while there was no clear trend in direction of change for DOC concentration observed in SCT samples (60% increase, 40% decrease, Fig. 2 a). Overall, the absolute changes of DOC concentration in peatland samples were lower than 4.7 mg C L^{-1} (< 23%) for EST samples and lower than 1.6 mg C L^{-1} (< 10%) for SCT samples. However, the changes of bulk DOC concentration due to freezing were not statistically significant in EST and SCT samples (Wilcoxon signed rank test, $p > 0.05$; Fig. 2). In contrast, the effects of freezing on DON concentration in peatland samples differed between EST and SCT samples (Fig. 2 b). Thereby decreases of DON concentration were observed for the EST samples (Wilcoxon rank signed test, $p < 0.05$), but not for the SCT samples (Wilcoxon rank signed test, $p > 0.05$), what resulted in an increase of the molar $C:N_{DOM}$ ratio for EST samples (Wilcoxon rank signed test, $p < 0.001$) but not for SCT samples (Wilcoxon rank signed test, $p > 0.05$). In total, changes of DON concentration due to freezing were higher than 10% of the original bulk DON concentration for more than the half of the peatland samples (EST: 82%, SCTL: 53%) and ranged from –0.2 to 0.17 mg N L^{-1}. DON concentration decreased significantly in the majority of EST samples (83%; Wilcoxon rank signed test, $p < 0.05$), while for

227 SCT samples no significant change of DON concentration (increase 60%, decrease 40%;
228 Wilcoxon rank signed test, $p > 0.05$) was observed.

229 **3.2. DOM composition**

230 Storage (cold storage, freezing) resulted in changes of DOM composition, while no effect was
231 observed for DOM source (lake, leachate) (PERMANOVA). Storage treatment explained 20% (R^2)
232 of the variance significantly (PERMANOVA, $p < 0.0001$). In particular for DOC_{HS} and DOC_{HMWS}
233 (Fig. 3 a, c), $C:N_{DOM}$ (Fig. 3 d) and β:α (Fig. 4 b) and HIX (Fig. 4 c) strong changes were observed
234 after cold storage. SEC fractions were stronger affected by cold storage than by freezing (Fig. 3
235 a – c). Overall there was no evidence for effects of freezing on DOC and DON SEC fractions for
236 lake and leachate samples (Mann Whitney U test, $p > 0.05$; Fig. 3). Changes of contributions of
237 DOC and DON SEC fractions after cold storage were only significant for DOC_{HMWS} in leachate
238 samples and DON_{HS} in lake samples (Mann Whitney U test, $p > 0.05$). In general changes of DOC
239 SEC fractions were more variable in leachate samples compared to lake samples (Fig. 2 a – d). In
240 the peatland samples significant changes of DOC in the individual SEC fractions were observed
241 for EST and SCT samples (Wilcoxon signed rank test, $p > 0.05$; Fig. 5). Thereby in EST samples
242 DOC_{HS} as well as DOC_{HMWS} decreased and DOC_{LMWS} increased, whereas in the SCT samples
243 DOC_{HMWS} decreased and DOC_{HS} increased (Wilcoxon signed rank test, $p < 0.05$). Overall changes
244 were stronger in EST samples compared to SCT samples (Fig. 5). Optical properties represented
245 by HIX, FI, β:α and $SUVA_{254}$ in lake and leachate samples were in most cases stronger affected
246 by storage in leachate compared to lake samples (Fig. 3 a – d). Thereby changes of FI and β:α
247 were not statistically significant (Mann Whitney U, $p > 0.05$), even though comparatively high
248 changes of β:α were observed in leachate samples after one week of storage at 4°C (Fig. 3 b).

Cold storage resulted in significant changes of HIX in lake and leachate samples (Mann Whitney U, $p < 0.05$). $SUVA_{254}$ was not affected by cold storage but decreased in leachate samples after freezing (Mann Whitney U, $p < 0.05$). In the EST pore water samples $SUVA_{254}$ did not change significantly (average change: 0.10 ± 0.23 L mg C m^{-1}; Wilcoxon rank signed test, $p > 0.05$), while in the SCT samples $SUVA_{254}$ was slightly increased after freezing (up to 17%, 0.5 cm^{-1}; average change 0.16 ± 0.22 L mg C m^{-1}; Wilcoxon rank signed test $p < 0.05$).

4. Discussion

We have selected three different types of DOM samples to test if freezing and storage at 4°C for one week alter DOC and DON concentration and DOM composition. We expected larger effects on leaf leachates compared to lake and peatland samples, since leachate DOM is not microbially processed so far, and thus supposed to be of more labile nature, i.e. more vulnerable to cold storage and freezing.

In accordance with our expectations effects on DOC and DON concentration were stronger for leachate than for lake samples, in particular after one week storage at 4°C with changes up to 64% of the initial DOC concentration in leachate samples. The change in optical properties towards a lower degree of humification (HIX) and aromaticity ($SUVA_{254}$), a higher contribution of recently produced fluorescent DOM (β:α), together with an increase of non-humic high molecular weight substances (DOC_{HMWS}) in the leachate sample after cold storage point to a microbial DOM as source for variation in DOC concentration. This is supported by findings of Wang et al. (2007) who showed that large fractions of bacteria cannot be retained by 0.45µm pore size filters. In accordance with previous studies, which reported only minor changes of

DOC concentration due to short-term storage for freshwater samples (Peacock et al. 2015, Carter et al. 2012) the lake samples were not affected by cold storage.

After freezing only minor changes of DOC and DON compared to the DOC and DON concentrations in the original sample were observed for lake (≤ 5%) and leachate (≤ 10%) samples, as well as for the majority of peatland samples (< 10%). Overall, changes of DON concentration after freezing were likewise low in lake and leachate samples (median: < detection limit, 0.01 mg N L^{-1}).

Our findings on the effects of freezing on DOC and DON concentration in lake and leachate samples are in accordance with the findings of Fellman et al. (2008) who observed no, or only minor changes of DOC concentration after freezing for samples with low DOC concentration (< 5 mg L^{-1}). However, although no overall change of DON concentration was observed, sometimes strong responses to freezing occurred for individual replicates.Although these changes were not statistically significant, we recommend that care should be taken for low initial DON concentration (lake: 0.19 ± 0.00 mg N L^{-1}, leachate: 0.13 ± 0.01 mg N L^{-1}). For stream samples and a range of terrestrial DOM sources, the magnitude of the effects of freezing can strongly depend on DOC concentration (Fellman et al., 2008; Thieme et al., 2016). Fellman et al. (2008) for example, reported decreasing DOC concentration as a result of abiotic particle formation during freezing. This is in contrast to our results for peatland samples, since despite high initial DOC concentration (7 – 40 mg C L^{-1}), changes of DOC concentration were lower than 10% in 70% of the peatland samples and overall not significant. This is in accordance with results from Peacock et al., (2015), who did not report a relationship between DOC concentration and the effects of freezing for surface water samples from peatlands. However,

292 in EST samples but not in SCT samples DON concentration and $C:N_{DOM}$ were altered due
293 freezing. Moreover, for samples from both regions effects of freezing on SEC fractions were
294 observed, whereby these effects were more pronounced in EST samples. In particular the
295 strong increases in low molecular weight DOC (DOC_{LMWS}) ongoing with decreases in high
296 molecular weight DOC (DOC_{HS} and DOC_{HMWS}) indicate, that freezing may result in physical
297 breakdown of high molecular weight substances into low molecular weight substances.
298 Previously it has been shown that DOM preferentially concentrates in the remaining liquid
299 phase during freezing (Belzile et al., 2002; Xue et al., 2015) and that concentration of DOM can
300 affect its macromolecular configuration (Ghosh and Schnitzer, 1980). Differences in partitioning
301 and concentration behavior were observed for individual DOM fractions (Xue et al., 2015).
302 Hence, partitioning and concentration during freezing and thawing (Belzile et al., 2002; Xue et
303 al., 2015) could have changed size fractioning of DOM and have a lasting effect on DOM
304 composition also after complete thawing of the sample. The different responses of SEC
305 fractions to freezing in lake and leachate with moderate DOC concentrations compared to
306 peatland samples with high DOC concentrations indicate that underlying processes are affected
307 by sample type and DOC concentration. Differences in the effects of freezing in peatland
308 samples from 2 different geographic regions show that magnitude and direction of change may
309 be also determined the sampling location.

310 Moreover, also the freezing temperature (Xue et al. 2015, Chen et al. 2016) or method
311 (Thieme et al. 2016) affect DOC concentration in the sample. Furthermore sampling site specific
312 sample characteristics as ionic strength (Mueller et al. 2011) can affect the freezing behaviour
313 of a sample, and the erichment of DOM in ice during freezing. The results of this study are

314 therefore study and sampling site specific and may not apply to samples from other samling
315 locations, or under other freezing conditions (temperature, method).

316 Strong variations in changes of optical properties and SEC fractions after storage at 4°C in
317 particular for leachate samples demonstrated that the DOM composition of leachate samples is
318 more likely affected by storage than DOM composition in lake samples. Overall, DOM
319 composition in lake and leachate samples was affected stronger by cold storage than by
320 freezing, whereby only HIX and $SUVA_{254}$ were altered due to freezing. In accordance with
321 previous studies (Peacock et al., 2015; Thieme et al., 2016) we do not recommend freezing of
322 samples for later analysis of optical properties.

323 ## 5. Conclusions

324 Overall, freezing, seemed to constitute an appropriate preservation method for the analysis
325 of bulk DOC concentration with SEC for lake, leachate and peatland samples analyzed here, but
326 maybe not apply for samples from other geographical regions or preserved under other
327 freezing conditions. If initial DOC concentrations in samples are high (> 7 mg C L^{-1}) e.g. in peat
328 samples, freezing can affect the individual SEC fractions as well as DON concentration and
329 should therefore be avoided. Likewise alterations of optical properties, in particular for HIX and
330 $SUVA_{254}$ due to freezing cannot be precluded and we recommend immediate analysis of
331 samples for spectral analysis. Further research should evaluate the effects of freezing on SEC
332 fractions of DOC and DON in different types of lake, leachate and pore water samples and with
333 different DOC and DON concentrations in order to develop a general guidance for sample
334 preparation of freshwater DOM.

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435

Tables

Table 1*. Brief summary of sample composition including optical properties and DOC and DON SEC fractions of the original lake (n=5), leachate (n=5) and peatland samples (peat-EST: n=18; SCT: n= 15). The contributions of the respective SEC fractions are given in percentage of bulk DON and DOC. $SUVA_{254}$ values are given in L mg C m^{-1}.*

Source	DOC_{HS}	DOC_{HMWS}	DOC_{LMWS}	DON_{HS}	DON_{HMWS}	$SUVA_{254}$	$C:N_{DOM}$
lake	74.7 (1.4)	9.5 (0.3)	15.6 (1.5)	80.7 (0.7)	19.3 (0.7)	2.3 (0.0)	9.0 (0.2)
leachate	72.3 (2.8)	3.1 (0.3)	25 (3.0)	95.7 (0.7)	4.3 (0.7)	3.3 (0.1)	7.4 (0.5)
peat-EST	94.2 (2.9)	5.1 (1.4)	0.6 (1.8)	76.3 (8.7)	23.7 (8.7)	3.4 (0.4)	35.9 (6.8)
peat-SCT	76.1 (5.2)	7.6 (1.5)	16.2 (4.8)	74.6 (5.7)	25.4 (5.7)	3.6 (0.6)	51.1 (11.4)

Table 1 *(continued).*

Source	HIX	FI	β:α
lake	0.9 (0.0)	1.6 (0.0)	0.8 (0.0)
leachate	0.8 (0.0)	1.4 (0.0)	0.5 (0.0)

Figures

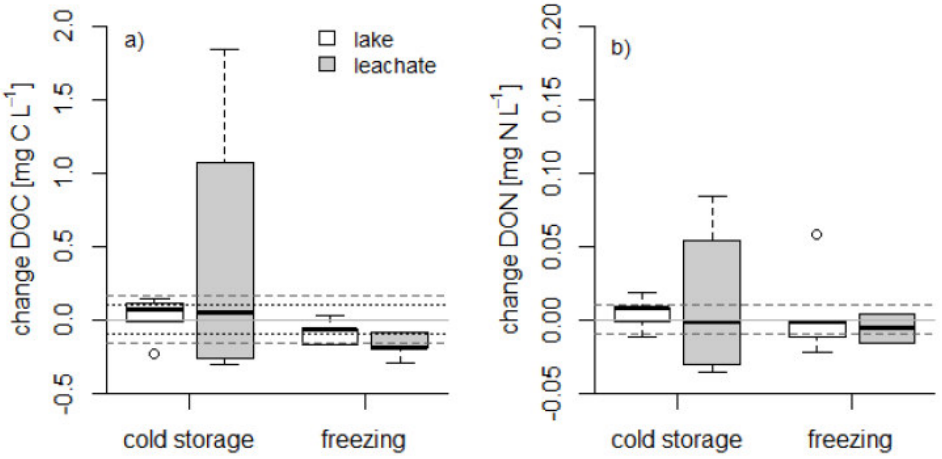
Figure 1. Total differences of DOC (a) and DON (b) concentration after cold storage 4 °C) and freezing (-20 °C) relative to the mean of original sample concentration (gray solid line). Data is shown for lake samples (white boxes) and leachate samples (light gray boxes). Dashed lines represent the standard deviation from the mean of the initial concentration for lake (dashed line) and leachate (dotted line) samples.

*Figure 2. Relative changes of DOC (a) and DON (b) concentration and molar C:N ratio of DOM (c) after freezing for peatland samples from Estonia (white) and Scotland (gray). Changes are shown as percentage of the initial concentration in the original sample. Asterisks mark significant differences to the initial concentration in the original sample (Wilcoxon signed rank test, p=0.05, p: * < 0.05, *** < 0.001).*

Figure 3. Total changes of the relative contributions SEC fractions to bulk DOM (a – c) and changes of molar $C:N_{DOM}$ (d) after one week of cold storage (4 °C) and freezing (-20 °C) relative to the mean of initial values in the original sample (gray solid zero line). White boxes represent lake samples and grey boxes leachate samples. Dashed lines represent the standard deviation from the mean of the initial concentration for lake (dashed line) and leachate (dotted line) samples. Asterisks mark significant differences to the initial concentration in the original sample (Mann-Whitney U test, p=0.05).

Figure 4. Total changes of optical properties, FI (a), freshness index β:α, HIX and $SUVA_{254}$ after one week of cold storage (4 °C) and freezing (-20 °C) relative to the mean of initial values in the original sample (gray solid zero line). White boxes represent lake samples and grey boxes leachate samples. Dashed lines represent the standard deviation from the mean of the initial concentration for lake (dashed line) and leachate (dotted line) samples. Asterisks mark significant differences to the initial concentration in the original sample (Mann-Whitney U test, p=0.05).

*Figure 5. Total changes of DOC in the humic-like (HS), non-humic high molecular weight (HMWS) and low molecular weight (LMWS) SEC fraction after freezing. White boxes represent peatland samples from Estonia, grey boxes samples from Scotland. Asterisks mark significant differences to the initial concentration in the original sample (Wilcoxon signed rank test, p=0.05, p: * < 0.05).*



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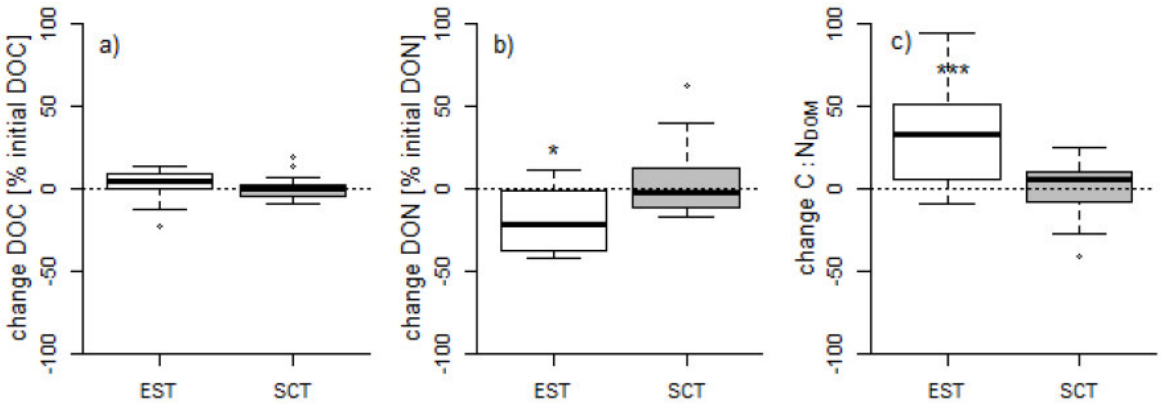

*Figure 2. Relative changes of DOC (a) and DON (b) concentration and molar C:N ratio of DOM (c) after freezing for samples from Estonia (white) and Scotland (gray). Changes are shown as percentage of the initial concentration of the original sample. Asterisks mark significant differences to the initial values in original sample (Wilcoxon signed rank test, p=0.05, p: * < 0.05, *** < 0.001).*

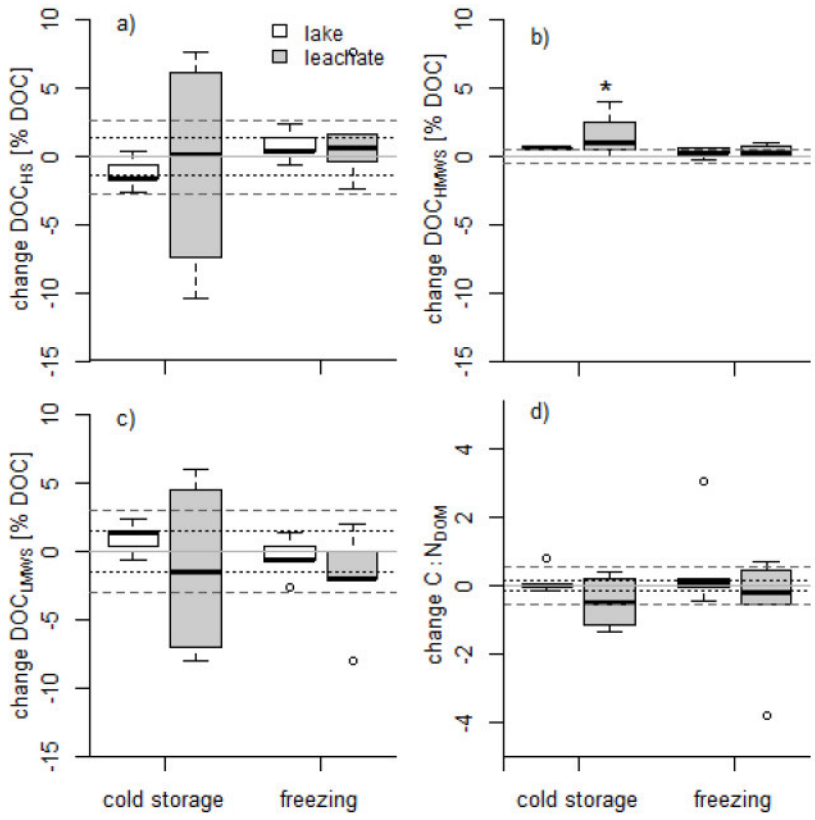

Figure 3. Total changes of the relative contributions SEC fractions to bulk DOM (a – c) and changes of molar C:N_{DOM} (d) after one week of cold storage (4 °C) and freezing (-20 °C) relative to the mean of initial values in the original sample (gray solid zero line). White boxes represent lake samples and grey boxes leachate samples. Dashed lines represent the standard deviation from the mean of the initial concentration for lake (dashed line) and leachate (dotted line) samples. Asterisks mark significant differences to the initial values in original sample (Mann-Whitney U test, p=0.05).

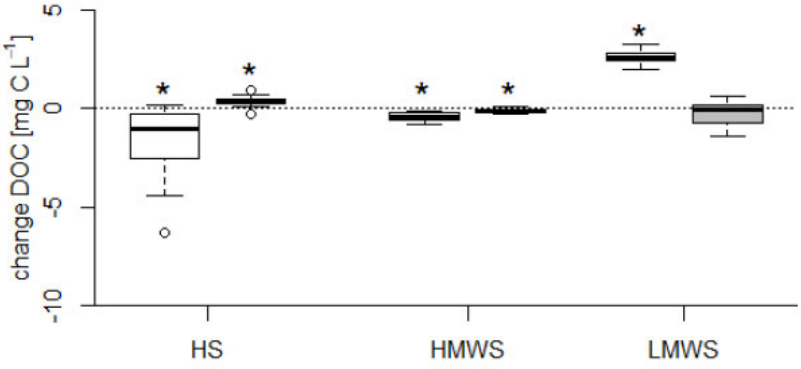

*Figure 5. Total changes of DOC in the humic-like (HS), non-humic high molecular weight (HMWS) and low molecular weight (LMWS) SEC fraction after freezing. White boxes represent samples from Estonia, grey boxes samples from Scotland. Asterisks mark significant differences to the initial values in original sample (Wilcoxon signed rank test, p=0.05, p: * < 0.05).*