EFFECTS OF AGRICULTURAL LAND USE ON DISSOLVED ORGANIC CARBON AND NITROGEN IN STREAMS

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Summary

Agricultural land use covers up to 40% of the terrestrial surface. Management practices associated with agricultural land use often alter the soil organic matter status and increase nutrient loads in soils and adjacent aquatic systems. In freshwaters dissolved organic matter (DOM) is the major form of organic matter and plays a key role in various ecological and biogeochemical processes. Thereby dissolved organic carbon (DOC) and nitrogen (DON) constitute main nutrient and energy sources to heterotrophic and autotrophic freshwater biota. As a consequence therof DOM, and in particular DON can contribute to eutrophication and support the growth of harmful phytoplankton. The identification of DOM and DON sources and composition as well as the determination of DOM degradability is therefore crucial e.g., for decision making in water conservation and resources management.

In this thesis I aimed to fill the knowledge gaps on the effects of agriculture on DOM export to streams and on DOM processing within streams, focusing in particular on dissolved organic nitrogen. In order to investigate DOM and DON export, two field studies, one at local and one at global scale, were conducted as parts of this thesis. At local scale, DOC and DON amount and composition was monitored in 12 agricultural and forest headwater streams, situated in the Northeastern German Lowlands over a one year period. On global scale, land use effects were evaluated for 75 agricultural and 45 reference streams in 5 different climate zones, including areas with intensive and extensive farming. Thereby, sampling was performed during two main seasons to assess possible temporal variations in DOC and DON amount and composition. Furthermore, in order to enhance the understanding how DOM is further processed, biodegradability of DOM from pristine and agricultural catchments was investigated and the combined effects of DOM composition and inorganic nutrient concentration were studied in a batch experiment with benthic stream biolfilm bacteria. In all field and laboratory studies within this thesis, SEC and optical measurement were applied in parallel for the characterization and quantification of DOM. No information were available on the vulnerability of DOM samples for later SEC analysis to storage effects. Therefore, in this thesis a laboratory experiment provided information on the effects of cold storage and freezing on DOC and DON concentration and size fractions determined by SEC analysis, and therefore contribute to fill these knowledge gaps.

Overall, this thesis revelealed that agricultural DOM was more microbially processed and had a much lower C:N ratio, and higher contributions of non-humic high molecular weight DOM than its forest counterpart. This was observed on local, as well as on global scale and presumably a consequence of accelarated organic matter processing in agricultural soils resulting from management processess, in particular fertilization. Strongly elevated DON loads from agricultural catchments indicate that agricultural soils can constitute important sources of DON and nitrogen-rich terrestrial DOM in streams. Even though the low C:N ratio of agricultural DOM was indicative of a lower content of refractory DOM sources in agricultural DOM, degradation of DOM was not evident during 81 days of laboratory experiment in this thesis. The absence of biodegradation of DOM by a benthic stream biofilm was likely not a result of the refractory character of DOM, but presumably can be attributed to the independence of the biofilm bacteria from external sources. However, from the results of the bioassay in this thesis no general conclusions on the biodegradability of DOM in freshwaters and its dependence on either DOM composition, or nutrient availability can be drawn. On the other hand, the findings indicated potentially different responses of planktonic versus benthic biofilm bacteria on DOM.

Finally the findings of this thesis call for the integration of DON losses from agricultural soils to considerations in catchment nutrient management, as well as in terrestrial, but also aquatic N budgets. To completely unravel the fate and role of terrestrial N-rich terrestrial DOM in freshwaters, further research should also focus on the availability of this DOM to stream biofilm and planktonic organisms.

Zusammenfassung

Landwirtschaftlich genutzte Fläche bedeckt bis zu 40% der globalen Landfläche. Die Bewirtschaftung dieser Flächen geht oft mit Veränderungen des organischen Materials, sowie mit Erhöhung der Nährstofffrachten in Böden, und weiterführend auch in Gewässern einher. In Süßwasser spielt gelöstes organisches Material (DOM) eine Schlüsselrolle in vielen ökologischen und biogeochemischen Prozessen. Dabei stellen gelöster organischer Kohlenstoff (DOC) und Stickstoff (DON) eine Hauptquelle für die Versorgung von heterotrophen und autotrophen Süßwasserorganismen mit Nährstoffen und Energie dar. Eine Folge davon ist, dass im speziellen DON stark zu Eutrophierung und dem vermehrten Wachstum von schädlichem Phytoplankton führen kann. Die Quellen und Zusammensetzung von DOM und DON zu identifizieren, ist daher von maßgeblicher Bedeutung, zum Beispiel zur Enscheidungsfindung im Gewässerschutz und der Wasserwirtschaft.

Mein Ziel war es, in der vorliegenden Arbeit bestehende Wissenslücken bezüglich des Einflusses von Landwirtschaft auf den Austrag und die Umsetzung von DOM und im speziellen DON, in Fließgewässern zu füllen. Der Austrag von DOM und DON wurde dabei im Rahmen zweier Feldstudien auf lokaler und globaler Ebene untersucht. Dazu wurden auf lokaler Ebene monatlich, für die Dauer eines Jahres, Bäche mit landwirtschaftlicher Nutzung oder Wald im Einzugsgebiet im Nordosten Deutschlands beprobt. Auf globaler Ebene wurden Landnutzungseffekte in Bächen mit landwirtschaftlicher Nutzung und Referenz-Bäche mit naturnaher Nutzung im Einzugsgebiet untersucht. Die auf globaler Ebene untersuchten Einzugsgebiete umfassten Gebiete mit intensiver, wie auch extensiver Landwirtschaft und waren über 5 Klimazonen verteilt. Mit dem Ziel Erkenntnisse über die Umsetzung von DOM und DON aus unterschiedlichen Landnutzungen zu erlangen wurde ein Abbauversuch mit DOM aus landwirtschaftlich genutzten und Waldeinzugsgebieten und unterschiedlich hohen Konzentrationen durchgeführt. In allen Untersuchungen innerhalb dieser Studie wurde zur Bestimmung der DOC und DON Konzentration und DOM Zusammensetzung Größenauschlusschromatographie (SEC) parallel mit der Analyse von Spektralen Eigenschaften des DOM durchgeführt. Die Ergebnisse eines Laborexperimentes zum Effekt von Probenlagerung auf DOC und DON Zusammensetzung, sowie Konzentration schließen

Wissenslücken bezüglich der Effekte von Probenlagerung auf die Größenfraktionierung mittels SEC.

Insgesamt demonstrierte diese Arbeit deutliche Unterschiede in der DOM Zusammensetzung und dem DON Austrag zwischen Landnutzungen. Dabei konnte herausgestellt werden, dass landwirtschaftliches DOM mikrobiell stärker umgesetzt und durch ein deutlich niedrigeres C:N Verhältnis sowie höhere Anteile an nicht-huminstoffartigen, hochmolekularen Substanzen gekennzeichnet war. Diese Unterschiede fanden sich auf lokaler und globaler Ebene wieder und sind wahrscheinlich das Resultat von verstärkter Umsetzung organischen Materials in landwirtschaftlich genutzten Böden durch Düngung. Die hohen DON und DOM Frachten zeigen, dass Böden eine bedeutende Quelle für Stickstoff-reiches organisches Material in Gewässern in landwirtschaftlichen Einzugsgebieten darstellen. Obwohl ein niedriges C:N Verhältnis auf eine höhere Verfügbarkeit hindeutete, lieferte ein Abbauversuch mit benthischen Biofilmbakterien keinerlei Hinweis auf den Abbau von landwirtschaftlichem DOM, während es Hinweise auf den Abbau durch pelagische Bakterien gab. Die Abwesenheit von Abbauprozesses war dabei weniger ein Resultat der DOM Zusammensetzung, sondern vermutlich eher ein Resultat der Unabhängigkeit von Biofilmbakterien von externen Nährstoffen. Obwohl auf der Grundlage dieser Erkenntnisse keine generellen Schlussfolgerungen bezüglich der Abbaubarkeit von DOM gezogen werden können, deuten die Ergebnisse auf unterschiedliche Reaktivität bezüglich DOM von Biofilmbakterien gegenüber planktonischen Bakterien hin.

Abschließend verdeutlicht diese Arbeit, dass der erhöhe DON Austrag aus landwirtschaftlichen Böden in terrestrische, aber auch aquatisch Stickstoffbilanzen, sowie bei der Entscheidungsfindung im Gewässerschutz und Wasserwirtschaft integriert werden sollte. Um die Rolle von N-reichem terrestrischen DOM aus landwirtschaftlichen Gebieten in Gewässern abschließend zu klären, sollten der Fokus künftiger Studien auf der Verfügbarkeit dieses DOM für planktonische und Biofilm-Organismen liegen.

Thesis outline

This thesis is a cumulative work of four manuscripts that are either published in peer-reviewed journals or ready to be submitted to peer-reviewed journals. Each manuscript constitutes an individual chapter of this thesis and includes its own introduction, methodology, results and discussion section. A general discussion section provides the general context of this study and the overall findings are discussed coherently in a general discussion section. As a consequence of this cumulative structure the general sections overlap to some degree with the content of the individual chapters. The references of the general introduction and discussion sections were merged in an overall reference section which can be found after the general discussion section.

Study 1

Heinz M., Graeber D., Zak D., Zwirnmann E., Gelbrecht J., Pusch M.T. (2015) Comparison of organic matter composition in agricultural versus forest affected headwaters with special emphasis on organic nitrogen, Environmental Science and Technology. DOI: 10.1021/es505146h

Author contributions

M. Heinz designed the study, organized and conducted field and laboratory work, analyzed the data, performed the statistics and compiled the manuscript. D. Graeber co-designed the study and contributed to statistics and to the text. Zak D. and contributed to the text. Zwirnmann E. co-performed laboratory work and contributed to the text. M. Pusch and J. Gelbrecht co-designed the study and contributed to the text.

Study 2

Graeber, D., Boëchat, I.G., Encina-Montoya, F., Esse, C., Gelbrecht, J., Goyenola, G., Gücker, B., **Heinz, M.**, Kronvang, B., Meerhoff, M., Nimptsch, J., Pusch, M.T., Silva, R.C.S., von Schiller, D., Zwirnmann, E., 2015 Global effects of agriculture on fluvial dissolved organic matter.(2015) Scientific reports, 5. DOI: 10.1038/srep16328

Author contributions

All authors worked on the sampling design and participated in writing and revising of the manuscript. In addition, D. Graeber. conducted field work, laboratory measurements and data analyses; **M. Heinz** conducted field work, laboratory measurements and GIS data analyses; I. Boëchat, B. Gücker, M. Meerhoff. and D. von Schiller conducted field work and participated in data analyses; J. Gelbrecht., F. Encina-Montoya, C. Esse, G. Goyenola and J. Nimptsch conducted field work and GIS data analyses; J. Gelbrecht and E. Zwirnmann conducted laboratory measurements and participated in data analyses; B.Kronvang and M.P. participated in data analyses; R. Silva conducted field work.

Study 3

Heinz M., Graeber D., von Schiller D., Pusch M. (to be submitted) Absence of dissolved organic matter degradation by stream biofilms in a laboratory experiment with different DOM composition and nutrient concentration scenarios.

Author contributions

M. Heinz designed the study, organized and performed field and laboratory work, analyzed the data, performed the statistics and compiled the manuscript. D. Graeber co-designed the study and contributed to statistics and to the text. M. Pusch and D. von Schiller co-designed the study and contributed to the text.

Study 4

Heinz M., Zak D. (to be submitted)

Storage effects on DOM analysis with size exclusion chromatography and fluorescence spectroscopy for lake water, leaf leachate and peat soil water

Author contributions

M. Heinz designed the study, organized and performed experiment and laboratory work, analyzed the data, performed the statistics and compiled the manuscript. Zak D. co-designed the study and contributed to the text.

General introduction

The role and function of dissolved organic matter in freshwater environments

In aquatic systems dissolved organic matter (DOM) occurs ubiquitously and plays a key role in various ecological and biogeochemical processes (Findlay and Sinsabaugh 2003). Operationally, DOM is defined as the fraction of organic matter in an aqueous solution that passes a 0.45µm filter (Thurman 1985) and structurally, bulk DOM is a heterogeneous mixture of various several thousand compounds (Leenheer and Croué 2003) and comprises organic forms of carbon (DOC), nitrogen (DON), sulfur (DOS) and phosphorus (DOP). Thereby DOC and DON constitute main nutrient and energy sources to heterotrophic and autotrophic freshwater biota (Stepanauskas et al. 1999; Stepanauskas et al. 2000; Brookshire et al. 2005; Boyer et al. 2006). In particular DON can contribute to eutrophication (Seitzinger and Sanders 1997; Petrone 2010) and support the growth of harmful phytoplankton (Mulholland et al. 2002; Berg et al. 2003). As sorbent and chelating agent DOM mediates the transport and processing of organic pollutants (e.g. Akkanen et al. 2004) as well as metals (Boyle et al. 1977; Aiken et al. 2011) and as a consequence thereof, significantly determines the fate of harmful substances in freshwaters. Moreover DOM, particularly nitrogen-rich DOM compounds can be precursors for the production of toxic disinfection byproducts (Lee et al. 2007; Chuang et al. 2013). Apart from this, DOM can substantially affect the physicochemical properties of water bodies, absorbing light from the water column (Ferrari et al. 1996) and influencing the attenuation of ultraviolet (UV) and photosynthetically active radiation (Scully and Lean 1994). As a consequence of strong involvement of DOM in the aforementioned processes, alterations of DOM and its composition can exert considerable impact on the health of aquatic ecosystems.

Effects of agricultural land use on fluvial dissolved organic matter export

Worldwide agricultural land use (croplands and pastures) covers up to 40% of the terrestrial surface (Foley et al. 2005). Intensification of agriculture due to progressive mechanization, as well as increased application of irrigation and fertilization practices has led to a growth of agricultural production between 2.5 and 3 times and to an increase of the global net cultivated area by 12% during the last 50 years (FAO 2011). This development

occurred at the expense of natural habitats and the quantity and quality of aquatic systems. In particular, the increased use of mineral fertilizers has elevated the nutrient loads in croplands and the transport of nitrogen to freshwater ecosystems. Apart from strongly elevated inorganic nitrogen loads, DON leaching from soils to streams can constitute a substantial (one-third of NO_3^- loss) part of nitrogen loss in agriculture systems (van Kessel et al. 2009).

Moreover, agriculture impacts the quality soil organic matter (SOM) (Balesdent et al. 2000; Kalbitz et al. 2000; Chantigny 2003) and this alterations potentially propagate to DOM in freshwaters dominated by terrestrial inputs. Especially in agricultural areas with subsurface drainage the hydrological pathway of DOM from soil to streams can be shortened (Blann et al. 2009; Dalzell et al. 2011) and DOM from the upper soil layers, predominantly affected by management practices, is delivered to the streams. So far, field studies in streams found no consistent effect of agriculture on either DOC (Cronan et al. 1999; Stedmon et al. 2006; Wilson and Xenopoulos 2008; Graeber et al. 2012b; Kronholm and Capel 2012) or DON concentration (Pellerin et al. 2006; Stedmon et al. 2006). This is perhaps due to the diversity of agricultural practices and their effects on terrestrial and aquatic carbon cycling (Stanley et al. 2011)

Since NO_3^- is the predominant form of nitrogen in agricultural systems comparably little attention has been paid to DON so far. In addition, determination of DON concentration can be biased when inorganic nitrogen dominates the dissolved nitrogen pool (Lee and Westerhoff 2005; Pellerin et al. 2006; Graeber et al. 2012a), which is the case for most agricultural streams (Stanley and Maxted 2008). Additionally to alterations of DOM amount, DOM composition in streams shifts to more microbial-derived and structural less complex organic matter with increasing contribution of agricultural land use in the catchment (Wilson and Xenopoulos 2009; Williams et al. 2010; Graeber et al. 2012b). In bigger streams, these alterations are often attributed to autochthonous production or anthropogenic inputs (Wilson and Xenopoulos 2009; Williams et al. 2010) whereas in small agricultural headwater streams terrestrial inputs, e.g. soil DOM are likely the prevailing sources for microbial-derived DOM (Graeber et al. 2012b). However, findings on the effect of agriculture on DOM composition in headwaters are restricted to chromophoric DOM and DOC, but information on DON in agricultural headwater streams are scarce.

Bioadegradability of dissolved organic matter from agricultural land use

Whether intrinsic poperies of DOM or environmental factors determine the bioavailability of DOM is currently debated in soil and aquatic sciences (Schmidt et al. 2011; Marin-Spiotta et al. 2014; Kellerman et al. 2015). However, it has been observed that changes of DOM composition due to agricultural land use in the catchment result in higher availability of DOM in these streams (Williams et al. 2010). In anthropogenic and urban catchments it was observed that the availability of DOC and in particular DON is high and can account up to 17% of total DOC and up to 44% of total DON (Petrone et al. 2009). Thereby, terrestrial plants were the main source for available DOC, whereas available DON derived from autochthonous and anthropogenic sources (Petrone et al. 2009). Further studies reported that DON is more likely consumed than DOC (Kaushal and Lewis, 2005). Moreover, the form in which DON and DOC are bound may determine their biodegradability. For example, DON bound to non-humic, microbial fractions of DOM has shown to be bioavailable (Kaushal and Lewis 2005). Besides this, more DOC was consumed in the high-molecular fraction (> 1 kDa) than in the low-molecular fraction (< 1 kDa) during bioavailability experiments using DOM from different aquatic sources (Amon and Benner 1996). In addition to DOM composition, environmental factors such as the availability of inorganic nutrients can affect the biodegradability of DOM (Mineau et al. 2013), with differences in magnitude and direction of the responses to increased inorganic nutrient supply for DOC and DON (Kaushal and Lewis 2005; Wymore et al. 2015). So far, the relationship between DOM biodegradability and inorganic nutrient concentration has been investigated in streams with low ambient inorganic nutrient concentrations and moderate nutrient elevations (< 1mg N L^{-1}, < 0.5 mg P L^{-1}; Brookshire et al. 2005; Kaushal and Lewis 2005; Mineau et al. 2013; Wymore et al. 2015) only. Information on DOC and DON availability under elevated inorganic nutrient concentrations, such as those typically observed in streams draining intensive agricultural catchments, are lacking.

Introduction to size exclusion chromatography and fluorescence analysis

Given the importance of DOC and DON in the global carbon and nitrogen cycle and the role DOM composition plays determining its fate in aquatic systems, a proper characterization of DOM composition is crucial. The inherent chemical complexity and heterogeneity of DOM results in a variety of different methods applied for the DOM analysis. Thereby the properties addressed and information derived (molecular size and mass, optical properties, polarity, elemental composition) are as various as the methods applied for DOC and DON measurement (Minor et al. 2014; Chen et al. 2015).

Fluorescence characterization of DOM is a rapid, precise and comparably inexpensive technique providing information on the source, redox state and biological reactivity (Fellman et al. 2010, and references herein) of DOM. Fluorescence is compound specific, and occurs when atoms or molecules are excited with energy and as a consequence thereof, an electron which was formerly loosely bound is transferred to a higher energy level absorbing energy. When the electron returns to its original energy level, energy is released in form of light and fluorescence occurs (Lakowicz 2006). Based on these mechanisms it can be differentiated between compounds which absorb light (chromophores, chromophoric DOM) and those which absorb and re-emit light energy (fluorophores, fluorescent DOM) (Mopper et al. 1996). The wavelength at which energy is absorbed (excitation wavelength, ex) and emitted (emission wavelength, em) is molecule specific (Lakowicz 2006). Combining a range of excitation wavelength with a range of emission wavelength produces a 3D map, the so called excitation emission matrix (EEM, Fig 1) which equals fingerprints, unique for individual fluorophores. To identify fluorophores occurring in a sample dataset parallel factor analysis (PARAFAC) can be used. PARAFAC is a multilinear method which decomposes 3-way data arrays (3D datasets as e.g excitation-emission matrixes) into single components, which represent fluorophores with specific excitation and emission spectra (Fig. 1). Furthermore, it determines the fluorescence intensity of each fluorophore and for each sample of a given data set, which is analogous to the sample-specific concentration of this fluorophore (Bro 1997; Fellman et al. 2010; Murphy et al. 2013). Based on their spectral form and peak position, the identity and characteristics of the PARAFAC components can be assessed (Fellman et al. 2010). Additionally, from excitation-emission data several indices can be calculated. These

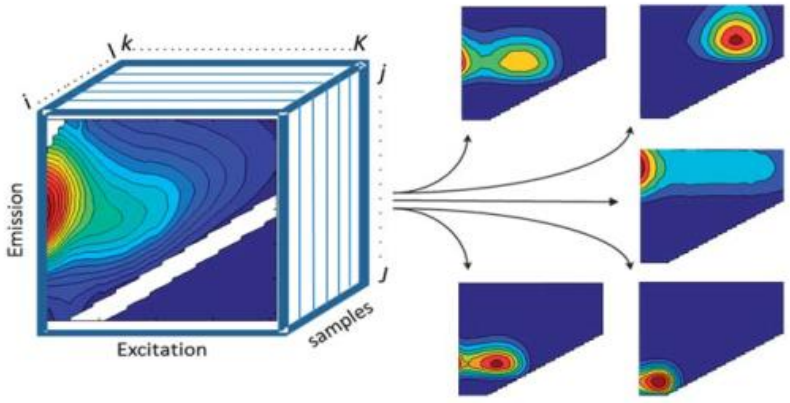

Figure 1. *EEM dataset arranged in a threeway structure and decomposed into five PARAFAC components (from Murphy et al. 2013)*

indicate for example: a) the degree of humification or humic substance content (HIX, after (Ohno 2002), b) the freshness of the material (freshness index β:α, 0.6-0.8 more terrestrial input, > 1 freshly produced and released to water; (Parlanti et al. 2000), and c) whether DOM is derived from more microbial (fluorescence index FI ~ 1.9) or terrestrial, higher plant (FI ~ 1.4) origin (Cory and McKnight 2005). A disadvantage of the method is that fluorescence and absorbance analysis is restricted to the fluorescent fraction of DOM.

In contrast to fluorescence, size exclusion chromatography (SEC) with organic carbon (OCD) and organic nitrogen detection (OND) (Huber et al. 2011), enables the determination of bulk DOC and DON concentration also for non-fluorescent DOM and provides direct information on the carbon and nitrogen content in different molecular size fractions. The method differentiates between DOC and DON bound in form of humic-like substances (HS), non-humic high molecular weight substances (HMWS) including e.g. proteins and polysaccharides and low molecular weight acids and neutrals (LMWS) (Huber et al. 2011).

In addition to the assessment of different molecular fractions of DOC and DON, the concentration of bulk DON is measured directly by SEC and, thus, overcomes the uncertainties of indirect DON determination as total dissolved nitrogen (TDN) minus dissolved inorganic nitrogen (DIN, the sum of NO_3^-, NO_2^- and NH_4^+) (Huber et al. 2011; Graeber et al. 2012b). This indirect determination is biased for DIN to TDN ratios higher than 0.6, above which large errors in DON assessment are to be expected (Lee and Westerhoff 2005; Graeber et al. 2012a;

Chen et al. 2015). This is problematic in many surface waters within agricultural landscapes and urban areas, where high NO_3^- concentrations commonly result in large DIN to TDN ratios (Graeber et al. 2012a; Chen et al. 2015). Under such conditions, the direct DON measurement by SEC results in much higher precision than the indirect determination of DON and therefore allows to explore the effects of human activity on DON biogeochemistry, which was very difficult before (Graeber et al. 2012b).

However, no information on the effects of commonly used preservation methods on SEC fractions are available (e.g. freezing and cold storage at 4°C).

Aims, approach and structure of this thesis

The aim of this thesis is to evaluate the effect of agricultural land use on the export and lability of DOM, especially DOC and DON. To address the export, I conducted two studies which investigate amount and molecular composition of DOM in agriculture relative to pristine reference catchments. Furthermore, in order to enhance the understanding how DOM is further processed in streams with agricultural land use, I investigate the biodegradability of DOM from pristine and agricultural catchments. This study also investigates the interaction of DOM processing with inorganic nutrient concentrations typically found in agricultural streams, as high nutrient concentrations may decisively modulate this processing. In all field and laboratory studies I conducted within this thesis, I applied SEC and optical measurement in parallel for characterization and quantification of DOM. Since recommendations for cold storage and freezing of samples for SEC analysis are lacking, I studied the effects of these preservation methods on optical properties, as well as DON and DOC measured with SEC. The specific objectives of the individual studies within this thesis are specified as follows:

The aim of **study 1** was to assess the influence of agricultural land use on the amount and composition of DOM and in particular DON in small streams of temperate regions. A further aim of this study was to identify seasonal patterns of DOM composition and quality. To achieve these aims, I conducted a monthly sampling of 6 forest and 6 agricultural headwater streams in the Northeastern German Lowlands. In these streams I monitored DOC and DON

concentration and loads, as well as DOM composition on a monthly base over a 1-year study period.

Study 2 aimed to evaluate whether land use effects on DOC and DON observed in temperate regions (study 1) also apply on the global scale. For this purpose, 75 agricultural and 45 reference streams in 5 different climate zones were sampled to investigate DOM composition as well as DOC and DON concentration in agricultural and near natural reference system. Regions with intensive (arable farming) and extensive (pasture and rangelands) farming practices were included and samples were taken during two main seasons to account for variation in agricultural land use intensity and temporal variations.

The overall aim of **study 3** was to unravel whether DOM composition or altered inorganic nutrient concentrations determine degradability of DOM from forest and agricultural streams. For this purpose I conducted a laboratory experiment, in which DOM from forested and agricultural streams with low and high inorganic nutrient additions was inoculated with benthic stream biofilm. I monitored ong- and short term changes of DOC, DON and inorganic nutrient concentration and DOM composition during 81 days of experiment.

The goal of **study 4** was to assess the vulnerability of SEC size fractions and optical properties to changes due to sample storage. In order to give recommendations for sample storage of samples for later SEC and spectral analysis I conducted a laboratory experiment exposing 3 different sample types to cold storage and freezing.

Study 1:

Comparison of organic matter composition in agricultural versus forest affected headwaters with special emphasis on organic nitrogen

Marlen Heinz, Daniel Graeber, Dominik Zak, Elke Zwirnmann, Jörg Gelbrecht, and Martin T. Pusch

(Environmental Science and Technology, http://dx.doi.org/10.1021/es505146h)

Comparison of organic matter composition in agricultural versus forest affected headwaters with special emphasis on organic nitrogen

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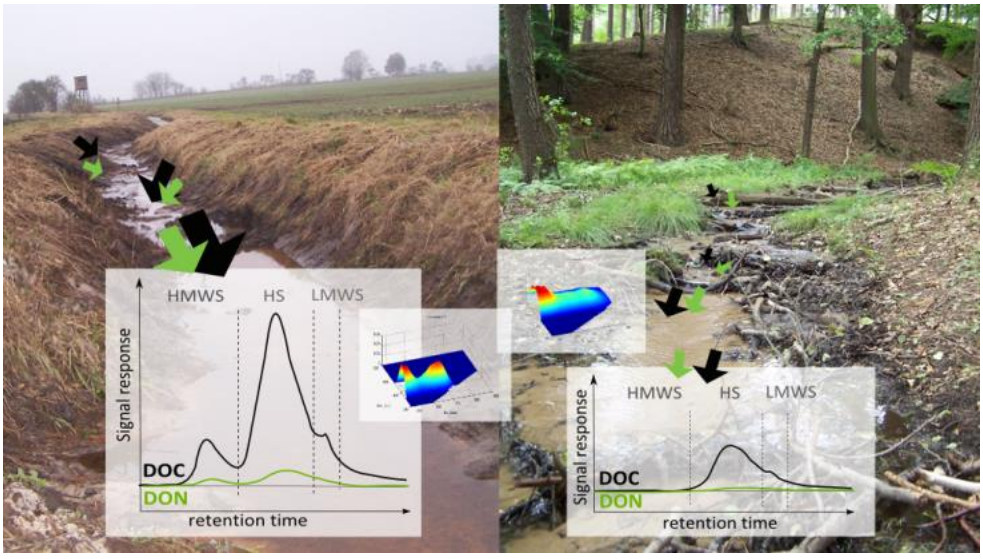
Keywords: DON quality, PARAFAC, land use, temporal variability, SEC

Graphical Abstract:



Abstract

Agricultural management practices promote organic matter (OM) turnover and thus alter both, the processing of dissolved organic matter (DOM) in soils and presumably also the export of DOM to headwater streams which intimately connect the terrestrial with the aquatic environment. Size exclusion chromatography in combination with absorbance and emission matrix fluorometry were applied to assess how agricultural land use alters the amount and composition of DOM, as well as dissolved organic nitrogen (DON) forms in headwater streams, including temporal variations, in a temperate region of NE Germany. By comparing six agriculturally and six forest-impacted headwater streams, we demonstrated that agriculture promotes increased DOC and DON concentrations, entailing an even more pronounced effect on DON. The major part of DOC and DON in agricultural and forest reference streams is exported in the form of humic-like material with high molecular weight, which indicates terrestrial, i.e. allochthonous sources. As an obvious difference in agricultural streams, the contribution of DOC and particularly DON occurring in the form of non-humic high molecular weight, presumably proteinous material, is clearly elevated. Altogether, DOM in agricultural headwaters is mainly complex soil derived and aromatic material with low C:N ratio which is more microbial processed than its counterpart from forest reference catchments. Our results emphasize the importance of agricultural land use on DOM loss from soils and identify agricultural soils as important DOC and particularly DON sources to headwater streams.

Introduction

Dissolved organic matter (DOM) represents the largest pool of reduced carbon in the biosphere and is a heterogeneous mixture of several thousand various dissolved organic carbon (DOC) and nitrogen (DON) forms. Since DOM composition is a key property determining its bioavailability,[1-3] changes of DOM composition in streams can have far-reaching effects on the whole stream ecosystem.

Recent findings indicate that soil organic matter (SOM) persistence is rather determined by environmental conditions than by intrinsic properties of the SOM.[4] Due to tillage, ploughing and fertilization, agricultural land use substantially alters environmental conditions in soils and results in qualitative changes of SOM, stimulation of SOM turnover and microbial decomposition and induces release of organic matter (OM) previously protected in soil aggregates.[5-7] Headwater streams intimately connect terrestrial and aquatic ecosystems, whereby catchment soils and vegetation constitute the main source of DOM.[8] Consequently, alterations of catchment characteristics potentially propagate to stream DOM composition.[9] Knowing the effects of agricultural land use on DOM composition and the forms and concentrations of DOC and DON in headwater streams is important for water resource management as small headwater streams markedly influence the downstream water quality[10] and dominate stream length and, with it riparian zones at the global scale.[11]

For streams with agriculture in the catchment, a uniform shift towards low molecular weight, reduced aromaticity, low redox state and increased lability in DOM was frequently found[12-14] irrespective of differences in agricultural management practices, soil types and hydrology. But as shown elsewhere for headwater streams, DOM from agriculture may be aromatic with low redox state and highly complex,[15] which fits the assumption that DOM leaving the soil is highly degraded by microbial processes.[9]

So far, most research has focused on DOC as surrogate for DOM, whereas DON has been of minor interest although it may be an important nutrient source.[16-19] Distinct DOC or DON compounds, respectively, are preferentially used during DOM metabolism; hence DOC and DON cycles are not necessarily coupled, which may result in changes of the DOC:DON ratio in soils and streams.[20-21] Generally, DON in freshwater environments has been much less examined than marine DON.[22] It seems that DON is more abundant in rivers and in the ocean

due to heterotrophic production, decreasing the C:N ratio of DOM relative to headwater streams which are governed by allochthonous sources.[23] To our knowledge, there are no studies on the quantitative importance of varying DON fractions and their sources in headwaters and how they may be influenced by agricultural land use in the catchment. In addition, it turns out that published data on DON concentrations might be flawed when inorganic nitrogen dominates the dissolved nitrogen pool.[20, 24, 25]

A method to overcome the uncertainties of classic DON determination is size exclusion chromatography (SEC) with organic carbon and organic nitrogen detection (LC-OCD-OND), which analyses DON concentration directly.[25, 26] Additionally, SEC provides direct information on the size distribution of bulk DOM and the carbon and nitrogen content in different molecular size fractions, enabling assessment of in which forms DOC and also DON are bound. The coupled approach of combining SEC with absorbance and excitation-emission matrix (EEM) fluorometry with subsequent parallel factor analysis (PARAFAC) provides a clearer picture about the sources and past processing of chromophoric DOM, including also the non-chromophoric part of DOM.

Using this approach, our main objective was to assess the impact of crop-based agricultural land use with sub-surface drainage on DOC, DON and bulk DOM exported from soils to headwater streams. Accordingly, we investigated 6 agricultural streams and compared the results with those from 6 forested reference streams over a one year period. We hypothesized that increased microbial turnover in agricultural soils concurred with i) increased DOC and DON losses from soils to streams, ii) not only DOC but also DON loss in the form of complex/terrestrial material in agricultural streams, iii) more microbial processed terrestrial derived DOM. Further, we expected iv) higher temporal variation in DOM concentrations and composition in agricultural headwaters relative to forested reference streams due to shortened hydrological pathways caused by tile drainage and thus more immediate responses to heavy precipitation.

Material and Methods

Study sites. The study area, which is located in the Northeastern German Lowlands, receives annual precipitation from 500 to 600 mm, with minimum precipitation during October to April and maximum during June to August. The annual average temperature is 8

to 9 °C, with the lowest temperature in January and February (-0.1 °C) and highest from July to August (19°C).[27] Agriculture (crop based, tile-drained and with conventional tillage) and forest land (mixed and coniferous) dominate the Federal State area (49% and 36%, respectively).[15] Small ditches and streams make up the majority (~ 80%) of the total length (32000 km) of the net of water courses.[28] For each of the two land use types, we selected six streams and specified the catchment area, land use, and soil types using QuantumGis (Version 1.8.0, Quantum GIS Development Team, 2012, Gnu General Public License), topographical maps (TK 10, Landesvermessung und Geobasisinformation Brandenburg 2013), Corine land cover data (European Environment Agency, 2007) and digital soil maps (State office for Mining, Geology and Resources, Federal State Brandenburg, 2007). Forested reference streams (F1-F6) refer to small headwater streams with > 70% forest in the catchment (Table S1). Agricultural streams (A1-A6) refer to drainage ditches with pure crop-based arable land use in the catchment, with the exception of streams A4 and A6, which contain small forest areas, pasture or settlement in their catchments (Table S1). The agricultural catchments are non-irrigated and tile-drained and have a heterogeneous cultivation pattern with the major crops being cereals, corn and canola. The catchment area soils are primarily sandy to loamy sandy mineral soils from glaciofluvial, periglacial and glacial deposits (Table S1). All selected catchments have an identical geological and pedological background, and do not include wetland or lake areas.

Sampling and analytics. Water sampling and discharge measurement in each stream were conducted on a monthly basis over a one-year period (142 samples in total), except for A3 and A6 that were not sampled in June due to seasonal dry-up or non-detectable water flow in these streams. Depending on hydrological conditions, we measured flow by slug addition with NaCl or with a flow velocimeter (MiniAir20, Schildknecht, Swiss precision, Gossan, Swiss). We stored the water samples in a dark refrigerator box for transport and at 5 °C in the dark until processing (maximum 24 h after sampling). Sample preparation and bulk analysis of nitrate and ammonia followed standard methods (S1). For determination of DOC and DON concentration and composition, we used size exclusion chromatography (SEC) combined with UV- and IR- organic carbon detection and UV-organic nitrogen detection (relative standard deviation DOC < 4%, DON < 18%).[25, 26] This procedure allowed us to measure the DON concentration directly and to differentiate between DOC and DON bound

in the form of non-humic high molecular weight substances (HMWS) of hydrophilic character (like polysaccharides and proteins), humic-like substances (HS) and between low-molecular weight acids and neutrals, which we combined as the low-molecular weight fraction in this study (LMWS).[25, 26] The fraction of low-molecular weight acids was consistently below detection limits, hence, with the notation low molecular weight substances (LMWS) we refer to neutral, hydrophilic to amphiphillic substances (aldehydes, sugars, amino acids).[26] The DON measured by SEC did not include the LMWS fraction, which in contrast to waste waters,[29] is negligible in natural freshwaters.[25] The terms $DOC_{\%HMWS}$, $DOC_{\%HS}$, $DOC_{\%LMWS}$, $DON_{\%HMWS}$ and $DON_{\%HS}$ indicate the relative contribution of DOC and DON bound in the respective size fraction. The detection limit of the individual SEC fractions was 0.01 mg L^{-1} for both carbon and nitrogen. Furthermore, we used SEC to measure $SUVA_{254}$ of DOC_{HS}, which is the specific absorbance of the sample at 254 nm, and an indicator of aromaticity.[26, 30] We measured absorbance and fluorescence to produce excitation-emission-matrices (EEMs) for parallel factor analysis (PARAFAC). We measured all samples at room temperature and preprocessed the absorbance and fluorescence data, including correction for spectral and instrumental biases, removal of scatter and normalization of the data before PARAFAC. A detailed description of absorbance and fluorescence measurement, preprocessing steps, PARAFAC modeling and software used is given in S2.[31-35] The PARAFAC modeling resulted in a five component model (Table S2) with fluorescence intensities described as percentage fluorescence contribution to total sample fluorescence (%C1, %C2, %C3, %C4, %C5). From the absorbance data, we calculated the slope ratio S_R, an indicator of molecular size (decreases with increasing molecular weight).[36] In addition, from the fluorescence data we calculated the following indices: the humification index (HIX),[37] the fluorescence index (FI), indicating if DOM was of more microbial (FI ~ 1.9) or terrestrial and higher plant (FI ~ 1.4) origin,[38] and the β:α ratio, indicating the material freshness (> 1 freshly produced and released to water, 0.6-0.8 more terrestrial input).[39]

Calculations. The dissolved inorganic nitrogen (DIN) concentration was calculated as the sum of nitrate and ammonia. The total dissolved nitrogen (TDN) concentration was calculated as the sum of DIN and DON concentrations. To assess the influence of discharge on DON and DOC concentrations, we calculated the discharge-weighted mean concentration (DWMC) as the ratio between the annual sum of loads and the annual sum of discharge. To

refer to differences in the catchment area, we calculated the specific DOC and DON loads (mg C s^{-1} km^{-2} and mg N s^{-1} km^{-2}) by multiplying the daily specific discharge with DOC and DON concentrations over the catchment area to enable comparison of DOC and DON yields per unit area between catchments with different sizes.[40] In Pleistocene landscapes, determination of the watershed area can be biased because surface water and groundwater watersheds often do not coincide.[41-42] Therefore, for the forest streams we additionally calculated the potential catchment area based on the average runoff of the investigation area (88 mm)[43] and the measured annual sum discharge of the individual streams. Hence, two values for catchment area are given for the forest streams (Table S1). However, specific loads for the forest catchments and specific loads calculated based on the potential catchment size were within the same ranges (Table 1, Table S1). Thus, in the assessment of the effects of agricultural compared to forest land use, the uncertainties regarding catchment size are negligible for the investigated streams. We are aware that the calculations of the specific loads are based only on 12 monthly samplings during one year and may therefore constitute only a point assessment. However, all the peak discharge events that we observed match the periods of highest precipitation during the investigation period, and we therefore assume that our calculations are valid for a general comparison between forest and agricultural land use. The C:N ratio was calculated as the molar C:N ratio for bulk DOM ($C:N_{DOM}$), and for DOM bound in the form of humic-like ($C:N_{HS}$) and non-humic high molecular weight substances ($C:N_{HMWS}$).

Statistical analysis. We performed all statistical analyses in R (Version 3.0.0, R Development Core Team, 2013). Because assumptions of normality and variance homogeneity were often not met for the data, we applied non-parametric ordination and tests for all statistical analyses. To determine the effect of land use on DOM concentrations and composition, we compared all variables characterizing DOC and DON concentration and DOM composition ($SUVA_{254}$, FI, HIX, β:α, S_R, $DOC_{\%HMWS}$, $DOC_{\%HS}$, $DOC_{\%LMWS}$ and $DON_{\%HMWS}$, $DON_{\%HS}$, $C:N_{HS}$, %C1 to %C5) between agricultural and forested reference streams applying a paired Monte-Carlo permutation test stratified by sampling date to account for potential temporal variations (oneway_test, package coin, 9999 iterations). We determined the main factors influencing DON and DOC composition, and the variables that best explained land use patterns applying non-metric multidimensional scaling (NMDS) using the metaMDS function

(Euclidean distances, 500 iterations, vegan package). The output of an NMDS is the dimensions, which represent a new, reduced set of variables derived from the original variables (DON and DOC composition variables).[44] Prior to NMDS, we tested whether land use explained the variation in DOM composition by performing a permutational multivariate analysis of variance (MANOVA, adonis function, package vegan, 9999 iterations, stratified by sampling date). To examine if differences among individual streams (spatial variation) or seasonal differences (temporal variation) were better at explaining the variability in DOM composition, we selected a permutational MANOVA, using sampling date (spatial variation) or stream (temporal variation) as stratification factors. To determine temporal and spatial variability in DON and DOC concentrations for agricultural and forest land use, we calculated the coefficient of variation (CV) for DOC and DON concentration as standard deviation by the mean. As a measure for temporal variability, we calculated the CV mean for each stream (CV_{temp}), and as a measure for spatial variability the CV mean for all agricultural and forest streams was calculated for each sampling date (CV_{spat}). Finally, we tested whether spatial or temporal differed significantly different between agricultural and forest land by analyzing the CVs with a Mann-Whitney-U test (Wilcox.test function, unpaired, package stats). To determine the strength of the relationship between DOC or DON concentrations and discharge, as well as between the results of SEC measurement and fluorescence analysis we calculated the Spearman rank correlation coefficient.

Results and Discussion

Previous studies have shown that DOC concentrations in freshwaters across Europe and North America were altered by human land use with ambiguous altitude and direction,[14] whereas only little is known about the effects on DON.[20] Here, we investigated the effect of agriculture on DOM composition and DOC and DON quantities at catchment scale. We found clear effects of agricultural land use on DOM composition, the portions of different DOC and DON size fractions and the amounts of DOC and DON exported from the catchment, and these will be discussed in detail in the following. Due to the high percentage of agriculture in the investigation area, it was difficult to find representative forest reference catchments. In consequence, we included also forest catchments (F1, F3, F6) with a low contribution (<30%) of agricultural area at the borders of their catchment. We did not observe any effects on DOM

in these streams, which is supported by previous findings showing that the adjacent land cover is more important than the average land cover.[45-46]

Effects of agriculture on DOC and DON concentrations and specific loads. In agricultural streams, DOC and DON concentrations and specific loads were higher than in the forest reference streams ($p < 0.001$), whereby this land use effect was more distinct for DON (on average 7-fold higher) than it was for DOC (on average 3-fold higher) concentrations. Despite higher DON concentrations in agricultural streams, similar to findings in previous reports,[47] the proportion of DON to TDN was lower compared to forest streams (Table 1).

Table 1: *Mean annual concentrations of DOC, DON and DIN (± 1SD); percentage of DON of TDN (TDN was calculated as sum of DIN and DON), annual sum loads of DOC and DON, discharge-weighted mean DOC and DON concentrations (DWMC) and the mean specific DOC and DON loads from the individual agricultural (A1 to A6) and forest (F1 to F6) catchments. For the forest streams, additionally to the specific DOC and DON loads, the specific DOC and DON loads based on the calculated potential catchment size are given in Table S3.*

Stream	**Mean annual concentration**				**DWMC**	
	DOC [mg L^{-1}] (± 1 SD)	DON [mg L^{-1}] (± 1 SD)	DIN [mg L^{-1}] (± 1 SD)	DON [% TDN]	DOC [mg L^{-1}]	DON [mg L^{-1}]
A1	6.59 (2.00)	0.43 (0.21)	10.6 (5.6)	8.5 (11.3)	7.09	0.50
A2	5.88 (1.78)	0.39 (0.20)	9.7 (5.0)	5.7 (7.8)	7.36	0.46
A3	5.42 (0.59)	0.45 (0.19)	9.3 (6.4)	11.1 (13.0)	5.50	0.50
A4	8.24 (1.83)	0.53 (0.13)	6.9 (5.7)	11.4 (9.7)	8.74	0.59
A5	7.85 (1.84)	0.57 (0.23)	10.7 (2.8)	5.5 (2.7)	7.60	0.62
A6	4.58 (1.43)	0.32 (0.11)	15.9 (7.8)	2.8 (2.1)	4.27	0.32
F1	3.05 (0.95)	0.08 (0.04)	0.1 (0.1)	42.9 (12.6)	3.10	0.08
F2	1.65 (0.68)	0.04 (0.02)	1.5 (0.2)	2.5 (1.2)	1.65	0.04
F3	2.45 (1.01)	0.09 (0.02)	0.2 (0.1)	38.3 (11.7)	2.48	0.09
F4	1.84 (0.48)	0.05 (0.02)	0.1 (0.0)	46.9 (7.1)	1.87	0.06
F5	2.22 (0.78)	0.05 (0.02)	0.1 (0.0)	51.3 (9.7)	2.28	0.05
F6	1.79 (0.45)	0.05 (0.01)	0.8 (0.1)	6.6 (2.3)	1.79	0.05

Table 1: (continued).

Stream	**Specific loads**		**Stream**	**Specific loads**	
	DOC [mg C s^{-1} km^{-2}]	DON [mg N s^{-1} km^{-2}]		DOC [mg C s^{-1} km^{-2}]	DON [mg N s^{-1} km^{-2}]
A1	19.0 (39.5)	1.38 (3.59)	F1	2.1 (0.8)	0.05 (0.03)
A2	25.3 (45.3)	1.55 (2.78)	F2	13.4 (5.8)	0.31 (0.17)
A3	4.7 (8.5)	0.43 (0.72)	F3	4.9 (2.4)	0.18 (0.07)
A4	25.4 (36.6)	1.71 (2.59)	F4	5.9 (2.1)	0.18 (0.09)
A5	10.0 (11.9)	0.83 (1.20)	F5	13.8 (6.2)	0.33 (0.17)
A6	4.8 (7.4)	0.37 (0.63)	F6	13.6 (3.7)	0.40 (0.09)

The low contribution of DON to TDN in forest streams F2 and F6 is due to the high DIN concentration relative to DON. We assume that groundwater polluted by agriculture is the explanation for these increased DIN concentrations in the respective forest catchments. The increased DOC and DON concentrations are partly in conflict with previous studies comparing DOC[15, 47, 48] and DON concentrations from agricultural and less disturbed reference catchments (Table 2). One explanation might be the presence of wetlands elsewhere, which, although being small (2-6%),[47] can increase the DON and DOC concentration in streams[53] and may superimpose agricultural land use effects. In addition in larger catchments with mixed land use, different DON sources, for example wetlands, urban areas and agriculture, interfere[49-52] and obscure the effect of agriculture on DON concentration in streams. In our study, the DON concentrations were within the range of concentrations recorded in urban and agricultural streams with catchments of similar size, whereas higher DON concentrations were observed in agricultural and urban catchments with larger catchments (Table 2). An explanation for these differences can be point sources or wastewater inputs from urban areas[20] and also the position in the fluvial network, as a more downstream position can increase DON concentrations due to higher in-stream DON production.[47] For the agricultural headwater streams of this study, point sources and wastewater inputs can be excluded as DON sources. The low deviation of DOC and DON concentration from DWMC, which was within one standard deviation for all agricultural and forest streams (Table 1), indicated that there was no dilution of DOC and DON concentrations by stream water, not even with increased discharge. The specific DOC and DON loads were, on average 2 and 4 times higher and the average area based specific discharge was lower in the agricultural catchments (median: 0.9 L s^{-1} km^{-2}) than in the forest catchments (median: 4.4 L s^{-1} km^{-2}). This suggests that despite tile drainage systems, less water was exported from the agricultural catchment soils and indicates higher DOC and DON losses from agricultural soils than from less managed forest soils which is in accordance with our first hypothesis i). We attribute this to increased SOM mineralization in agricultural soils, facilitated by agricultural management practices: The additional nutrient supply due to fertilization accelerates microbial processing of SOM; consequently more DOM is released to underlying soil layers.[54] Moreover, ploughing incorporates OM into deeper soil layers and destroys soil aggregates, whereby formerly physically protected OM is released and can fuel OM turnover.[5]

Table 2: *Overview of the influence of agricultural land use on DON concentration in freshwaters*

Main findings	DON [mg*L^{-1}]	DOC:DON	Catchment area [km²]	Land use	Reference
DON concentration in headwater streams not strongly affected by urban/agricultural land use, increased DON concentration related to wastewater and wetlands	0.2 – 0.8		0.5 – 4.2	Gradient: agricultural/urban – wetland/forest	Pellerin et al.[49]
DON concentration lower in agricultural streams, DON autochthonously produced in lakes	0 - 224µmolL-1	9.3	11.9 – 179.3	Agriculture vs. forest	Stedmon et al.[47]
DON concentration increased in agricultural/urban streams relative to forest/wetland streams	0.01 - 4.3		1 – 12 665	Gradient: agriculture/urban – forest/wetland	Stanley and Maxted[50]
No effect of urban/agricultural land use DON concentration	0.1 – 1.9		2.5 – 84.1	Mixed: rural, agriculture, forest, wetland	Aitkenhead-Peterson et al.[51]
DON concentration increased with agricultral land use (Wales, Finland) with exceptions (Denmark, France)	0.1 – 3.2	11 – 20	1.3 – 49 400	Gradient: agriculture – forest/wetland	Mattsson et al.[52]
DON concentration in agricultural/urban streams increased	0.2 – 1.2	13.5 – 24.8	10 – 119 035	Gradient: agriculture/urban - forest	Petrone et al.[1]
No effect of agricultural land use on DON concentration, DON concentration related to sandy soils	0.0 – 4.3		153 - 842	Mixed: >65% agriculture, forest, pasture	Wohlfart et al.[40]
DON concentration increased in agriculture relative to forest streams	0.02 – 1.04	4.8 – 90.7	0.1 – 8.8	Agriculture vs. forest	This study

Soil tile drainage intensifies this effect on stream DOM because it shortens the retention time of soil water in the soil column and delivers DOM directly from surface soil layers, richer in SOM and DOM and most exposed to tillage and fertilization practices. A consequence of this increased export of DOM, and particularly of DOM with high nitrogen content from agriculture, is the higher likelihood of formation of toxic DBPs.[55, 56]

Effects of agriculture on DOM composition. For both systems, fluorescence analysis revealed that DOM was highly humified (HIX)[37] and aromatic ($SUVA_{254}$)[30], and it showed an overall high contribution of humic-like fluorescence components (~95%, sum %C1 to %C4). Likewise, confirming our second hypothesis ii), SEC revealed that the major part of DOC, but

also DON (> 75% on average), was bound in the form of humic-like substances ($DOC_{\%HS}$, $DON_{\%HS}$), whereby $DOC_{\%HS}$ and $DON_{\%HS}$ were higher in the forest streams ($p < 0.05$, $p < 0.001$, respectively; Fig. 1 j, k). This is in contrast to systems dominated by urban and autochthonous sources where DON was mainly present in the hydrophilic (non-humic) fraction. The contribution of low-molecular weight substances (15.8 ± 5.5%, $DOC_{\%LMWS}$) was comparatively low in all agricultural and forest streams and did not significantly differ between agriculture and forest streams ($p > 0.05$). Similar $SUVA_{254}$ values and contributions of fluorescence components and SEC size fractions have been observed for DOM in mineral soils[37, 57-58] or small creeks receiving DOM mainly from surrounding soils,[26] which distinguishes DOM in this study from DOM in lakes and larger streams with increased in-stream production and from DOM sourced in organic pollution.[26]

However, according to permutational MANOVA, streams with agricultural land use and forest land differed significantly in DOM composition (R^2=0.18, $p < 0.001$). NMDS of the composition data (chromophoric DOM, DOC and DON SEC fraction) generated a 4 dimensional model (stress: 0.074; R^2 non-metric fit: 0.995; R^2 linear fit: 0.964; Fig. 2). Samples exhibited a clear distribution in NMDS space and clustered according to their land use along the first and second dimension (axes NMDS1 and NMDS2, Fig. 2 a, b), which represented gradients of $DOC_{\%HS}$, $DON_{\%HS}$, %C3, S_R, FI, β:α and $C:N_{HS}$ for NMDS1 and gradients of %C1 and %C2 for NMDS2. The third and fourth dimensions (axes nMDS3 and nMDS4) represented primarily $DOC_{\%HMWS}$, $DOC_{\%HS}$, %C1, %C5 and HIX gradients, but the sample distribution did not exhibit any pattern related to land use in these dimension (Fig. S1 a, b).

In agricultural streams, the contribution of C3, a humic-like component[57] associated with microbial transformed material,[13] was higher compared to forest streams (Fig. 1 g; $p < 0.001$), whereas the contribution of C2, a fulvic acid-like component C2 corresponding to higher plant material,[59-61] was lower (Fig. 1 f; $p < 0.001$). Higher FI values and β:α for DOM in agricultural streams (Fig. 1 a, b; $p < 0.001$) indicate microbial-derived material or fluorophores surrounded by microbial-released material[60] and a greater contribution of more recently produced materials.[39] The higher $SUVA_{254}$ and the lower S_R for the agricultural DOM (Fig. 1 c; $p < 0.001$) can be indicative of a higher degree of microbial transformation of agricultural DOM, as increased aromaticity or a higher molecular weight of chromophoric DOM in soils were associated with progressing stages of SOM and DOM degradation.[36, 57, 62-63]

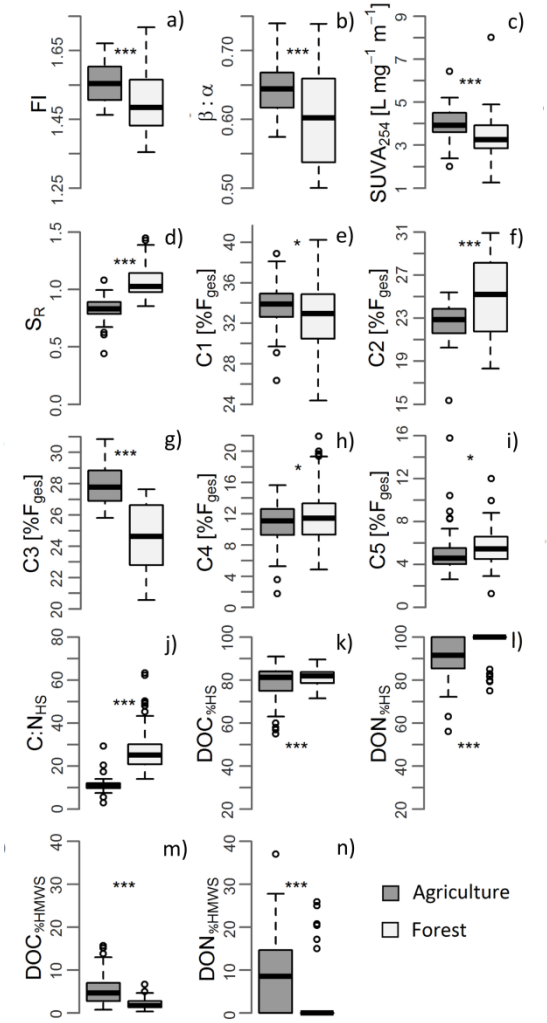


***Figure 1**. Boxplots of fluorescence (a, b) and absorbance (c, d) indices, PARAFAC components (e, f, g, h, i), C:NHS (j) and relative contributions of DOC (k, m) and DON (l, n) in the form of humic-like substances (k, l), high molecular weight substances (m, n) for agricultural land use (dark grey) and forest (light grey). Statistical significance of differences (Monte-Carlo permutation test, 9999 permutations, with sampling data as stratum) between agriculture and forest streams are marked with asterisks (*: p < 0.05, ***: p < 0.001). The individual mean, SD, median, minimum and maximum values for each parameter are given in Table S5.*

Results from SEC showed that $DOC_{\%HMWS}$ in agricultural stream samples (5.7 ± 3.7%) was more than twice as high as the forest stream $DOC_{\%HMWS}$ (2.1 ± 1.3%, $p < 0.001$), and $DON_{\%HMWS}$ was approximately five times higher in agricultural streams ($p < 0.001$, Fig. 1 m, n). $DOC_{\%HMWS}$ and $DON_{\%HMWS}$ determined by SEC include non-humic high molecular weight substances of hydrophilic character with apparent molecular weights > 10kDa as, for example, polysaccharides and proteins.[26] In soils, those compounds can be produced during microbial degradation and can be stabilized.[64-66] Otherwise, Malik and Gleixner[58] attributed the increased contribution of 'very high molecular weight' substances (> 10kDa; corresponding to

$DOC_{\%HMWS}$ and $DON_{\%HMWS}$ in the present study) in the uppermost 20 cm of a mineral soil to fresh plant-derived OM, presumably in the form of carbohydrates, alkenes and aliphatics, while old SOM-derived, microbial-transformed materials of high and low molecular weight (< 0.4, 0.4 – 10kDa) dominating in deeper soil layers.[58] Furthermore, in agricultural soils tillage can increase the release of formerly stabilized OM due to destruction of protecting soil aggregates[5]. The low $C:N_{HMWS}$ (agriculture (n = 51): 7.5 ± 5.3, forest (n=8): 4.1±2.1, others fell below detection limit) and the positive relationship between %C5 and $DOC_{\%HMWS}$ or $DON_{\%HMWS}$ (Spearman rank correlation coefficient: 0.65; 0.55) indicate a more proteinous character of the HMW DOM[67] and support the idea that increased DOC_{HMWS} and DON_{HMWS} concentrations in the studied agricultural streams are due to elevated release of HMWS of proteinous character as a result of increased OM degradation and/or release of formerly protected material from soil aggregates. The C:N ratio of DOM indicates the origin of DOM in streams[68] but also differences in the DOC and DON response to DOM processing in soil.[69] The average molar $C:N_{HS}$ and $C:N_{DOM}$ ratio were roughly two times lower ($p < 0.001$) for agricultural (11.4 ± 3.2, 13.7 ± 12.9)) than for forest streams (27.3 ± 10.1, 33.7 ± 12,9; Fig. 1 j). A possible reason for these differences in the C:N ratio can be different sources for DOC and DON in soils. While DOC release is determined mainly by the SOM content, DON release is independent of the SOM content and rather affected by the pool of inorganic nitrogen in the soil. Thereby, inorganic N fertilizer addition in agricultural soils can result in preferential release of DON from SOM.[69] Another reason for the low $C:N_{DOM}$ ratio of agricultural DOM can be that progressive OM degradation decreases the C:N ratio of SOM and DOM.[57, 66] Furthermore, in agricultural streams, organic amendments applied to the soil and washed into the streams, or in situ production of nitrogen-rich compounds, can also lower $C:N_{DOM}$.[20] Considering that the $C:N_{DOM}$ and $C:N_{HS}$ ratios in the agricultural streams were consistently low (Fig. S2), it is unlikely that they result from pulse inputs of material with a low C:N ratio added to agricultural soils or from in-stream production. The effects of organic amendments on soil DOM are short-lived and do not affect deeper soil layers,[7, 70] and are therefore unlikely to permanently influence stream DOM.

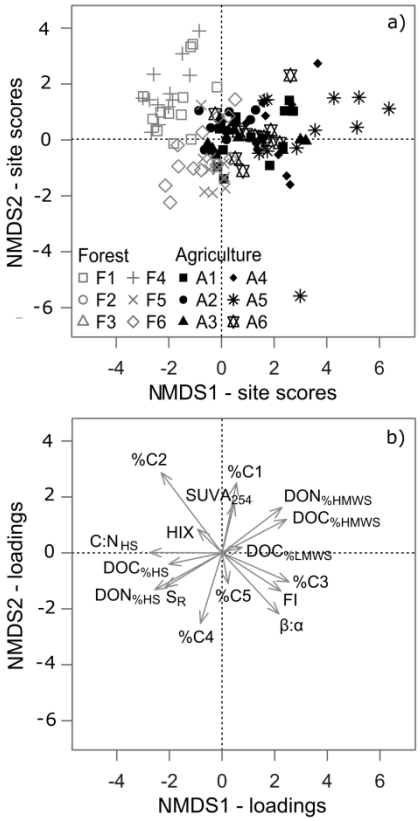


Figure 2. *Results of the NMDS ordination. The scores for dimensions 1 and 2 are shown in panel a) with agricultural samples marked in black and forest samples in grey. The factor loadings for dimensions 1 and 2 are shown in the panel b) with the length of the arrows indicating the importance/contribution of the respective variable to the dimension. Scores and loadings for dimensions 3 and 4 are shown in the supplementary data (Fig. S1).*

Higher rates of primary production would produce low C:N ratios during times of high productivity[71] and result in lower complexity of DOM. Therefore, in accordance with Accoe et al.[72], we conclude that low C:N ratios of DOM or HS are the result of long-term microbial degradation processes in agricultural soils. Altogether, DOM in agricultural headwaters appeared to be mainly complex soil-derived and aromatic material with a low C:N ratio, which is in accordance with our hypothesis iii) that more microbial processed than its counterpart from forest reference catchments. Previously, the shift of stream DOM to a more microbial character, along with decreased DOM complexity, has been attributed to both increased autochthonous DOM production and allochthonous DOM input.[12-14] Here, we identified agricultural soils as being the main source of complex, microbial-processed, nitrogen-rich DOM in the investigated headwater streams.

Seasonal patterns of DOC and DON concentration, specific loads and DOM composition. During the one-year observation period, DOC and DON concentrations in agricultural and forest streams exhibited no consistent temporal pattern (Fig. 3 a, c) and increased at the highest discharge levels only. Furthermore, we observed no, or only weak, correlations between DOC and DON concentration and discharge (Spearman rank correlation coefficient < 0.03) in agricultural and forest streams. Only the DON concentration in forest streams correlated slightly with discharge (Spearman rank correlation coefficient: 0.59), which needs further investigation.

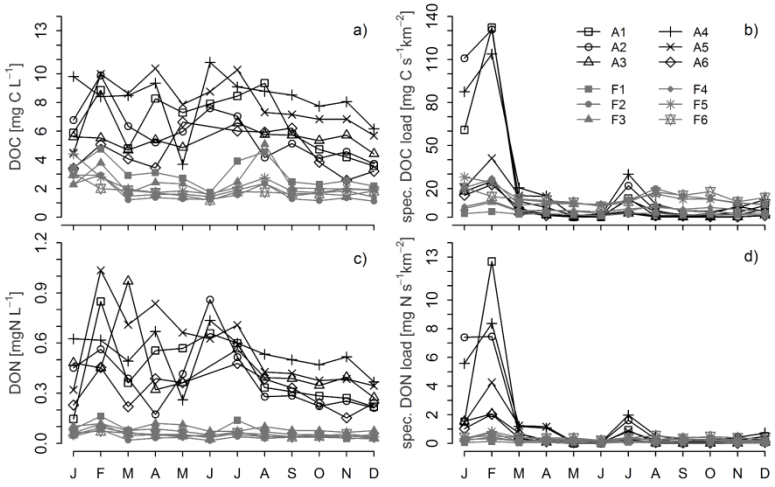


Figure 3. *Seasonal variation in DOC (top) and DON (bottom) concentrations (a,c) and specific load (b,d) over a one-year period for agriculture (black) and forest (grey) land use. Specific loads refer to yields (mass of DOC and DON exported) per catchment area unit. A1-A6 and F1-F6 represent the individual agricultural and forest streams.*

Overall, no differences in temporal variability of DOC and DON concentrations (CV_{temp}) were observed between agricultural (DOC: 0.25, DON: 0.40) and forest streams (DOC: 0.33, DON: 0.35) ($p > 0.05$). Similarly, spatial variability in DOC and DON concentrations (CV_{spat}) between agricultural (DOC: 0.28, DON: 0.32) and forest streams (DOC: 0.28, DON: 0.38) did not differ ($p > 0.05$). In contrast to the DOC and DON concentrations, we found a temporal pattern for the specific DOC and DON loads, which differed between agricultural and forested reference streams (Fig. 3 b, d). In agricultural streams, we observed the highest specific DOC and DON loads during February (DOC: 25.2 – 132.3 mg C s^{-1} km^{-2}, DON: 2.0 – 12.7 mg N s^{-1} km^{-2}) which were higher than the annual average (4.7 – 19.0 mg C s^{-1} km^{-2}, 0.4 – 1.7 mg N s^{-1} km^{-2}). Also discharge was higher in February (median for all streams, February: 22.8 L s^{-1}) compared to the annual median value (2.3 L s^{-1}) in agricultural streams, whereas the

DOC and DON concentrations did not change. This indicates that the variations of specific loads are mainly discharge driven. In forested reference streams, the variability of the specific loads over the year was much lower (Fig. 3 b, d). The high specific loads observed only during very short time periods, particularly in the agricultural streams, are consistent with results of Dalzell et al.[73], who reported that 70 to 85% of the total annual organic carbon was exported during 20% of the time in an agricultural catchment. We attribute the increased variability in specific DOC and DON loads to tile drainage, resulting in a shortcut of the hydrological pathway and, consequently a more immediate response to precipitation events.

In contrast to our expectations, DOM composition did not exhibit a consistent temporal pattern in agricultural streams. NMDS analysis and MANOVA showed that spatial variability (R^2=0.47, $p < 0.001$) explained the variation in DOM composition better than temporal variability ($R^2 = 0.17$, $p < 0.001$). Hence, land use and differences among individual streams had a stronger effect on variation in DOM composition than any temporal changes of environmental conditions (e.g. temperature). In detail, we did not observe any consistent temporal pattern for chromophoric DOM composition in the agricultural streams (CV < 0.5), except for %C1 and %C4 which covaried as in the forest streams (Fig. S1). In the forest streams, some parameters for chromophoric DOM composition (HIX, $SUVA_{254}$, %C1, %C4) showed a temporal pattern, while others varied without a consistent pattern or did not vary at all (FI, β:α, %C2, %C3, %C5, S_R). During late summer (August, September), the DOM composition in the forest streams shifted to a less humified (HIX), less aromatic ($SUVA_{254}$) character and a higher C:N ratio (Fig. S1), which suggests input of fresh plant material due to leaf senescence. The relative contributions of the different DOC and DON SEC fractions did not exhibit a consistent pattern in either the forest streams, or the agricultural streams. In the agricultural streams, we observed a higher variability in SEC DOC and DON fractions, particularly $DOC_{\%HMWS}$ and $DON_{\%HMWS}$. The absence of a distinct temporal pattern for DOM composition and DOC and DON concentrations in the agricultural streams in this study contrast our hypothesis iv) and a range of previous studies,[74-76] and may be explained by the various forms of agricultural management practices (including irrigation practices),[75-76] climatic conditions, or the position of the sites in the fluvial network of the different studies. The absence of a pronounced temporal pattern for DOM composition in this study contrasts with the results of Royer and David[74] based on a shift from mainly allochthonous DOM sources from winter to

early summer, which were delivered by tile drainage system to streams, to more autochthonous DOM sources from summer to late autumn. A consistent temporal variability pattern in protein-like fluorescence or $DOC_{\%LMWS}$ for both agricultural and forest streams would indicate the influence of in-stream DOM production during periods of increased primary production.[77-78] The absence of such a pattern supports the idea of terrestrial sources being dominant in shaping the character of DOM consistently over seasons and suggests that DOM in the investigated agricultural streams was more closely associated with residuals and metabolites derived from microbial OM processing in the soil, than being the result of in-stream production.

Associated content

Supporting Information.

Details about sample processing, nitrate and ammonia analysis (S1); details about absorbance and fluorescence measurement, preprocessing of raw fluorescence and absorbance data and PARAFAC analysis (S2); figures of scores and loadings of NMDS dimensions 3 and 4 and the temporal variation in DOM composition (Figure S2); detailed information on catchment characteristics (land use, soil, catchment area, discharge) (Table S1), characterization of the modelled PARAFAC components (Table S2); specific DOC and DON loads for the forest streams based on the calculated potential catchment size (Table S3): details on DOC, DON, DIN, DWMC and specific loads for the individual streams during individual sampling campaigns (Table S4). This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations

DOM, dissolved organic matter; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; OM, organic matter; SOM, soil organic matter; TDN, total dissolved nitrogen; SEC, size exclusion chromatography; PARAFAC, parallel factor analysis; EEM, excitation-emission matrix; DWMC, discharge-weighted mean concentration; HS, humic-like substances; HMWS, high molecular weight substances; LMWS, low molecular weight substances.

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Study 2:

Global effects of agriculture on fluvial dissolved organic matter

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Global effects of agriculture on fluvial dissolved organic matter

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Agricultural land covers approximately 40% of Earth's land surface and affects hydromorphological, biogeochemical and ecological characteristics of fluvial networks. In the northern temperate region, agriculture also strongly affects the amount and molecular composition of dissolved organic matter (DOM), which constitutes the main vector of carbon transport from soils to fluvial networks and to the sea, and is involved in a large variety of biogeochemical processes. Here, we provide first evidence about the wider occurrence of agricultural impacts on the concentration and composition of fluvial DOM across climate zones of the northern and southern hemispheres. Both extensive and intensive farming altered fluvial DOM towards a more microbial and less plant-derived composition. Moreover, intensive farming significantly increased dissolved organic nitrogen (DON) concentrations. The DOM composition change and DON concentration increase differed among climate zones and could be related to the intensity of current and historical nitrogen fertilizer use. As a result of agriculture intensification, increased DON concentrations and a more microbial-like DOM composition likely will enhance the reactivity of catchment DOM emissions, thereby fuelling the biogeochemical processing in fluvial networks, and resulting in higher ecosystem productivity and CO_2 outgassing.

The environmentally safe operating space of humanity on Earth is limited and some Earth-system processes, such as the nitrogen cycle and the climate system are already beyond their limits[1]. The climate system[2], as well as the nitrogen and other biogeochemical cycles[3] are significantly affected by the global carbon transport from soils to freshwaters, and human activities have altered this transport from approximately 1.1 Pg yr^{-1} to 1.9 Pg yr^{-1}[4]. This carbon flux is mainly organic[2], and the largest part of it is DOM[5]. The molecular composition and DON content of this DOM can be heavily altered by changes and intensification in agricultural land use[6–8].

Changes in quantity and composition of DOM exported from soils can strongly affect the receiving aquatic ecosystems, by changing their metabolism, light regime, as well as by modulating the activity of other chemicals and biological processes[9,10]. Furthermore, DOM reactivity in fluvial networks determines the DOM quantity and composition entering marine environments and the carbon reaching the atmosphere[11,12]. In fact, a recent study suggests that the outgassing of CO_2 from fluvial networks and lakes to

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the atmosphere is approximately twice as high as previously thought and calls for better understanding of the sources of this CO_2 [13].

Agricultural practices can alter both DOM amount and composition in temperate fluvial networks through alterations of hydrological flow paths, changes of the soil organic matter pool and altered aquatic microbial processing[10]. Relative to pristine, undisturbed catchments, DOM exported from agricultural catchments is often altered in a way which enables it to be potentially more reactive in aquatic ecosystems[6–8,14], and therefore it may increase productivity, outgassing and burial of carbon in fluvial networks[11,12]. However, so far it remains unclear if the effect of agriculture is a global phenomenon, relevant to the global carbon cycle and coupled elemental cycles[3].

To address this issue, we sampled headwater streams draining 45 reference and 75 agricultural catchments situated within five climate zones in the northern and southern hemispheres. The chosen climate zones include some of the largest and most rapidly intensifying areas of agriculture worldwide[15]. We tested, if agricultural land use results in similar effects on fluvial DOM, independent of the global region in which the samples have been taken. Two major types of agriculture, as well as reference catchments were investigated: (i) arable farming with soil tillage, artificial fertilization and with partial drainage (intensive farming), which covers approximately 12% of Earth's land surface area, (ii) livestock production on permanent grasslands (extensive farming), which covers approximately 26% of Earth's land surface area[15] and (iii) pristine, reference catchments with natural vegetation but otherwise similar characteristics as the agricultural catchments. Fluvial DOM samples for intensive farming were taken in headwater catchments situated in northern temperate (lowlands of Germany and Denmark), Mediterranean (North-east Spain), subtropical (grasslands of Uruguay) and tropical (transition zone between the Brazilian Cerrado and the Atlantic Forest) climate. Samples for extensive farming were taken in northern temperate, subtropical and tropical climate, and also in southern temperate climate (lowlands of North Patagonia, Chile). In each of the climate zones, samples were taken in each main season (winter and summer or wet and dry season). Dissolved organic carbon (DOC) and DON concentration and composition were measured by size-exclusion chromatography and by fluorescence measurements[16–18]. Based on the fluorescence measurements, five fluorophores were modelled (C1-C5) by parallel factor analysis[18]. Dissolved inorganic nitrogen (DIN, nitrate + nitrite and ammonium) concentration was also measured in all samples.

Results

Across climate zones, intensive farming resulted in a general significant increase of DON and DIN concentrations relative to the reference catchments ($p < 0.001$, Monte-Carlo resampling test). Separate analyses of the individual climate zones show that the effect of intensive farming on DON and DIN concentrations was largely driven by the catchments in northern temperate and Mediterranean climate (Fig. 1a,b). Moreover, DIN concentration increased significantly in catchments with extensive farming, but to a lesser extend than for intensive farming ($p < 0.001$, Fig. 1c). No general effect of intensive farming on DOC concentrations or of extensive farming on DOC or DON concentrations was found ($p > 0.05$).

Both intensive farming ($p < 0.001$, permutational MANOVA) and extensive farming ($p = 0.034$) affected the molecular composition of DOM across climate zones. Separate tests of the individual climate zones show that intensive farming affected in-stream DOM composition in the northern temperate and Mediterranean climate ($p < 0.001$ and $p = 0.002$), and extensive farming affected in-stream DOM composition in the northern temperate and subtropical climate ($p = 0.002$ and $p = 0.010$). The described effects were found in both main seasons, indicating a stable source of altered fluvial DOM in the agricultural catchments.

Across climate zones, fluvial DOM from agricultural catchments was generally more microbial in character and less characteristic of higher terrestrial plant sources than DOM from reference catchments. For intensive farming, this was evident from i) a higher fluorescence index (FI, $p < 0.001$, Monte-Carlo resampling test, Fig. 2a), indicating a more microbial source[19]; ii) a higher freshness index (FreshIndex, $p < 0.001$), indicating a rather recent, microbial DOM source[19]; iii) a lower humification index (HIX, $p = 0.001$), indicating less complex material[19]; iv) a lower C:N ratio ($p < 0.001$), indicating a lower content of refractory carbon from higher-plant sources[20]; v) more carbon in the proteinuous/polysacharide, high-molecular weight chromatographic fraction (HMWS-C, $p < 0.001$) and less carbon in the humic-like chromatographic fraction (HS-C, $p = 0.019$), indicating a shift from plant to microbial origin[21]; vi) more protein-like fluorescent DOM (fluorophore C5, $p = 0.028$) and a shift from plant-derived (fluorophore C2, $p < 0.001$) to microbially-derived fluorescent DOM (fluorophore C3, $p < 0.001$, Fig. 2b)[19]. For extensive farming, the effect was similar and evident from i) a higher FI ($p = 0.028$, Fig. 2b); ii) a higher FreshIndex ($p = 0.013$); iii) a lower C:N ratio ($p = 0.013$) and a shift from fluorophore C2 ($p = 0.026$) to fluorophore C3 ($p = 0.035$).

The aforementioned higher DON concentrations and the microbial-like character of fluvial DOM from intensive farming catchments was correlated to higher DIN concentrations (permutational MANOVA, $p < 0.001$, Fig. 2), whereas no overall relationship for the catchments with extensive farming was found ($p = 0.42$, Fig. 2). In detail, the changes of DOM composition and DON concentration in intensive farming were positively correlated to DIN concentrations in the subtropical, northern temperate and Mediterranean climates ($p < 0.05$, Spearman rank correlation, Fig. 2a). For extensive farming,

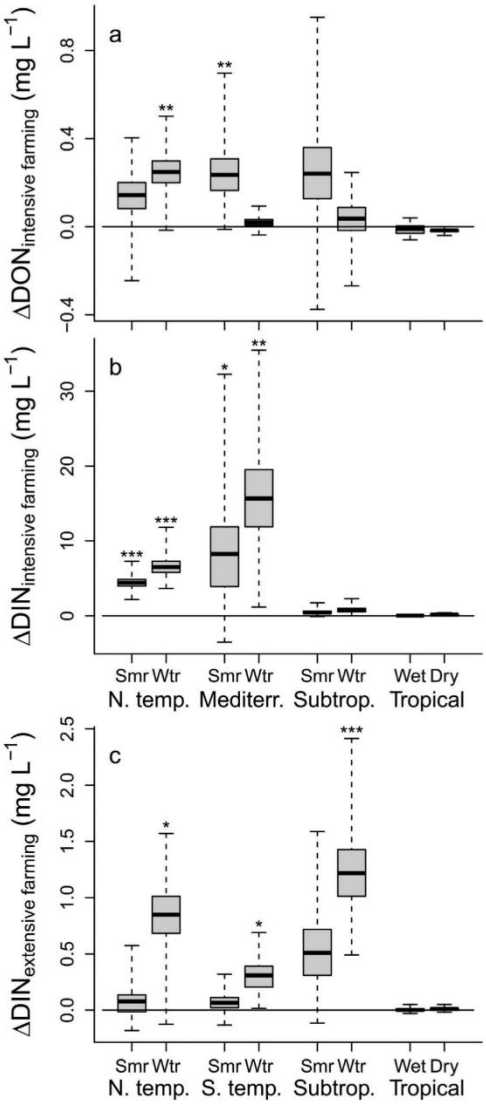

Figure 1. Effects of agriculture on fluvial dissolved nitrogen concentrations. Effect of intensive farming on dissolved organic nitrogen concentrations (panel (**a**)) and both intensive farming and extensive farming on dissolved inorganic nitrogen concentrations (panels (**b**,**c**) DIN = sum of nitrate and ammonium) concentrations. Due to lacking significance, the effects on DON are not shown for extensive farming. Boxplots show median (line), interquartile range (box) and data extremes (whiskers). The errors of the differences were calculated as bootstrap standard errors. p were calculated by Monte-Carlo resampling tests: $^{***}p < 0.001$ $^{**}p < 0.01$, $^{*}p < 0.05$; Smr = Summer, Wtr = Winter, N. temp. = northern temperate, S. temp. = southern temperate, Mediterr. = Mediterranean, Subtrop. = subtropical.

these changes were only positively correlated to DIN concentrations in the subtropical climate zone ($p < 0.05$, Fig. 2b).

The observed variation of the effect of agriculture on DIN and DOM (Figs 1 and 2) may be explained by the current and historical intensity of nitrogen fertilizer application in the investigated climate zones. This is supported by strong differences in the general temporal development of the intensity of intensive farming in the different countries (Fig. 3): The magnitude of the nitrogen fertiliser application in agriculture peaked in the 1980s for the countries sampled in the northern temperate climate (Germany and Denmark, Fig. 3). In all other countries a continuing increase is apparent, with Chile being the most extreme case (Fig. 3).

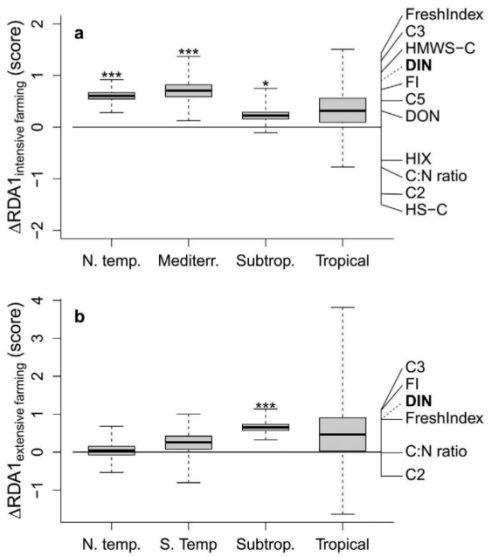

Figure 2. Effects of agriculture on fluvial dissolved organic matter composition. The shown scores and loadings are based on the first axis of a redundancy analysis (RDA1). Boxplots show median (line), interquartile range (box) and extremes (whiskers) of the effects for intensive farming (panel (**a**)) and extensive farming (panel (**b**)) on RDA1 scores for DOM. Stacked lines on the right show the loadings of DOM composition variables on RDA1 and the correlation to nitrate and ammonium (marked in bold, with dotted line). The errors of the differences were calculated as bootstrap standard errors. Asterisks indicate the climate zones, in which the scores of RDA1 where significantly affected by DIN: $^{***}p < 0.001$, $^{*}p < 0.05$ (Spearman correlation). N. temp. = northern temperate, S. temp. = southern temperate, Mediterr. = Mediterranean, Subtrop. = subtropical, C2, C3, C5 = contribution of PARAFAC components 2, 3 & 5 to total sample fluorescence, HIX = humification index, FreshIndex = freshness index, HS-C = contribution of humic substances to DOC, HMWS-C = contribution of non-humic high-molecular weight substances to DOC, C:N ratio = C:N ratio of bulk DOM.

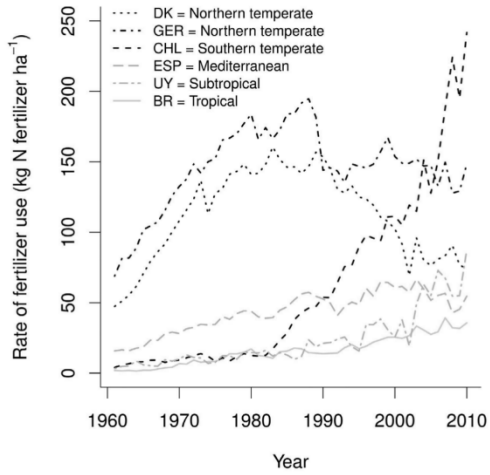

Figure 3. Intensity of total nitrogen fertilizer application in arable farming from 1961–2010. Data from country-wise data base of the FAO (http://faostat.fao.org). DK = Denmark, GER = Germany, CHL = Chile, ESP = Spain, URU = Uruguay, BR = Brazil.

Discussion

Intensive farming resulted in a concomitant increase of both DIN and DON concentrations in the investigated catchments. Furthermore, fluvial DOM from agricultural catchments was generally more microbial in character and less characteristic of higher terrestrial plant sources than DOM from reference catchments. Based on this, we conclude that the effect of agriculture on DIN concentrations is correlated to the effect of agriculture on DON concentrations and DOM composition. Here, higher DIN concentrations may not directly drive the changes in fluvial DOM. However, our results suggest that mechanisms related to intensification of agriculture could be the source of the relationship between increased DIN concentrations and changed character of fluvial DOM. Intensified soil tillage, drainage and fertilization, either as separate mechanisms or in combination, could affect the DOM exported to fluvial networks[7,8].

The past and current fertilizer use intensity was high in the same regions in which we found the strongest effects of intensive farming on fluvial DIN concentrations, DON concentrations and DOM composition. This data supports the aforementioned idea that intensive agricultural management, including intensive fertilizer, the use of heavy machinery, intensive soil tillage, drainage and the intensive use of pesticides alters the soil microbial processing of DOM. Moreover, a further intensification of agriculture is expected in developing countries, since in addition to population growth, the per capita food demand will increase with increasing gross-domestic product in the future[22]. Therefore, the strong effects of intensive farming on fluvial DOM composition which we found in the northern temperate climate are a potential future scenario in regions with a currently lower intensification of agriculture.

Due to the spatial extent of agriculture[15] and its contribution to anthropogenic carbon losses[4,5], it can be assumed that fluvial DOM from agriculture is a major carbon source to aquatic ecosystems. Moreover, according to laboratory studies it is likely that agricultural DOM with higher contents of DON is of higher reactivity and will be mineralized faster than DOM from comparable reference catchments[6,14]. Hence, global intensification of agriculture may result in the release of large amounts of biogeochemically reactive DOM to fluvial networks, thereby altering the biogeochemical cycles related to DOM and increasing the productivity, respiration and outgassing of CO_2 from fluvial networks on a global scale.

Methods

Sampling. We sampled headwater streams in catchments with a size ranging between 0.1–46.6 km^2 (Supplementary Catchment Data). Catchments selected for dominant arable farming or livestock production, exhibited the respective land use on >50% of their area (Supplementary Catchment Data). In reference catchments, the reference vegetation type covered ≥60% of the area (Supplementary Catchment Data). Water samples were immediately filtered through 0.45 μm filters and frozen within 24 h for transport. All samples were analysed at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries in Berlin.

Spectroscopic analyses of DOM. Excitation was measured from 240–450 nm in 5 nm steps and emission was measured from 300–600 nm in 2 nm steps. Both were measured with a bandwidth of 5 nm and a speed of 700–1500 nm s^{-1} depending on the sample concentration, using a Perkin-Elmer LS-50B fluorescence spectrometer (Rodgau, Germany). All samples were measured at room temperature. To correct for inner-filter effects, absorbance was measured on a Shimadzu UV-2401 UV/Vis spectrophotometer (Duisburg, Germany), using the same 1 cm quartz glass cuvettes as used for the fluorescence measurements.

We used the drEEM toolbox to standardise all measured excitation-emission-matrixes (EEMs)[18]: In detail, spectral correction was conducted based on instrument-specific values for excitation and by a correction kit for emission (BAM fluorescence calibration kit)[23]. Inner-filter effect correction was conducted based on absorbance measurements[18]. All samples were Raman-normalized based on measurements of the Raman peak at 350 nm.

Based on the fluorescence measurements, three indices were calculated: i) the fluorescence index (FI), which indicates a more microbial (FI ~ 1.9) or a more terrestrial higher plant (FI ~ 1.4) origin of the DOM[19], ii) the freshness index (FreshIndex), which indicates the freshness of the material with values >1 representing freshly produced DOM, and values of 0.6–0.8 representing rather decomposed DOM and iii) the humification index for which higher values indicate more humified DOM[19]. A parallel factor analysis (PARAFAC) model with five components was validated by using residual and sum-of-squared-error investigation, as well as split-half validation (Supplementary Fig. S1) and random initialisation with 20 iterations[18].

The character of the components was interpreted based on the fluorescence maxima and spectra (Supplementary Fig. S1 and Supplementary Table S1). C1 and C4 resembled terrestrial humic-like fluorophores exported ubiquitously from catchments[7,24] and potentially susceptible to photodegradation[25]. C2 resembled a ubiquitous fulvic-like fluorophore and C3 a humic-like fluorophore dominating agricultural DOM[7,24]. The ratio of C2 and C3 was shown to indicate higher-plant (C2) or microbial (C3) sources of DOM[26]. A component similar to C3 was also linked to bacterial production and arable farming in a Canadian study[6]. C5 resembled a tryptophan-like fluorophore and is part of the protein-like fluorescence, which is positively related to the microbial availability of DOM[19,24].

Chromatographic analysis of DOM. Size-exclusion chromatography (SEC) was applied to analyse the molecular-size composition of DOC and DON, and the sum of the DOC and DON molecular-size fractions was used to represent the DOC and DON concentrations. The system used in this study was developed by Huber *et al.* (2011)[16] and the direct measurement of DON with high accuracy was demonstrated in freshwaters for this SEC system by Graeber *et al.* (2012)[17].

In SEC, a combination of UV- and IR- organic carbon detection and UV- organic nitrogen detection was used[16,17]. This procedure differentiated between non-humic high molecular weight substances (HMWS) of hydrophilic character (polysaccharides, proteins, amino sugars), humic-like substances (HS) with higher aromaticity based on UV measurements at 254 nm, and between low-molecular weight acids and neutrals which were combined as the low-molecular weight fraction in this study (Supplementary Fig. S2, LMWS)[16,17]. LMWS referred to neutral, hydrophilic to amphiphillic substances (alcoholes, aldehydes, ketones, sugars, amino acids)[16]. The humic-like substance fraction in SEC had a similar column retention time as humic and fulvic substance extracts provided by the International Humic Substance Society[16]. The DON measured by SEC did not include the LMWS fraction, since it could not accurately be differentiated from nitrate[16]. This fraction contains very little DON in natural freshwaters, usually not affecting DON determination with SEC[17].

Analysis of dissolved inorganic nitrogen. Nitrate (measured as nitrate plus nitrite) and ammonium concentrations were measured by standard spectrophotometric methods (ISO 13395 and ISO 11732).

Statistics. Since assumptions for parametric statistics often were not fulfilled, non-parametric tests were conducted.

Monte–Carlo resampling tests of the effect of land use on DIN, DOC and DON concentrations and single variables of DOM composition were conducted with the coin package[27] in R (version 3.0[28]) using the interaction of climate zone and season as stratum (block). Permutational MANOVAs of the effect of land use on DOM composition were conducted with the vegan package[29] in R, based on Euclidean distances and climate zone and season as strata. When the effects of land use in single climate zones were analysed by Monte–Carlo resampling tests and permutational MANOVAs, season was used as stratum. All tests were conducted with 9999 iterations.

The redundancy analysis (RDA) on the relationship of DOM composition to the DIN concentration (sum of nitrate + nitrite and ammonium) was performed with the rda function (vegan package) as partial RDA, with climate zone and season as constraints. Significance of the RDA model, axes and terms was tested with permutational ANOVAs of the vegan package[29]. Permutational Spearman tests were conducted on the RDA site scores from RDA axis 1 with Monte–Carlo resampling (coin package). All tests were conducted with 9999 iterations.

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Author Contributions

All authors worked on the sampling design and participated in writing and revising of the manuscript. In addition, D.G. conducted field work, laboratory measurements and data analyses; M.H. conducted field work, laboratory measurements and GIS data analyses; I.B., B.G., M.M. and D.S. conducted field work and participated in data analyses; J.G., F.E., C.E., G.G. and J.N. conducted field work and GIS data analysis; J.G. and E.Z. conducted laboratory measurements and participated in data analyses; B.K. and M.P. participated in data analyses; R.S. conducted field work.

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Study 3:

Absence of dissolved organic matter degradation by stream biofilms in a laboratory experiment with different DOM composition and nutrient concentration scenarios

Marlen Heinz, Daniel Graeber, Daniel von Schiller und Martin T. Pusch

Absence of dissolved organic matter degradation by a stream biofilm in a laboratory experiment with different DOM composition and nutrient concentration scenarios

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Abstract

Dissolved organic matter (DOM) plays a central role in aquatic ecosystems, and its source and composition can strongly alter its biodegradability. Moreover, additional inorganic nutrient supply can affect the biodegradability of DOM and the associated organic nitrogen (DON) and carbon (DOC) subpools. However, the relevance of the DOM composition and additional inorganic nutrient supply for DOC and DON biodegradation is not yet completely elucidated, in particular lacking for streams with high inorganic nutrient concentrations. In a controlled laboratory experiment, we exposed a heterotrophic benthic river biofilm to different sources of DOM (agricultural and forest) and low and high concentrations of inorganic nutrients. Changes in DOC and DON concentration as well as in DOM composition were recorded during an 81 day period using size exclusion chromatography (SEC) and fluorescence and absorbance analysis. Our results show that, independent of the source of DOM and level of inorganic nutrient concentration, neither DOC nor DON was significantly biodegraded during the experiment, presumably due a notable lack of dependence on external sources.

Introduction

Agricultural land use covers 40% of the global land surface (Foley et al., 2005), and recent studies have demonstrated the strong influence of agricultural land use on the composition and concentrations of stream dissolved organic matter (DOM) (Graeber et al., 2012, 2015; Heinz et al., 2015). In aquatic systems, DOM plays a key role for ecosystem metabolism and the carbon (C) cycle, as well as for regulating the fluxes of dissolved nutrients and the bioavailability of metals and organic pollutants (Prairie, 2008; Stanley et al., 2011).

Information on whether increased inputs of inorganic nutrients, an altered DOM composition, or a combination of these determine DOM availability in agricultural streams will contribute to the prediction of changes in C cycling, in the downstream fate and processing of DOM, as well as in nutrients and harmful substances associated to DOM. Also, it and will help water managers to decide on which tools to implement to mitigate the potential adverse effects of DOM on aquatic ecosystems.

The composition of DOM in streams varies with land use, and with higher agricultural land use in the catchment DOM composition attains a more microbial-derived and structurally less complex character (Daniel Graeber et al., 2012; Heinz et al., 2015; Williams et al., 2010; Wilson and Xenopoulos, 2009). In terms of elemental composition, DOM mainly consists of dissolved organic carbon (DOC) and nitrogen (DON). The C:N ratio of DOM ($C:N_{DOM}$) tends to be lower in agricultural streams with intensive farming than in natural steams (Graeber et al., 2015a, 2015b; Heinz et al., 2015). This is indicative of increases in the DON concentration relative to the DOC concentration and, accordingly, different responses of DON and DOC to agricultural land use.

Bacteria often consume DON more likely than DOC (Kaushal and Lewis, 2005; Petrone et al., 2009), whereby they utilize DON-rich DOM for efficient growth, whereas DOC-rich DOM is mostly used for respiration (Wiegner et al., 2006; Wiegner and Seitzinger, 2004). The form in which DON and DOC are bound may determine their biodegradability as DON bound to non-humic, microbial fractions of DOM has been shown to be bioavailable (Kaushal and Lewis, 2005). Moreover, in bioavailability experiments using DOM from different aquatic sources more DOC was consumed in the high-molecular fraction (> 1 kDa) than in the low-molecular fraction (< 1 kDa) (Amon and Benner, 1996). In small forest and agricultural headwater streams, more than 75% of DON and DOC can be found in the humic-like fraction with a molecular size of ~ 1 – 10 kDa, with the humic-like fraction in the agricultural streams having significantly lower C:N ratios (Graeber et al., 2015b; Heinz et al., 2015). We propose that the lower C:N ratio of DOM in agricultural streams results in higher microbial uptake of DOC and especially of DON out of this humic-like fraction.

In addition to the effects of DOM composition on its degradability (Volk et al., 1997; Fellman et al., 2008; Williams et al., 2010), recent findings within soil and aquatic sciences suggest that environmental conditions control the biodegradability of DOM rather than

properties of DOM (Marín-Spiotta et al. 2014; Schmidt et al. 2011). For instance, additional inorganic nutrient supply in forest headwater streams has been shown to enhance DOC consumption, even that of humic-like DOM (Mineau et al., 2013). Furthermore, in small North American natural mountain streams DON was either consumed at low availability, or generated at high availability of inorganic nutrients, while DOC remained unimpacted (Kaushal and Lewis, 2005). Similarly, in low nitrate streams in North America, DON acted as a nutrient source when dissolved inorganic nitrogen (DIN) was added, whereas DOC showed a weaker response to elevated dissolved inorganic nutrient levels (Wymore et al., 2015). This result was attributed to reduced consumption of DON in favor of more available DIN (Wymore et al., 2015). So far, the relationship between DOM biodegradability and inorganic nutrient concentrations has been investigated in streams with low ambient inorganic nutrient concentrations or moderate nutrient increases (< 1mg N L^{-1}, < 0.5 mg P L^{-1}; Brookshire et al. 2005; Kaushal and Lewis 2005; Mineau et al. 2013, Wymore et al., 2015). Information on DOC and DON availability under conditions with elevated inorganic nutrient concentrations, such as those typically observed in agricultural streams, are lacking.

Here, we aim to assess the relevance of DOM composition and inorganic nutrient availability for DOM biodegradability. To achieve this, we set up a laboratory experiment in which we exposed a heterotrophic benthic river biofilm to different sources of DOM (agricultural and forest) and low and high concentrations of inorganic nutrients. During the 81 days of the experiment, we monitored the changes occurring in DOC and DON concentrations and DOM composition. We hypothesized that: i) Agricultural DOM (higher DON concentration and lower $C:N_{DOM}$) is more degradable by heterotrophic stream biofilms than forest-derived DOM (lower DON concentration and higher C:N ratio). ii) Addition of DIN reduces the uptake of DON but increase the uptake of DOC due to more easily available nitrogen and phosphorus, allowing higher uptake of DOC and reducing the need to take up nitrogen from DON. iii) DON and the non-humic/less aromatic fraction of DOM are preferentially consumed relative to DOC, resulting in a higher $C:N_{DOM}$ and increased complexity and aromaticity of the remaining DOM over time.

Methods

Experimental setup and sampling

To assess the effects of DOM source and inorganic nutrient (nitrogen (N) and phosphorous (P)) concentrations on DOM biodegradability, we incubated DOM from two different sources (agriculture (A), forest (F)) together with a benthic stream biofilm and added dissolved inorganic nutrients (measured as dissolved inorganic nitrogen (DIN) and soluble reactive phosphorous (SRP)) at low (AL and FL: 1 mg L^{-1} DIN, 0.03 mg L^{-1} SRP) and high (AH and FH: 10 mg l^{-1} DIN, 0.1 mg l^{-1} SRP) concentrations. Agricultural and forest DOM sources were prepared concentrating composite stream water samples of forest and agricultural streams located within the catchment of the River Spree (NE Germany) using tangential flow filtration (1 kDa cutoff). Further details on the preparation of DOM sources and the treatments can be found in the supplementary material (S1). The biofilm used for the bioassays was previously grown during 8 weeks of incubation on small (~1 cm^2) marble mosaic tiles in the River Spree and conditioned for 2 weeks in the dark at ambient stream water temperatures (average annual temperature in River Spree, 11°C) in a climate chamber. The River Spree is a heterotrophic system with the highest bacterial activity per volume occurring in the benthic zone (Fischer and Pusch, 2001), and it is the receiving system for the headwater streams from which the source of the composite agricultural and forest DOM samples were derived. At the start of the experiment, we placed 5 randomly collected tiles in each of the sterile 450-mL cell culture flasks (PS, TPP) containing a 250 mL sample of the respective treatment (AL, AH, FL, and FH). We additionally set up controls (AB and FB; only DOM 0.45µm filtered and sterile tiles) with 5 replicates per sampling to account for abiotic factors (absorption to flask, precipitation, photodegradation, etc.) and possible degradation of DOM by residual bacteria remaining in the DOM sample after 0.45µm filtering. The flasks were closed with a filter screw cap and shaken on an orbital shaker (90 rpm) to enable aeration.

We sampled the treatments and the control at the start (day 0) and after 4, 9, 27 and 81 days of the experiment to assess short- and long-term changes in DOM concentrations and composition. On each sampling occasion, we removed 5 flasks for each treatment and control; i.e. the samples were independent of each other over time. For the agricultural control (AB) on sampling day 4, only 3 of 5 replicates were available due to accidental loss of incubation flasks during sampling. From the water column, 150 mL sample water were taken

and filtered through a 0.45µm syringe filter (cellulose actetate (CA), Minisart, Sartorius) for DOC, DON, DIN and SRP determination as well as absorbance and fluorescence measurements. Before sampling the filter was rinsed with 150 ml deionized water and 50 ml sample. Samples for DON, DON and DIN analysis were frozen until measurement, while SRP and optical measurements were performed no later than after 12 h storage at 5°C. For each replicate of the treatments, we preserved 3 tiles in formalin for bacterial abundance analysis. For the control no biofilm was sampled, since there was no biofilm available on the tiles.

Laboratory analyses

We colometrically determined DIN and SRP concentrations (details can be found in supplementary material, S2). For the determination of DOC and DON concentrations and respective fractions, we used size exclusion chromatography (SEC) combined with UV- and IR-organic carbon detection and UV-organic nitrogen detection (D. Graeber et al., 2012; Huber et al., 2011). Based on SEC, we differentiate between the fractions of non-humic high molecular weight substances (DOC_{HMWS} and DON_{HMWS}) of hydrophilic character (e.g. polysaccharides and proteins); low-molecular neutral, hydrophilic to amphiphilic substances such as aldehydes, sugars, and amino acids (DOC_{LMWS}); and humic-like substances (DOC_{HS} and DON_{HS}) (Huber et al., 2011). The low molecular weight fraction of DON was not included in the SEC analysis and is negligible in natural freshwaters (D. Graeber et al., 2012). Furthermore, we used SEC to calculate the molar C:N ratio of bulk DOM ($C:N_{DOM}$), humic-like ($C:N_{HS}$), and non-humic high-molecular weight substances ($C:N_{HMWS}$), as well as to measure $SUVA_{254}$ of DOM, the specific absorbance of the sample at 254 nm which is a measure for aromaticity (Weishaar et al., 2003; Huber et al., 2011).

Absorbance was measured at room temperature using a Shimadzu UV-2401 UV/VIS spectrometer (Duisburg, Germany) and fluorescence measurement was performed using an Aqualog (Horiba, USA). Further details on spectral measurements and corrections can be found in supplementary material (S2). From the absorbance data, we calculated the slope ratio S_R, an indicator of molecular size which decreases with increasing molecular weight (Helms et al., 2008). We used the fluorescence data to calculate the following indices: the humification index (HIX) indicating the level of complexity and aromaticity of DOM (Ohno, 2002); the fluorescence index (FI), an indicator of DOM origin (more microbial (FI ~ 1.9) or terrestrial and higher plant (FI ~ 1.4) origins) (Cory and McKnight, 2005); 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).

Biofilm was removed from the tiles by scratching and dissolved in UV sterilized, 0.2µm filtered water for bacterial abundance analysis. Cell counts for bacterial abundance analysis were performed using DAPI (Carl Roth, Karlsruhe) staining according to Porter and Feig (1980) using black PC (polycarbonate) filters (0.2 µm, Sartorius) and an epifluorescence microscope (Axioskop, Carl Zeiss, Jena).

Statistical analyses

All statistical analyses were performed using 'R' (2016, Version 3.3.1, The R Foundation for Statistical Computing) except for the Dunnett's and Steel's tests with control, which were performed using JMP Pro (Version 11.0.0, SAS Institute Inc. 2003). The statistical analysis aimed to assess the effects of the DOM source on the behavior of DOM treatments relative to the control from the same source (AL and AF relative to AB or FL and FH relative to FB). Therefore, all the following statistics (except for the statistics on the controls AB and FB) were performed on data corrected by the control of the two different DOM sources. To achieve this, we subtracted the mean value of the control (AB or FB) at each sampling date from the value of each replicate within the respective nutrient treatment (AL and AH or FL and FH) at the same sampling date. This was done for each of the five sampling dates and for all DOM variables (bulk DOC concentration, bulk DON concentration, DOC and DON SEC fractions, $C:N_{DOM}$, $C:N_{HS}$, $C:N_{HMWS}$, HIX, FI, β:α, S_R and $SUVA_{254}$).

To test for the main and interaction effects of DOM source and sampling date on DOC and DON concentration, we used a permutational 3-way ANOVA (factors: DOM source, nutrients, sampling date, 10000 iterations) with interactions (based on aov(), package 'stats', R). Sampling date was used as factor, since independent replicates exist for each sampling date. We used a permutational 3-way ANOVA, as the assumptions of variance homogeneity and normal distribution of residuals were not met for DOC and DON concentrations. The function of the permutational 3-way ANOVA was written for this purpose and can be found in the supplementary material (S3). To assess the main and interaction effects of DOM source and inorganic nutrient concentration on DOM composition and sampling date, we applied a permutational MANOVA (PERMANOVA) on DOM composition (DOC and DON SEC fractions, $C:N_{DOM}$, $C:N_{HS}$, $C:N_{HMWS}$, HIX, FI, β:α, S_R and $SUVA_{254}$) testing the factors DOM source, nutrients

and sampling date (adonis(), package 'vegan', Euclidean distance, 999 iterations (Oksanen et al., 2015)). To display the results of the PERMANOVA, we calculated Principal Response Curves (PRC; prc(), package 'vegan') separately for agricultural and forest DOM in order to analyze the effects of treatment and their interaction effect with time on DOM composition. PRC is a special case of Redundancy Analysis (RDA; rda(), package 'vegan') for multivariate responses with repeated observations in time, which enables to focus the analysis on time-dependent treatment effects (Van den Brink and Ter Braak, 1999). Within the prc() function we used contrasts against a sampling-time specific control, revealing the effects of the treatments (AL and AH or FL and FH) at each sampling date relative to the control at the same sampling date (AB or FB). To test if the PRC models and PRC axes significantly explained the data, we used an ANOVA-like permutation test (anova.cca() within the 'vegan' package).

To assess changes of DOC and DON concentrations and DOM composition in the controls (AB and FB) over time, we applied a one-way ANOVA using sampling date as factor (aov(), package 'stats'). The assumptions of normal distribution and variance homogeneity were met. We further applied Dunnett's test using start concentration (day 0) as control group to test at which sampling date DOC and DON concentration differed from the start value. We applied PERMANOVA on optical DOM properties (HIX, FI, β:α, S_R and $SUVA_{254}$) for agricultural and forest DOM to test for changes of DOM composition with time. We used Steel's test (with start as control group) for individual optical parameters of DOM composition (HIX, FI, β:α, S_R and $SUVA_{254}$) to test for significant differences between the individual sampling dates compared to start values. The differences between start and end values of inorganic nutrients (SRP and DIN) and bacterial cell counts were tested for significance with the Mann-Whitney U test (wilcox.test(), package 'stats').

Results

The composition of DOM differed between agricultural and forest streams. In particular, C:N ratios were lower and $SUVA_{254}$ was higher in agricultural DOM relative to forest DOM (Table 1). In addition, at the start of the experiment $SUVA_{254}$ was higher and S_R was lower in the high nutrient treatments (AH, FH) relative to the control and the low nutrient treatments (AB, FB, AL, FL) (Table 1).

Table 1. *Mean (n=5) and standard deviation (±1SD) of all SEC and optical parameters measured at start of the experiment in all treatments and the control. Concentration of DOC and DON and the respective SEC fractions are given in mg C L^{-1} and mg N L^{-1}. $SUVA_{254}$ values are given in L mg C m^{-1}.*

	agriculture			**forest**		
	control	low N+P	high N+P	control	low N+P	high N+P
	AB	**AL**	**AH**	**FB**	**FL**	**FH**
DOC	4.03 (0.12)	4.10 (0.07)	4.22 (0.01)	4.23 (0.00)	4.38 (0.00)	4.29 (0.00)
DON	0.27 (0.02)	0.28 (0.00)	0.27 (0.02)	0.13 (0.12)	0.15 (0.06)	0.14 (0.21)
DOC_{HS}	3.70 (0.10)	3.80 (0.05)	3.79 (0.10)	3.46 (0.01)	3.52 (0.01)	3.55 (0.01)
DOC_{HMWS}	0.08 (0.01)	0.09 (0.01)	0.10 (0.01)	0.11 (0.05)	0.15 (0.25)	0.13 (0.08)
DOC_{LMWS}	0.25 (0.05)	0.24 (0.00)	0.33 (0.06)	0.60 (0.01)	0.60 (0.03)	0.62 (0.01)
DON_{HS}	0.26 (0.02)	0.26 (0.00)	0.25 (0.02)	0.11 (0.02)	0.13 (0.02)	0.11 (0.14)
DON_{HMWS}	0.01 (0.00)	0.01 (0.09)	0.02 (0.00)	0.02 (0.01)	0.02 (0.01)	0.03 (0.01)
$C:N_{DOM}$	12.8 (0.7)	12.6 (0.1)	13.4 (1.2)	29.1 (3.0)	25.3 (1.7)	26.5 (1.6)
$C:N_{HS}$	12.3 (0.7)	12.3 (0.1)	12.9 (1.3)	27.3 (1.9)	23.6 (1.9)	27.1 (2.5)
$C:N_{HMWS}$	6.3 (1.5)	5.4 (1.2)	4.9 (1.2)	5.8 (1.1)	6.3 (0.5)	4.2 (0.7)
HIX	0.94 (0.01)	0.94 (0.00)	0.94 (0.00)	0.93 (0.01)	0.92 (0.01)	0.93 (0.01)
FI	1.57 (0.01)	1.57 (0.00)	1.57 (0.00)	1.50 (0.02)	1.51 (0.01)	1.52 (0.04)
β:α	0.63 (0.00)	0.63 (0.00)	0.63 (0.00)	0.59 (0.02)	0.59 (0.00)	0.59 (0.01)
$SUVA_{254}$	3.6 (0.1)	3.6 (0.1)	3.8 (0.1)	2.9 (0.1)	2.8 (0.0)	3.2 (0.0)
SR	0.91 (0.01)	0.93 (0.01)	0.80 (0.02)	0.95 (0.01)	0.98 (0.02)	0.82 (0.01)

Table 2. *P-values and significance for permutational ANOVAs calculated for DOC and DON concentration and p-values with significance and explained variance (R^2) for permutational MANOVA (PERMANOVA) calculated for DOM composition. Significance code: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.'*

	3-way perm. ANOVA				**PERMANOVA**		
	DOC		DON		DOM composition		R^2
source	0.0876		0.4832		0.001	***	0.078
nutrient	0.1334		0.1285		0.001	***	0.041
sampling	<0.0001	***	<0.0001	***	0.001	***	0.314
source*nutrients	0.3502		0.0696		0.141		0.008
source*sampling	0.3906		0.0373	*	0.001	***	0.071
nutrients*sampling	0.7066		0.2957		0.002	**	0.044
source*nutrients*sampling	0.5432		0.726		0.263		0.024
						residuals	0.420

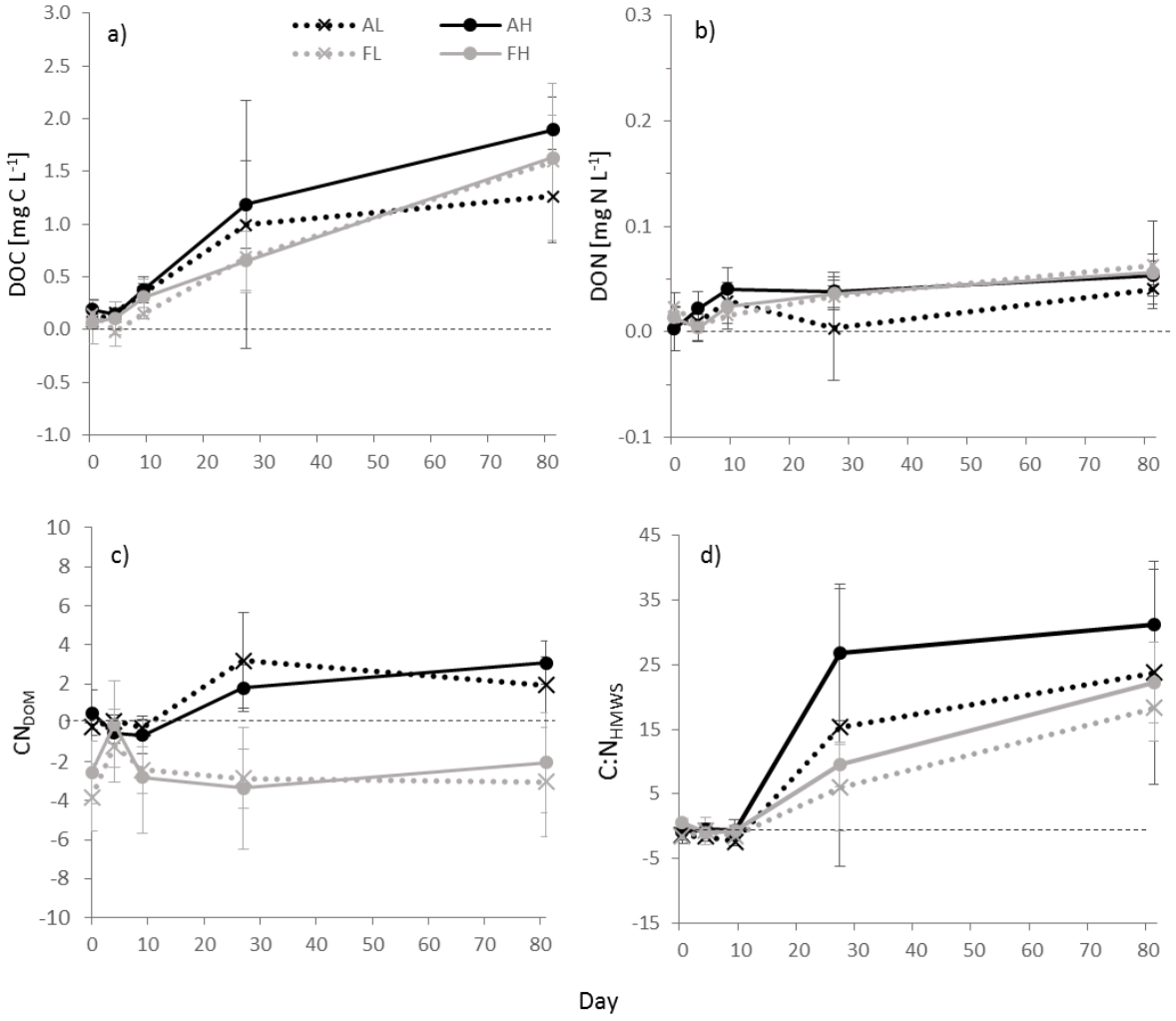

Figure 1. *Changes of DOC (a) and DON (b) concentration, C:N_{DOM} (c) and C:N_{HMWS} (d) for agricultural (A, black lines) and forest (F, grey lines) DOM, with low (L, dotted lines) and high (H, solid lines) inorganic nutrient additions. Shown are mean (symbol) and standard deviation (whiskers) of changes relative to control mean (zero line).*

DOM source and concentrations of inorganic nutrients did not affect DOC or DON concentration during the experiment, whereas sampling date had a significant effect on both (permutational ANOVA, Table 2). Specifically, DOC and DON concentrations increased relative to the control from the start to the end of the experiment for all treatments (Fig. 1 a, b), with the highest increases in humic-like (DOC_{HS}: on average 0.7 ± 0.2 mg C L^{-1}) and non-humic high molecular weight (DOC_{HMWS}: on average 0.9 ± 0.4 mg C L^{-1}) fractions (Fig. 2 a- c). For DOC concentration, there were no interaction effects between DOM source, nutrients and sampling date (permutational ANOVA, Table 2). Regarding the DON concentration, an interaction effect between DOM source and sampling date occurred, and consequently the effect of sampling date differed between DOM sources (permutational ANOVA, Table 2).

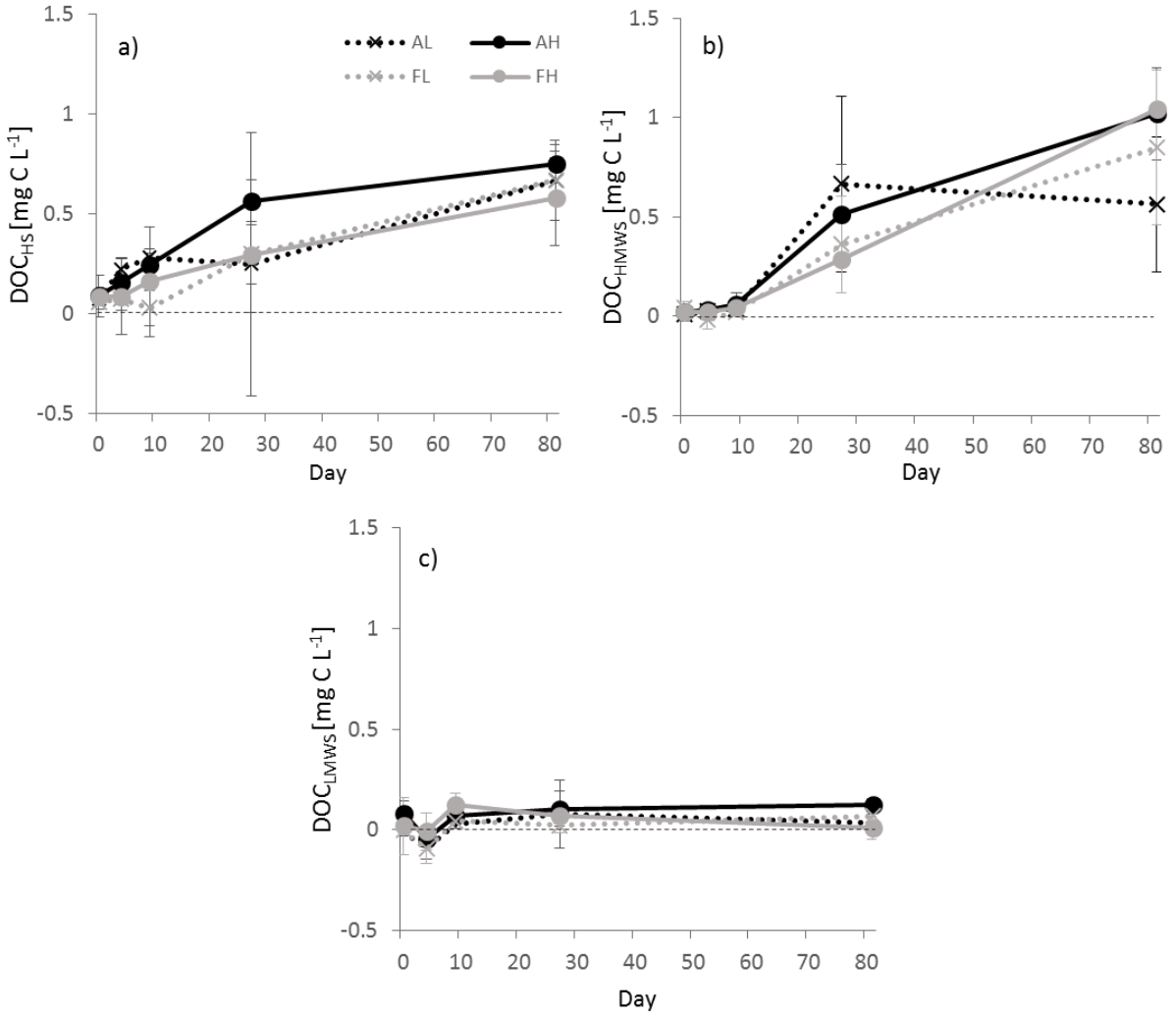

Figure 2. *Changes of the concentration of HS (a), HMWS (b) and LMWS (c) SEC fractions for agricultural (A, black lines) and forest (F, grey lines) DOM, with low (L, symbol: cross) and high (H, symbol: circle) inorganic nutrient additions. Shown are mean (symbol) and standard deviation (whiskers) of changes relative to control mean (zero line).*

DOM source, nutrients, and sampling date affected the DOM composition, and interaction effects occurred for sampling date with DOM source and nutrients (PERMANOVA, Table 2). Sampling date explained 31% (R^2) of the variance, while the variance explained by DOM source and its interactions with sampling as well as that explained by nutrients and their interactions with sampling date were less than 10% (PERMANOVA, Table 2).

The PRC models calculated for agricultural and forest DOM significantly ($p < 0.001$) explained 29.4 and 34.4% of the total variance in composition data. The first axis of the PRC explained 18% ($p < 0.001$) and 20% ($p < 0.001$) of the total variance for agricultural and forest DOM composition, respectively. This PRC axis showed an interaction effect of the treatments with time and a stronger effect for high relative to low nutrient levels for agricultural DOM

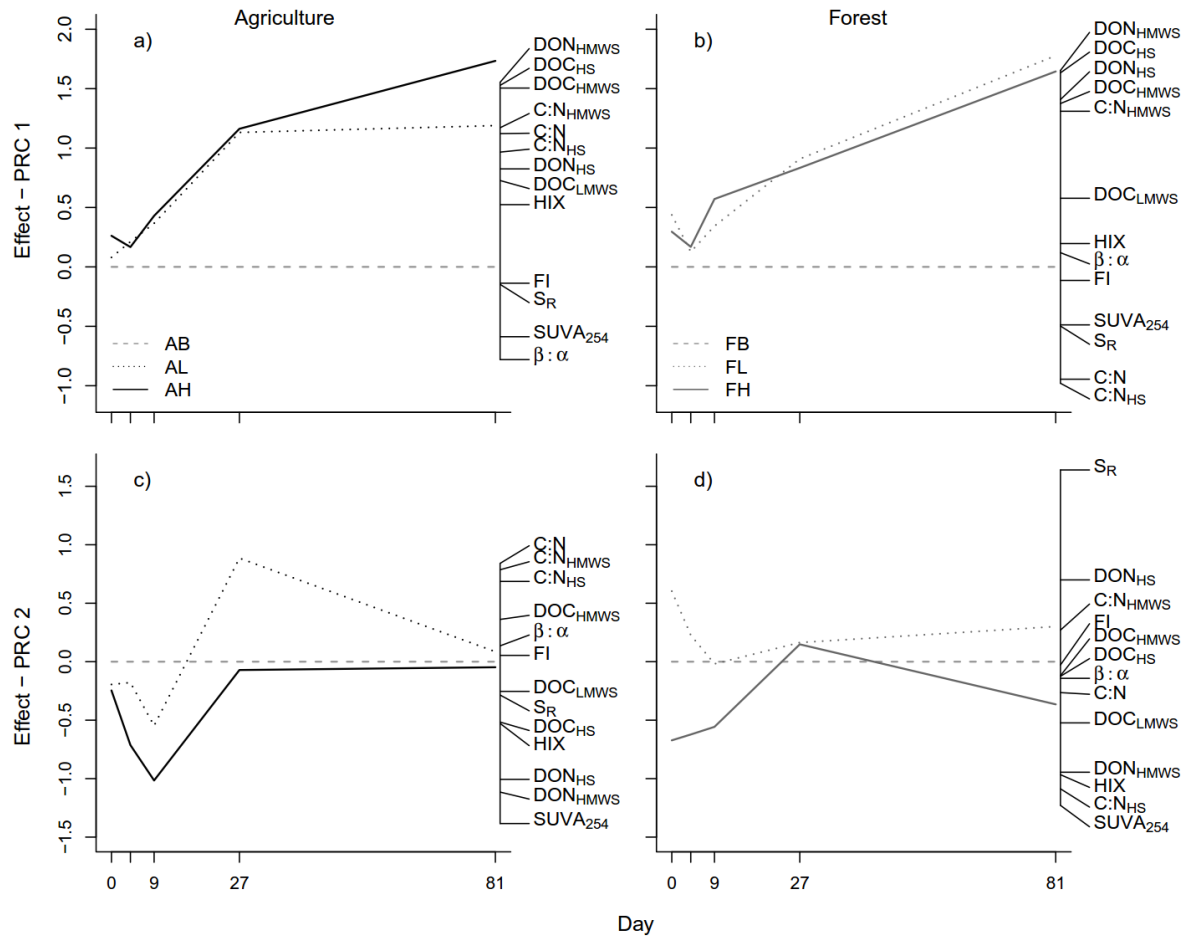

Figure 3. *Principal response curves (PRC) with species weights (right) for DOM composition variables. Shown are results for the first (PRC1, a, b) and second (PRC2, c, d) PRC axis for agricultural (a, c) and forest (b, d) landuse. PRC1 indicates a strong effect of time with the treatments and PRC2 indicates a smaller nutrient treatment effect for agricultural and forest DOM.*

between sampling days 27 and 81 (Fig. 3 a). In contrast, both nutrient treatments were stable for forest DOM between these sampling days (Fig. 3 b). For both agricultural DOM and forest DOM, the two treatments exhibited higher DOC_{HMWS}, DOC_{HS}, DON_{HMWS}, and $C:N_{HMWS}$ values than the control (Fig. 3 a, b). Moreover, in both cases, the treatments showed reduced values of $SUVA_{254}$ (Fig. 3 a, b) and lower values of β:α for the DOM from agricultural streams (Fig. 3 a) and lower values of C:N and $C:N_{Hs}$ for the DOM from forest streams (Fig. 3 b). The most pronounced changes of $C:N_{DOM}$; and in particular $C:N_{HMWS}$, occurred after day 9 of the experiment (Fig. 1 c, d). On average, $C:N_{HMWS}$ increased from 5.6 to 21.5 for agricultural DOM and from 5.4 to 18.8 for forest DOM, and maximum $C:N_{HMWS}$ values up to 59.7 for agricultural DOM and 34.7 for forest DOM were reached. The second axis of the PRC explained 5% ($p <$

Table 3. *Mean (n=5) and standard deviation (±1SD) DIN and SRP concentration and DAPI cell counts at start and end of the bioassay, for high and low nutrient treatment of agricultural/low C:N_{DOM} and forest/high C:N_{DOM}*

			DIN [mg N L^{-1}]	SRP [µg L^{-1}]	cell counts [10^8 cells cm^2]
agriculture	control	start	0.76 (0.05)	37.8 (1.1)	-
		end	0.77 (0.18)	6.2 (2.8)	-
	low N+P	start	0.68 (0.05)	34.9 (0.4)	2.01 (0.42)
		end	0.77 (0.11)	13.1 (5.1)	1.22 (0.15)
	high N+P	start	10.28 (0.41)	79.1 (1.8)	1.12 (0.13)
		end	11.37 (0.52)	16.5 (8.4)	1.40 (0.41)
forest	control	start	0.85 (0.03)	25.1 (1.5)	-
		end	0.82 (0.09)	1.4 (1.3)	-
	low N+P	start	0.86 (0.02)	24.0 (0.0)	1.13 (0.25)
		end	0.88 (0.09)	10.6 (3.6)	1.10 (0.27)
	high N+P	start	9.74 (0.45)	114.4 (1.4)	1.09 (*)
		end	9.50 (0.96)	32.6 (14.4)	1.12 (0.25)

0.001) and 7% ($p < 0.001$) of the total variance for agricultural and forest DOM composition, respectively. This axis demonstrated treatment effects for the treatments with inorganic nutrients (AH and FH), which were not visible on PRC axis 1 (Fig. 3 c, d). On the second axis, the change of DOM composition within the low nutrient treatments was related to $SUVA_{254}$ and DOC_{HMWS} for both, agricultural and forest DOM (Fig. 3 c, d). Furthermore, the high nutrient treatment showed high values of DON_{HS} for agricultural DOM (Fig. 3 c) and high values of HIX and $C:N_{HS}$ for forest DOM (Fig. 3 d). For agricultural DOM, the low nutrient treatment also exhibited higher values of C:N, $C:N_{HMWS}$, and $C:N_{HS}$ than both the control and the high nutrient treatment (Fig. 3 c). In contrast, the low nutrient treatment exhibited higher values of S_R for forest DOM (Fig. 3 d). The third PRC axis was significant ($p < 0.05$) but not included in the further analysis, as it explained only 3% of the total variance for agricultural and forest DOM composition and did not represent any distinct treatment or sampling date effect.

The concentration of DIN did not change (Mann-Whitney U test, $p > 0.05$) except for the increase of DIN in the high nutrient treatment of agricultural DOM (Mann-Whitney U test, $p < 0.05$; Table 3). The SRP concentration decreased during the course of the experiment in all treatments and in the control (Table 3), with decreases by more than 50% of the initial SRP

concentration in the high and low nutrient treatments and decreases by more than 80% of the initial SRP concentration in the control. Bacterial cell counts did not change from start to

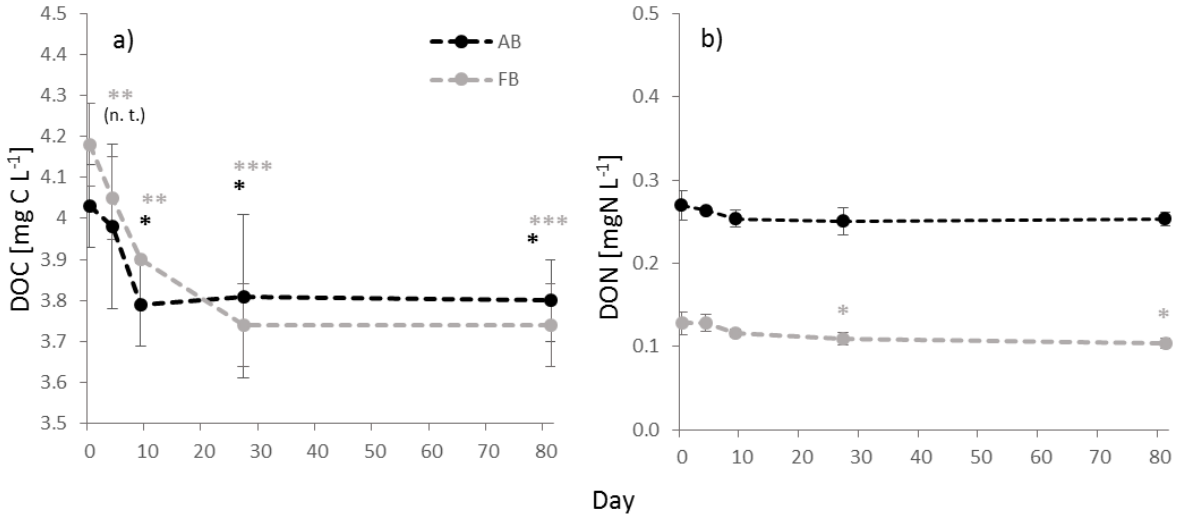

Figure 4. *Changes of DOC (a) and DON (b) concentration in the control for agricultural (A, black lines) and forest (F, grey lines) DOM. Shown are mean (symbol) and standard deviation (whiskers) of concentration per sampling date. Asterisks indicate significant differences to the start values (day 0) (Dunnetts test, p: < 0.05*, < 0.001**, < 0.0001***, (n.t.) not tested).*

until the end of the experiment and did not differ between the treatments at the end of the experiment (Mann-Whitney U test, $p > 0.05$; Table 3).

In the controls sampling date exhibited a strong effect on DOC concentration for forest DOM (perm. ANOVA, $p < 0.001$) and a weak effect on agricultural DOM (perm. ANOVA, $p < 0.042$). Results of Dunnett's test using the DOC and DON concentrations at the start of the experiment as control showed that the DOC concentration decreased significantly over the course of the experiment in the control for agricultural and forest DOM (Fig. 4 a). On average, the DOC concentration decreased by around 6% and 11% of the initial DOC concentration for the agricultural and forest controls, respectively. In contrast for DON an effect of sampling date was only observed for forest DOM (perm. ANOVA, $p < 0.01$), but not for agricultural DON (perm. ANOVA, $p > 0.05$). Specifically, DON concentration decreased slightly in the control for forest DOM after day 27, while DON concentration did not change significantly in the control for agricultural DOM (Fig. 4 b).

In both controls (AB, FB), some optical properties significantly differed between sampling dates (PERMANOVA, $p < 0.001$). Specifically, β:α (Fig. 5) and FI increased during the

experiment (Steels test, $p < 0.05$), while S_R differed between sampling dates and the start (Steels test, $p < 0.05$), the trend being inconsistent, however.

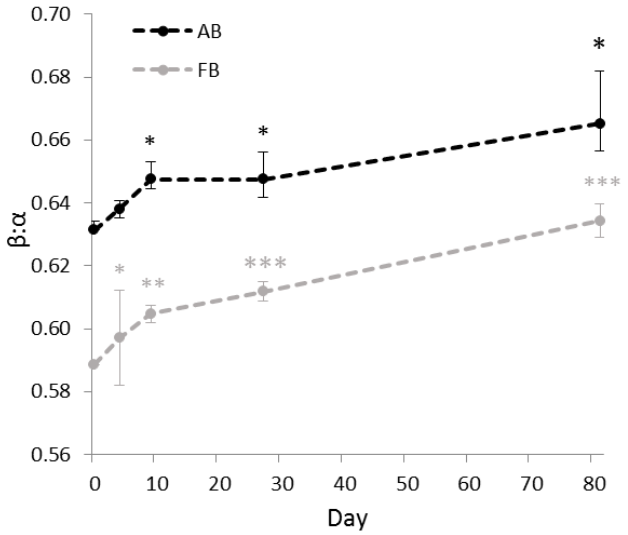

Figure 5. *Changes of β:α values in the control for agricultural (A, black lines) and forest (F, grey lines) DOM. Shown are mean (symbol) and standard deviation (whiskers) of concentration per sampling date. Asterisks indicate significant differences to the start values (day 0) (Steels test, p: < 0.05*, < 0.001**, < 0.0001***).*

Discussion

The aim of this study was to assess the effects of DOM composition and inorganic nutrient availability on DOM biodegradability by heterotrophic benthic stream biofilms. In contrast to our expectations, neither DOC nor DON concentrations declined but rather increased during the 81 days of experiment. In addition, the changes in DOC and DON concentrations were independent of DOM source and inorganic nutrient concentrations. In parallel, DOM composition changed over time with differences depending on DOM source and inorganic nutrients. However, no clear indication of biofilm degradation was found. Therefore, based on the absence of DOM degradation and the lack of response of DOC and DON to DOM source and inorganic nutrients we must reject all of our hypotheses.

The absence of DOM degradation in this study contrasts previous findings reporting strong biodegradation of DOC (Fasching et al., 2014; Volk et al., 1997; Wickland et al., 2012) and DON (Wiegner et al., 2006) in freshwaters. We propose two explanations for this result. Either, i)

DOM was not consumed or consumed only to a non-detectable degree because the provided DOM was refractory and, hence, not degradable within 81 days and/ or ii) biofilm microorganisms did not need carbon and nutrients from water column DOM. Based on the temporal development of the controls (without biofilm), we infer that the DOM provided was not refractory. The control was filtered with 0.45 µm, to ensure identical DOM pretreatment conditions for the treatments and the control, and the DOC concentration decreased by around 6% and 11% of the initial DOC concentration for agricultural and forest DOM. Thus, it is likely that, although no biofilm was added to the control, DOC was degraded by residual planktonic bacteria remaining in the DOM after 0.45 µm filtration, since a large percentage of freshwater microbial communities is not retained by 0.45 µm pore size filters (Wang et al., 2007). Furthermore, DOM processing by planktonic bacteria in the control is indicated by increasing FI and β:α values over the course of the experiment, showing an increasing contribution of recently produced microbial derived DOM (McKnight et al., 2001; Parlanti et al., 2000). In addition, it has been previously demonstrated that complex and aromatic terrestrial DOM can be degraded by planktonic bacteria (Fasching et al., 2014; Guillemette and del Giorgio, 2012; Ward et al., 2013). Altogether, it is likely that DOM was degraded within the controls without biofilm and that this was either counteracted by DOM release in the treatments with biofilms or DOM degradation was completely absent in the treatments.

Biofilms are key sites of organic matter processing in streams (Battin et al., 2016, 2008; Romaní et al., 2004) and bacterial production in biofilms constitutes an important process of organic matter consumption in lotic systems (Pusch et al., 1998). Therefore, in order to obtain a realistic picture of DOM processing in streams, in our experiment we decided to use biofilms and not planktonic bacteria, as otherwise commonly used in biodegradability studies (Fasching et al., 2014; Wickland et al., 2012; Wiegner et al., 2006). However, there is rising evidence that biofilm bacteria may be less dependent on external DOM supply from the water column than planktonic bacteria. For instance, Kamjunke et al. (2015) reported a strong response of planktonic bacteria to changes in DOM quantity or composition, while no clear response was found for biofilm bacteria. In contrast to planktonic microorganisms, which depend strongly on external nutrient supply, the matrix of extracellular polymeric substances (EPS) in which biofilm bacteria are embedded may be the dominant DOM source within biofilms, providing DOM adsorbed to/ or incorporated in the biofilm matrix (Freeman and

Lock, 1995). Hence, the absence of DOM degradation in our study may be explained by the lack of dependence of the biofilm on external DOM supply.

The absence of an effect of inorganic nutrients on DOC and DON concentrations and the constant DIN concentrations during the course of the experiment further support the idea that biofilm microorganisms were independent of external nutrient sources. However, the observed decline in SRP concentration may be explained by consumption or abiotic adsorption (e. g. at the surfaces of the cell culture flask). The decline of SRP in the control without biofilm indicates that it is unlikely that the SRP decline can be ascribed to consumption by the benthic biofilm bacteria alone. More likely, the decrease of SRP was due to abiotic adsorption to cell culture flasks or consumption by residual bacteria in the water column.

Whether the lack of response by biofilm bacteria to DOM quality is due to recalcitrance of the DOM added, or independency from external nutrient sources cannot be resolved conclusively by the results of this study.

Increasing DOC and DON concentrations relative to the control over the course of the experiment indicate that in this study the dominating process was DOM release and not DOM consumption by the benthic stream biofilm. The increases in DOC and DON concentrations were accompanied by changes in DOM composition over time. Specifically, $C:N_{HMWS}$ and the concentrations of the DOC_{HMWS} increased, while aromaticity decreased. The non-humic high molecular weight fraction identified by SEC includes polysaccharides and proteins (Huber et al., 2011). These are main constituents of EPS and build up the biofilm matrix (Stewart et al., 2013). EPS can contain up to 40-95% polysaccharides and up to 60% proteins (Flemming and Wingender, 2001). The increase of $C:N_{HMWS}$ from around 5 at the start up to around 21 at the end of the experiment indicates that DOM_{HMWS} changed from protein to polysaccharide dominance during the experiment (Kroll et al., 2014; Stewart et al., 2013). Benthic diatoms can produce free carbohydrates and EPS under light conditions and in the short term (up to 3 days) also under dark conditions (Smith and Underwood, 2000). However, increases of DOC_{HMWS} and DON_{HMWS} in this study occurred mainly after day 9 of the experiment. Besides, the biofilm used in this study was incubated in the dark during the experiment and conditioned in the dark for 2 weeks prior to the experiment, so photosynthetic activity of benthic diatoms was inhibited and can be excluded as the source of DOM_{HMWS}. Neu and

Lawrence (1997) observed that humic substances and detrital material ("colloidal and particulate organic matter") were integrated into the biofilm during 57 days of growth in stream water and contributed significantly to the structure of biofilms. In contrast, in the EPS of newly colonized biofilms only building blocks of humic substances were identified by SEC analysis, while no humic-like substances were integrated in the EPS (Kroll et al., 2014; Neu and Lawrence, 1997). In the present study, biofilm grown in stream water for 8 weeks was used and it is therefore possible that humic-like material was incorporated into the EPS of the biofilm during growth. From our observations, we conclude that polysaccharides and humic-like substances were washed out from the EPS matrix and enriched in the surrounding water column during the course of the experiment.

Further, $SUVA_{254}$ was higher in the high N+P treatments than in the respective low N+P treatments or the control at the start of the experiment (Table 1). This was due to the high nitrate concentrations that may interfere with the determination of $SUVA_{254}$ at high concentrations (>40 mg NO_3 L^{-1} $^{-}$ ≙ 9.04 mg N L^{-1}); thus, the tail of the absorbance peak of nitrate (210 bzw. 222 nm) can extend to 254 nm, which is the absorbance wavelength used for the calculation of $SUVA_{254}$ (Weishaar et al., 2003). Likewise, S_R, which is the ratio of the slopes from 275 to 295 and 350 to 400, may be influenced by the high NO_3^- concentration since NO_3^- absorption bands in aqueous solution can extend from 270 to 340 nm (Gaffney et al., 1992). However, since the DIN concentration did not change during the experiment, the observed changes in DOM composition are presumably not a consequence of changing NO_3^- concentration.

Our results show that, independent of the source of DOM and level of inorganic nutrient concentration, neither DOC nor DON was significantly biodegraded during the experiment, presumably due a notable lack of dependence on external sources.

In our study, neither terrestrial DOC nor DON was significantly biodegraded by benthic biofilm bacteria. The absence of biodegradation of this terrestrially derived DOM in the water column was independent of the source of DOM and level of inorganic nutrient concentration and presumably due to a notable lack of dependence on external sources bythe benthic biofilm bacteria. Further research should also focus on planktonic organisms and systems with high autotrophic activity to completely elucidate the biodegradability of terrestrial N-rich DOM in freshwaters.

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Study 4:

Storage effects on DOM analysis with size exclusion chromatography and fluorescence spectroscopy for lake water, leaf leachate and peat soil water

Marlen Heinz, Dominik Zak

Storage effects on DOM analysis with size exclusion chromatography and fluorescence spectroscopy for 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 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. 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.

Introduction

Dissolved organic matter (DOM) is a mixture of various soluble compounds differing in their molecular weight, structure and complexity (Leenheer and Croué, 2003). 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). To arrest biological activity during cold

storage samples can be acidified (Schneider-Zapp et al., 2013). While samples are acidified for later analysis of bulk dissolved organic carbon (DOC), it is not recommended for later fluorescence and absorbance analysis (Schneider-Zapp et al., 2013; Spencer et al., 2007) or analysis with size exclusion chromatography (SEC; Sandron et al., 2015) due to 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 inconsistent effects on chromophoric DOM composition and 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. In contrast Peacock et al. (2015) reported that DOC concentrations in peatland samples were 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. Given the variety of DOM sample types investigated, the various effects observed indicate direction and intensity of DOM alterations are affected by source and composition of samples (Peacock et al. 2015, Spencer et al. 2007, Hudson e al. 2010). Furthermore, it has been reported for stream samples and a range of terrestrial DOM sources, that the magnitude of the effects of freezing strongly depends on DOC concentration (Fellman et al., 2008; Thieme et al., 2016), and changes were attributed to particle formation (Giesy and Briese, 1978). In contrast Peacock et al., (2015) did not report a relationship between DOC concentration and the effects of freezing for pore and surface water samples of peatlands .

Fast freezing with liquid nitrogen can minimize the changes in bulk DOC concentration due to reduction of freezing time, but still, alterations of DOM fluorescence and absorbance properties could not be prevented (Thieme et al., 2016). This demonstrate that even if bulk concentration is not affected, alterations of DOM structure and hence optical properties cannot be precluded. Size exclusion chromatography (SEC) can be used to determine bulk

DOC and DON concentration and identify the main size fraction of DOC and DON in a sample (Huber et al., 2011). Applied in parallel with fluorescence and absorbance analysis (Graeber et al., 2015; Graeber et al., 2012a; Heinz et al., 2015) it enables to track changes of DOM composition on the elemental level (e.g C:N ratio of DOM), regarding the distribution of DOC and DON in different size classes of DOM and from the perspective of spectral properties of DOM. 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. 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.

In order to present a recommendation for storage and preservation of DOM samples for later SEC analysis as well as fluorescence and absorbance analysis, this study aims to evaluate the effects of freezing and storage for one week at 4°C on bulk DOC and DON concentration and SEC fractions. To account for differences in DOM composition we analyzed storage effects for three different sample types. A sample from Lake Müggelsee was used to represent a freshwater DOM sample including allochthonous as well as autochthonous DOM sources and a leaf litter leachate of *Phragmites australis* representing a purely terrestrial, but microbially unaltered, 'fresh' DOM source. To assess also the effects of freezing on DOC and DON concentration and SEC fractions for a set of different samples of the same sample type, but covering a range of DOM concentrations, we analyzed soil water samples of oligotrophic nutrient poor bogs from 2 different geographic regions.

We expect that leaf leachates are more vulnerable to storage and acidification than lake samples due to the more 'labile' nature of leachate samples. Further, we expect that the bog samples constituting a less reactive humic sample type behave more or less conservative, independent from the geographical region where they derive from. In our study we aim to give a recommendation for storage and sample preservation for three different types of natural samples.

Methods

Sampling and preparation of the leaf leachate

To test storage and acidification effects on different types of DOM samples we used water from Lake Müggelsee (for lake details see Recknagel et al., 2016) representing a freshwater DOM source (hereafter referred to as lake sample) and a leaf leachate from *Phragmites australis* representing a terrestrial DOM source (hereafter referred to as leachate sample). The lake sample was taken at the lakeshore of Lake Müggelsee and filtered with a 0.45µm 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 mostly of leaves which were cut in 5-10 cm pieces to improve handling before of the 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 rinsed with 100ml deionized water and preconditioned with 20ml sample to minimize filter effects.

Lake and leachate samples had similar DOC and DON concentrations but differed DOC and DON SEC-fractions and optical properties (Table 1) with higher contributions of low-molecular weight DOC (DOC_{LMWS}) and higher aromaticity ($SUVA_{254}$) but less contribution of recently, microbial produced DOM (FI, β:α) and hence more terrestrial character of the leachate sample 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 a 5 peeper) and Estland (EST; 6 sites a 3 peeper) 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) with chambers filled with de-ionised water. Prior to insertion, 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.

***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)

After degassing, vessels were sealed with airtight cups for transportation to the sampling sites. Between three and five dialysis samplers with 14 spaced chambers were always inserted completely into the upper horizon of the peat (0–60 cm) within the peat sampling area (six sites in Estonia and three sites in Scotland). Three samplers were used per site 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.Samples of dialysis sampler were not 0.45µm 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.

Experimental setup and laboratory analyses

To test the effects of storage, freezing and acidification 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 dark (cold storage), or frozen at -20°C (freezing). Additionally 5 replicate blank samples (deionized water), each subjected to the same storage treatments (one week cold

storage and freezing) as lake and leachate samples were analyzed. To test for the effects of freezing on the DOC and DON SEC fractions of peatland pore water samples, samples from the two different geographical regions were analyzed before (original) and after freezing at - 20°C.

All samples were stored in 25 ml polypropylene (PP) vessels (washed with 10% HCl before usage) during storage and analyzed at the same day after removing them from the refrigerator or thawing. The DOC and DON concentration and respective size fractions we determined using size exclusion chromatography (SEC) combined with UV- and IR- organic carbon detection and UV-organic nitrogen detection (Huber et al., 2011; D. Graeber et al., 2012a). SEC enables to differentiate between DOC and DON in form of non-humic high molecular weight substances of hydrophilic character (DOC_{HMWS}, DON_{HMWS}; e.g. polysaccharides and proteins), humic-like substances (DOC_{HS}, DON_{HS}) and low molecular weight neutral, hydrophilic to amphiphillic substances (DOC_{LWMS}; e.g. aldehydes, sugars, amino acids). The C:N ratio of bulk DOM ($C:N_{DOM}$) was calculated as the molar ratio of DOC to DON. Absorbance and fluorescence properties were measured using an Aqualog spectrophotometer (Horiba, USA). An excitation wavelength range from 230 to 600 nm with a 5 nm increment was used. Emission spectra were collected for the wavelength range 214.1 – 619.3 nm with a 1.6 nm increment, using 1 s integration time, a pixel bin of 4 and medium detector gain. Absorbance spectra were collected from 230 to 600 nm in 5nm steps. Absorbance and fluorescence were measured at room 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. $SUVA_{254}$ of lake and leachate samples was calculated from absorbance data and DOC measured with C/N analyzer (multi N/C 2100, Jena Analytics). Here DOC measured with C/N analyzer but not SEC were used to enable the evaluation of the effect of acidification on $SUVA_{254}$, since there were no DOC measurements performed with SEC on acidified samples. 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).

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. The function of the permutational 3-way ANOVA after Heinz et al. (study 3 in this thesis) was modified for the application with 2-factors.

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

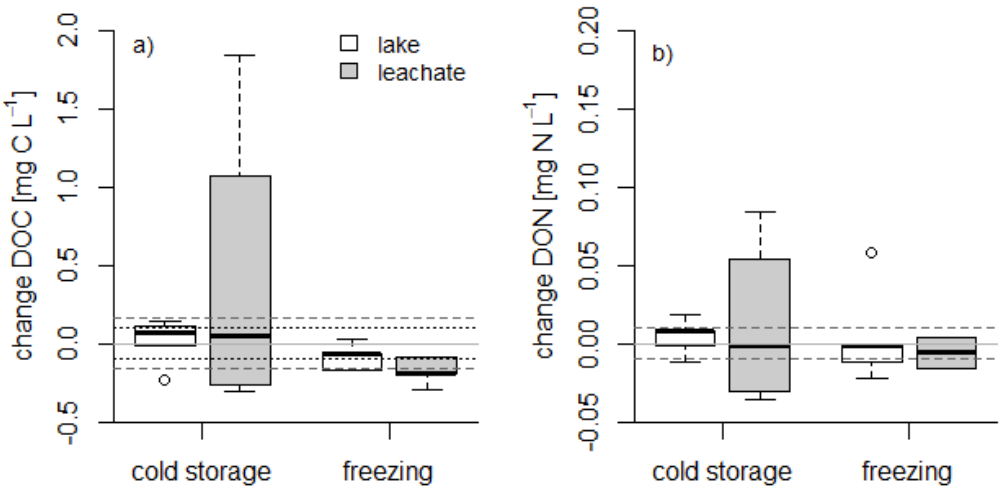

***Figure 1**. Total differences of DOC (a) and DON (b) concentration after cold storage (4 °C) and freezing (-20 °C) relative to the mean of original sample concentration (gray solid line). Data is shown for lake samples (white boxes) and leachate samples (light gray boxes). Dashed lines represent the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

Results

DOC and DON concentration

Permutational ANOVA revealed neither effects of DOM source nor of storage treatment on changes of DOC and DON concentration (perm. ANOVA, $p < 0.05$). Overall, changes of DOC and DON concentration due to freezing were lower than for cold storage, in particular for leachate samples where comparatively high changes occurred after cold storage (Fig. 1 a, b). In the leachate samples the changes of DOC and DON concentration after cold storage were more variable compared to lake samples (Fig. 1 a, b) ranging from -0.31 to 1.84 mg C * L^{-1} (-11 to 64 %) in leachate samples and from -0.23 to 0.14 mg C * L^{-1} (-7 to 4 %) in lake samples (Fig. 1a). Freezing of lake and leachate samples resulted in minor decreases of DOC (lake: ≤ 5% 0.2 mg C L^{-1}; leachate: ≤ 10%, ≤ 0.3 mg C L^{-1}) 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, $p > 0.05$).

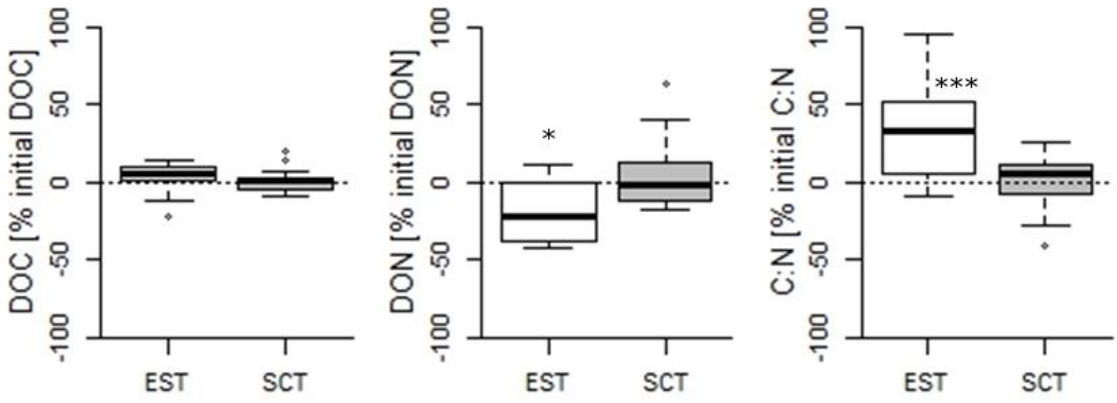

Figure 2*. Relative changes of DOC (a) and DON (b) concentration and molar C:N ratio of DOM after freezing for samples from Estonia (white) and Scotland (gray). Changes are shown as percentage of the initial concentration of the original sample. Asterisks mark significant differences to the initial values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

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 (total: 70%, 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} for EST samples and lower than 1.6 mg C L^{-1} for SCT samples which accounted for up to 23% and 10% of the bulk DOC concentration in the original EST and SCT samples. However, the changes for bulk DOC 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, $p > 0.05$). Consequently, the molar $C:N_{DOM}$ ratio increased in EST samples (Wilcoxon rank signed test, $p < 0.001$) but not in 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 DOC concentration for more than the half of the peatland samples (EST: 82%, SCTL: 53%) and ranged from –0.2 to 0.17 mg C L^{-1}. DON concentration decreased significantly in the majority of EST samples (83%; Wilcoxon rank signed test, $p < 0.05$), while for SCT samples no significant change of DON concentration (increase 60%, decrease 40%; Wilcoxon rank signed test, $p > 0.05$) was observed.

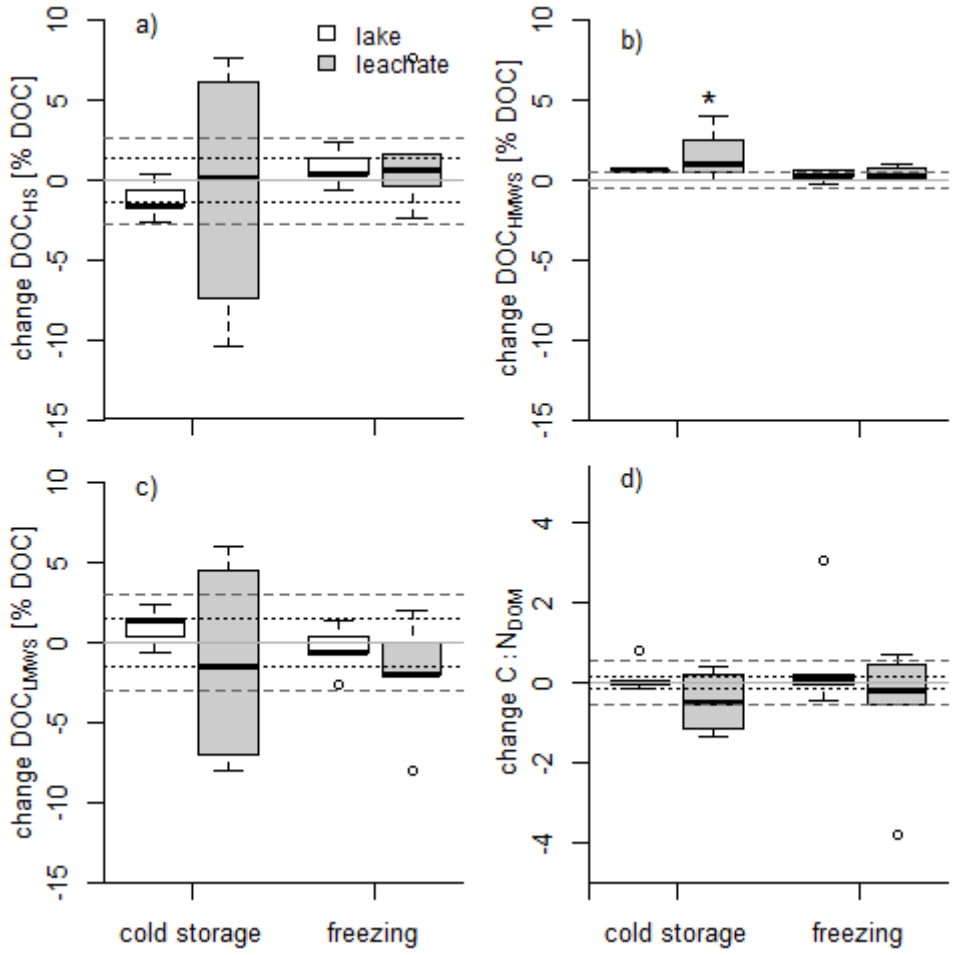

Figure 3. *Total changes of the relative contributions SEC fractions to bulk DOM (a – c) and changes of molar C:N$_{DOM}$ (d) after one week of cold storage (4 °C) and freezing (-20 °C) relative to the mean of initial values in the original sample (gray solid zero line). White boxes represent lake samples 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).*

DOM composition

Storage (cold storage, freezing) affected changes of DOM composition, while no effects for DOM source (lake, leachate) was observed (PERMANOVA). Storage treatment explained 20% (R^2) of the variance significantly (PERMANOVA, $p < 0.0001$). High changes after one week of cold storage were observed in particular for DOC$_{HS}$ and DOC$_{HMWS}$ to bulk DOC (Fig. 3 a, c), C:N$_{DOM}$ (Fig. 3 d) and β:α (Fig. 4 b) and HIX (Fig. 4 c). For the SEC fractions changes due to cold storage were higher than changes due to freezing (Fig. 2 a – c). Overall there was no evidence for effects of freezing on DOC and DON SEC fractions for lake and leachate samples (Mann

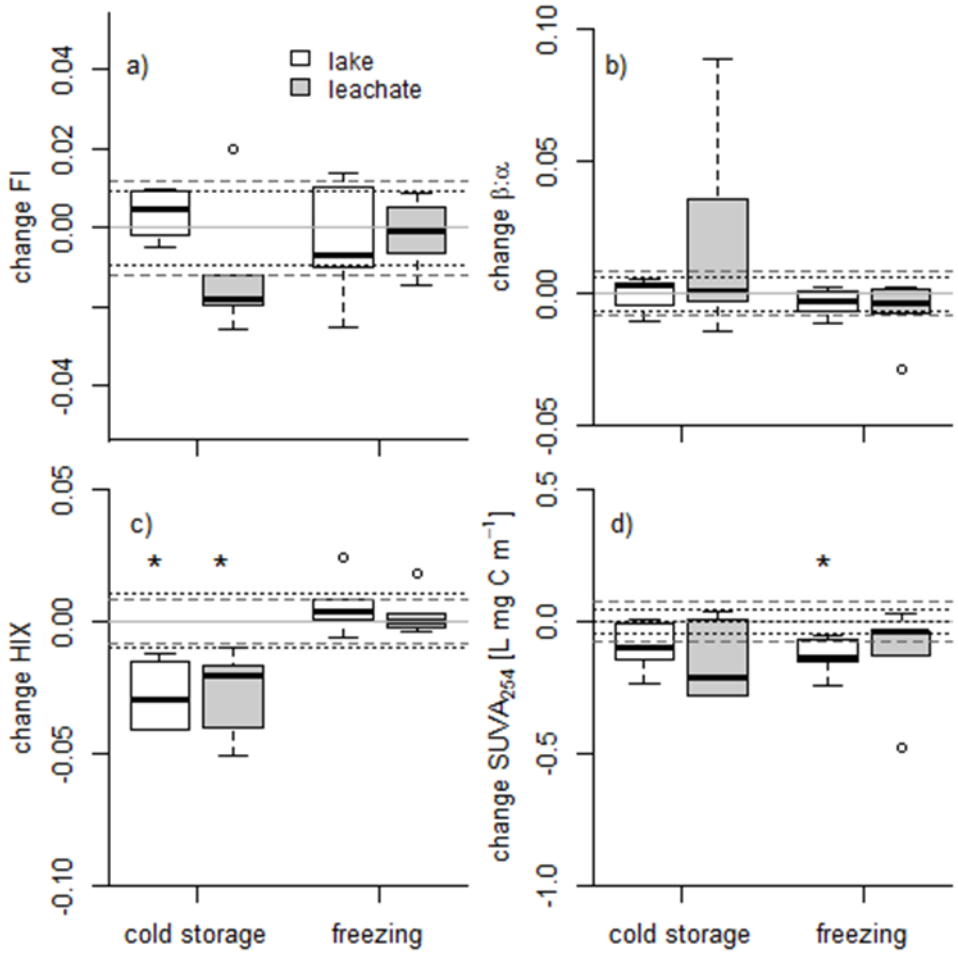

Figure 4. *Total changes of FI (a), β:α (b), HIX (c) and $SUVA_{254}$ (c) after one week of cold storage (4 °C) and freezing (-20 °C) relative to the mean of initial values in the original sample (gray solid zero line). Lake samples are represented by white boxes and leachate samples by gray boxes. Dashed 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).*

Whitney U test, p > 0.05; Fig. 2). The changes of contributions of DOC and DON SEC after cold storage were only significant for DOC_{HMWS} in leachate samples and DON_{HS} in lake samples (Mann Whitney U test, p > 0.05). In general changes of DOC SEC fraction were more variable in leachate samples compared to lake samples (Fig. 2 a – d). In the peatland samples significant changes of DOC in the individual SEC fractions were observed for EST and SCT samples (Wilcoxon signed rank test, p > 0.05; Fig. 5). Thereby in EST samples DOC_{HS} as well as DOC_{HMWS} decreased and DOC_{LMWS} increased, whereas in the SCT samples DOC_{HMWS} decreased and DOC_{HS} increased (Wilcoxon signed rank test, p < 0.05). Overall changes were stronger in EST samples compared to SCT samples (Fig. 5).

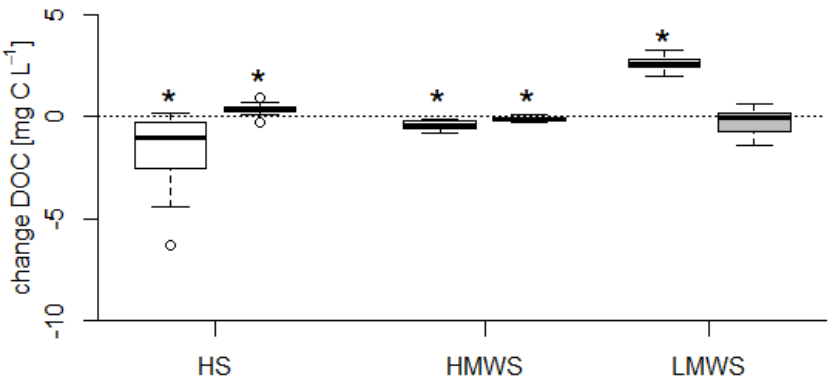

***Figure 5**. Total changes of DOC in the humic-like (HS), non-humic high molecular weight (HMWS) and low molecular weight (LMWS) SEC fraction after freezing. White boxes represent samples from Estonia, grey boxes samples from Scotland. Asterisks mark significant differences to the initial values in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

Optical properties represented by HIX, FI, β:α and $SUVA_{254}$ in lake and leachate samples were in most cases stronger affected by storage in leachate compared to lake samples (Fig. 3 a – d). Thereby changes of FI and β:α were not statistically significant (Mann Whitney U, $p > 0.05$), even though comparatively high 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, $p < 0.05$). In contrast, $SUVA_{254}$ was not affected by cold storage but decreased in leachate samples (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 $p < 0.05$).

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 storage and freezing.

According to 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. 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}). Similarly, strong variations in changes of optical properties and SEC fractions in leachate samples demonstrated that the DOM composition of leachate samples is more likely affected by storage than DOM composition in lake samples. Overall, DOM composition in lake and leachate samples was affected stronger by cold storage than by freezing, whereby only HIX and $SUVA_{254}$ were altered due to freezing. In peatland samples which were expected to be most robust against disturbance by preservation, the magnitude and direction of change differed for samples from different geographical regions.

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 and 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 samples with high DOC concentration Fellman et al. (2008) 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. However, in EST samples but not in SCT samples DON concentration and $C:N_{DOM}$ were altered due freezing. Moreover, for samples from both regions effects of freezing on SEC fractions were observed, whereby these effects were more pronounced in EST samples. In particular the strong increases in low molecular weight DOC (DOC_{LMWS}) ongoing with decreases in high molecular weight DOC (DOC_{HS} and DOC_{HMWS}) indicate, that freezing may result in physical breakdown of high molecular weight substances into low molecular weight substances. Moreover, it has been shown that DOM preferentially concentrates in the remaining liquid phase during freezing (Belzile et al., 2002; Xue et al.,

2015) and that concentration of DOM can affect its macromolecular configuration (Ghosh and Schnitzer, 1980). Differences in partitioning and concentration behavior were observed for individual DOM fractions (Xue et al., 2015). Hence, partitioning and concentration during freezing and thawing (Belzile et al., 2002; Xue et al., 2015) could have changed size fractioning of DOM continuously also after complete thawing of the sample. However, the different responses of SEC fractions to freezing in lake and leachate with moderate DOC concentrations compared to peatland samples with high DOC concentrations indicate that underlying processes are affected by sample type and DOC concentration.

Overall, freezing, seemed to constitute an appropriate preservation method for later SEC analysis of DOC concentration in lake, leachate and peatland samples. If initial DOC concentrations in samples are high (> 7 mg C L^{-1}), freezing can affect the individual SEC fractions and should therefore be avoided. Likewise alterations of optical properties, in particular for HIX and $SUVA_{254}$ due to freezing cannot be excluded and in accordance with previous studies (Peacock et al., 2015; Thieme et al., 2016) we recommend immediate analysis of samples for spectral analysis.

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General discussion

Agricultural land use in the catchment substantially impacts aquatic systems, modifying hydrology, nutrient cycling and physical conditions of water bodies, which has far reaching consequences for aquatic ecosystems and their faunal and floral aquatic communities (Harding et al. 1999; Blann et al. 2009). Only recently, Jansen et al. (2014) highlighted the need to study the sources and processing of fluvial DOM to understand the dynamics of DOM as a key player in linking aquatic and terrestrial systems. So far it has been shown that agriculture and anthropogenic activities affect DOM composition (Williams et al. 2010; Graeber et al. 2012b) and change DOC concentration in streams (Stanley et al. 2011; Graeber et al. 2012b). Comparatively little is known about the effects of agriculture on DON composition and DON processing in agricultural streams. In this thesis I aimed to fill the knowledge gaps on the effects of agriculture on DOM export to streams and DOM processing within streams with special emphasis on DON.

In this thesis I clearly demonstrated that agricultural land use in the catchment alters DOM composition and increases DOC, and in particular DON concentration and export in headwater streams (study 1). The higher DON concentration observed in agricultural streams compared to less disturbed reference catchments in this thesis (study 1) are only partly in accordance with previous findings on the effects of agricultural land use in the catchment: Mattsson et al. 2009 reported higher DON concentration in most of the agricultural streams along a climate gradient in Europe, while Stedmon et al. (2006) reported lower DON concentration in agricultural compared to forest streams. High DON concentrations were also reported for agro-urban catchments (Pellerin et al. 2006; Stanley and Maxted 2008; Petrone et al. 2009), whereby high DON concentrations in human altered catchments can be related to waste water inputs (Pellerin et al. 2004). Moreover, also high inorganic nutrient loads (as they are common in agricultural systems), can bias the determination of DON concentration (Lee and Westerhoff 2005; Graeber et al. 2012a; Chen et al. 2015), and may account partly to the inconsistent findings on DON concentrations published so far. Interestingly, DON concentrations in agricultural streams in this thesis were within the range of DON concentrations reported for agricultural and urban catchments with similar size (Pellerin et al. 2006; Aitkenhead-Peterson et al. 2009), but lower than in larger catchments (Stanley and Maxted 2008; Mattsson et al. 2009; Wohlfart et al. 2012). This could be an effect of

accumulation of 'recalcitrant' DON in streams during downstream transport, a result of increasing contribution of autochthonous production of DON in more downstream reaches, or be due to elevated anthropogenic inputs of DON in bigger catchments. However, from the data available so far, this cannot be finally resolved.

A further important outcome of this thesis is that I identified soils as the major DOC and DON source of fluvial DOM in agricultural headwater streams (study 1 and 2). Based on the calculation of the DOC and DON yields per catchment area in agricultural and forest streams, I demonstrated that in addition to higher DOC and DON concentrations, DOC and DON losses from agricultural soils were higher than from less managed forest soils. The overall terrestrial character of fluvial DOM and the absence of a seasonal pattern in DOM composition support the hypothesis, that DOC and DON in agricultural and forest streams are derived from catchment soils. Thereby major parts of DOC and DON (up to 74%) were exported in the form of HS-like material. The optical properties of DOM likewise showed high contributions of humic-like, terrestrial derived DOM with high aromaticity and high degree of humification (study 1 and 2). Taken together, based on these findings I could reaffirm findings gained from a meta-analysis of DON losses from the perspective of agricultural soils (van Kessel et al. 2009) which indicated that leaching from soils is an important pathway of N loss from agriculture.

Another major finding of this thesis is that, in addition to increased amounts of DOM and particular DON, agriculture in the catchment has a strong impact on the composition of the DOM and DON exported in headwater streams. Along with higher increases of DON concentrations relative to DOC concentrations, the C:N ratio of DOM exported from agriculture is much lower than the C:N of DOM in reference streams (study 1 and 2). Despite the overall terrestrial and humic character of DOM in agricultural and forest streams, the DOM composition in agricultural streams was shifted to a more microbial derived and processed character of DOM. Moreover the contributions of presumably proteinous, non-humic high molecular weight DON (DON_{HMWS}), material to bulk DON were approximately five times higher in agricultural compared to forest streams in this study (study 1). In soils DON is more vulnerable to changes of inorganic nitrogen than DOC, fertilization can result in the preferential release of DON from soil organic matter (Kalbitz and Geyer 2002). On the other hand, the decreased C:N ratio of DOM can be a result of progressive organic matter degradation in soils (Ohno et al. 2010; Knicker 2011). Based on these information I was able

to link the observed alterations of fluvial DOM in agricultural streams to changes of organic matter processing in soil due to agricultural land use. The shift to a more microbial derived and processed DOM with agriculture has been described (Williams et al. 2010; Graeber et al. 2012b) and linked to soil processes earlier (Graeber et al. 2012b). Based on the findings of this thesis I could extend this knowledge now to alterations of DON and of the elemental composition of DOM reflected by its C:N ratio.

It is important to highlight that the aforementioned effects of agriculture on DOM composition and DON are not only a local phenomenon (study 1). Rather it applies also at the global scale, which w by the results of a comprehensive global survey of DOM and DON in headwater streams (study 2). Another important finding of this global monitoring covering different climate zones was, that the intensity of agricultural land use, and in particular of fertilization practices exerts strong effect on the amount of fluvial DON in headwater streams worldwide (study 2). Similar to the absence of a seasonal pattern on the local scale (study 1), the alterations of agricultural DOM composition occurred in both main seasons and demonstrated stable sources of altered fluvial DOM in agricultural streams on local as well as on global scale.

The alterations of DOM composition and DON concentration which I demonstrated in the first part of this thesis (study 1 and 2) may have far-reaching consequences on further processing of DOM in streams. The low C:N ratio of DOM is indicative of a lower content of refractory DOM sources (Sun et al. 1997) and hence, higher bioavailability of agricultural DOM. Whether intrinsic DOM properties, namely DOM composition or environmental conditions, as e.g. inorganic nutrient concentration, determine degradability of DOM is currently under strong debate (Schmidt et al. 2011; Marin-Spiotta et al. 2014; Kellerman et al. 2015). Therefore, with the experiment in the second part of this thesis (study 3) I aimed to identify the dominant factor determining DOC and DON degradability. The results from the long-term bioassay (81 days) suggest that neither composition nor inorganic nutrient concentration affected biodegradability of DOM. In fact, increasing DOC and DON concentrations were observed, which is in strong contrast to other studies reporting up to 44% and 70% of DON and 17% and 30% of DOC can be bioavailable in small mountain streams (Kaushal and Lewis 2005) and anthropogenic influenced estuaries (Petrone et al. 2009). This unexpected outcome of the bioassay experiment can presumably be explained by one major

difference in the experimental setup in this thesis. In contrast to previous studies that used planktonic bacteria (Wiegner et al. 2006; Wickland et al. 2012; Fasching et al. 2014) or suspended sediment bacteria (Kaushal and Lewis 2003; Kaushal and Lewis 2005; Petrone et al. 2009) for biodegradability studies, in this thesis a benthic biofilm was used. The initial intention of using a biofilm instead of 'free or suspended' bacteria was to take advantage of the ability of biofilms to use also complex DOM compounds, making them available by the use of extracellular enzymes (Battin et al. 2016). Besides, the use of benthic biofilms seemed appropriate since biofilms constitute key sites of organic matter processing in streams (Romaní et al. 2004; Battin et al. 2008; Battin et al. 2016) and specifically the production of bacteria which are important consumers of organic matter in lotic systems occurs in biofilms and to lower extend in the water column (Pusch et al. 1998). Consequently, biofilms should constitute a representative ecosystem compartment for studying bacterial DOM degradation. However, no degradation effect was observed, independent from DOM source and inorganic nutrient availability. This presumably can be attributed to the independence of the benthic biofilm bacteria from external sources due to internal nutrient supply from the EPS matrix of the biofilm (Freeman and Lock 1995). Recent findings of Kamjunke et al. (2015) further support this finding, showing responses to DOM composition for planktonic bacteria but not for benthic biofilm bacteria. Moreover, it seems unlikely that the DOM provided in the experiment was completely refractory within 81 days of experiment, since slightly decreasing DOM concentrations and increasing contribution of recently produced, microbial derived chromophoric DOM in the control treatment (DOM without biofilm) indicate that DOM was consumed by planktonic bacteria which presumably remained in the water column after 0.45µm filtration (Wang et al. 2007). Hence, in this thesis the missing biodegradation of DOM by the biofilms is likely not a result of the refractory character of DOM. Finally, from the results of the bioassay in this thesis I cannot general conclusions on the biodegradability of DOM in freshwaters and its dependence on either DOM composition or nutrient availability. Nevertheless, the findings I presented in this thesis, along with recent findings of Kamjunke et al. (2015) suggest that there is a need to distinguish between benthic and planktonic bacteria when investigating DOM processing in streams.

Field studies (study 1 and 2) and laboratory experiments (study 3) within this thesis demonstrate that the application of fluorescence analysis and PARAFAC in parallel with SEC

is an informative combination of methods to study fluvial DOM and DON dynamics. Within this thesis I provide information on the effects of cold storage and freezing on DOC and DON concentration and size fractions determined by SEC analysis (study 4), and therefore contribute to fill the gap of knowledge on the vulnerability of DOM samples for later SEC analysis to alterations due to storage.

Further perspectives

In this thesis I demonstrated that agricultural land use in the catchment alters DOM composition and increases DOC, and in particular DON concentration and export in headwater streams (Fig. 1). Based on these findings I was able to affirm earlier findings from soil science which indicated that leaching from soils is an important pathway of N loss from agriculture (van Kessel et al. 2009). This emphasizes the high connectivity of aquatic and terrestrial systems and therefore highly I recommend the integration of DON losses from agricultural soils to considerations in catchment nutrient management, as well as in terrestrial, but also aquatic N budgets.

Degradation of DOM by biofilm bacteria was not indicated in this study but there was some evidence for degradation due to planktonic bacteria. In fact, recent findings demonstrated that even old and terrestrial 'refractory' DOM can be respired by bacteria in streams and lakes, and potentially contribute to CO_2 outgassing from aquatic systems (McCallister and del Giorgio 2012; Fasching et al. 2014). Therefore, further investigations on the availability of DON and DOM with microbially altered structure and low C:N ratio for planktonic bacteria are recommended. Moreover, the findings of this thesis showed that elevated DON concentration and altered DOM composition with intensive agricultural land use occur across different climate zones. Future scenarios predict a further increase of fertilizer use in line with projected increases in crop production in the next 50 years (Alexandratos and Bruinsma 2012). Thereby strongest increases of agricultural production are projected for South Asia and Sub-Saharan Africa (Alexandratos and Bruinsma 2012), regions within the subtropical and tropical climate zone. Hence, future research should also focus on the effects of agricultural land use on DOM and DON processing under tropical and subtropical climate conditions.

Since DON constitutes an important nutrient source for phytoplankton, its composition and availability can influence species composition of freshwater and marine phytoplankton

assemblages (Berman and Bronk 2003; Fiedler et al. 2015), hence DON can contribute to eutrophication (Seitzinger and Sanders 1997). There is some evidence that even high molecular weight and humic DOM and DON can be available for phytoplankton (Berg et al. 2003; See et al. 2006). Although there is some evidence for the availability of humic DOM to phytoplankton from an experiment with comparatively fresh humic DON, the degradability of nitrogen-rich, soil derived DOM as it is exported in the agricultural streams investigated in this thesis, is largely unkown. In particular with respect to the currently changing perception on the recalcitrance of terrestrial DOM in aquatic systems (McCallister and del Giorgio 2012; Fasching et al. 2014) I recommend the further investigation of the potential bioavailability of nitrogen-rich, terrestrial DOM for phytoplankton specifically in aquatic systems further downstream from headwaters where autotrophic and planktonic processes are of higher importance (Vannote et al. 1980).

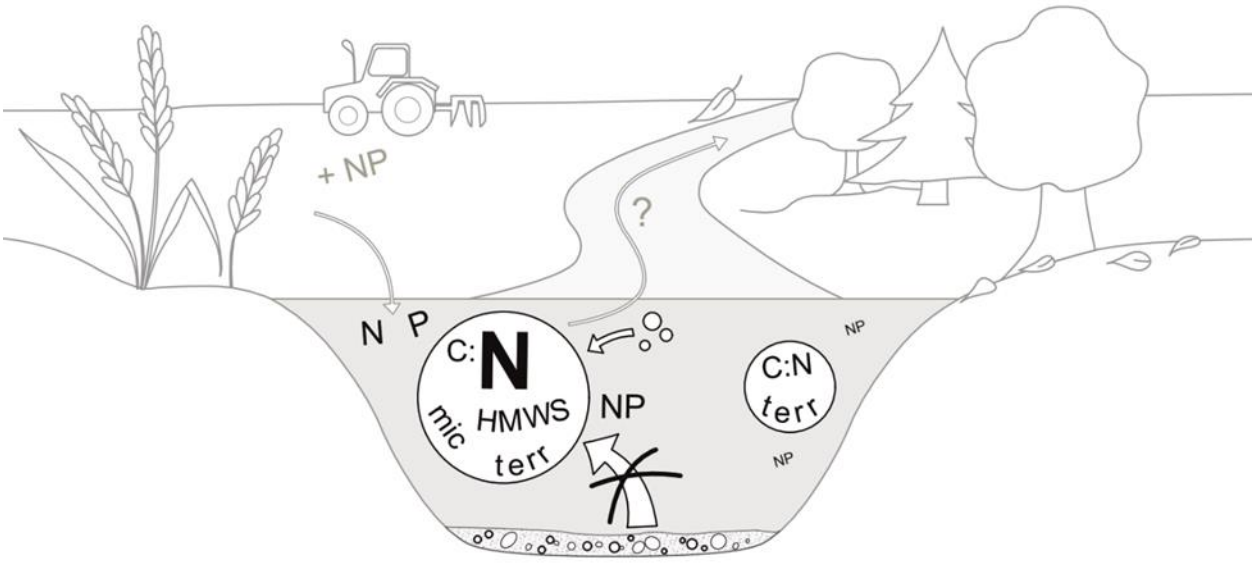

Figure 1. *Major findings of this thesis: Agriculture results in strong changes of DOM amount (represented by size of bubbles) and DOM composition, whereby strongest changes are highlighted in bold letters. The question marks represent a potential future research objective rising from this study, directing towards research on the fate of terrestrial N-rich DOM exported from agricultural headwaters in further downstream aquatic systems.*

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Statement of academic integrity

I hereby certify that the submitted thesis “Effects of agricultural land use on dissolved organic carbon and nitrogen in streams” is my own work, and that all published or other sources of material consulted in its preparation have been indicated. All collaboration that has taken place with other researchers is indicated and I have clearly stated my own personal share in those investigations in the Thesis Outline. I confirm that this work has not been submitted to any other university or examining body in an earlier doctoral procedure in the same or a similar form, or has been judged to be insufficient.

Berlin, 27.10.16

Marlen Heinz

Supplementary Information

Supplementary Information - Study 1

A comparison of organic matter composition in agricultural versus forest affected headwaters with special emphasis on organic nitrogen

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Contents:

S1 Processing of samples and bulk analysis of nitrate and ammonia

Prior to measurements, we filtered the samples with prerinsed (1 L deionized water and 300 ml sample) 8.0 µm cellulose acetate and 0.45 µm cellulose nitrate filters (Sartorius). For the measurement of NO_3^-/NH_4^+ we acidified the samples with 0.1N HCl to pH 2-3. Immediately after filtering we measured fluorescence on non-acidified samples, whereas for NO_3^-/NH_4^+ analysis, and DOC and DON determination the samples were stored at -20°C up to one month before measurement. Dissolved inorganic nitrogen (DIN) concentrations comprised of ammonium, nitrate, and nitrite we determined colorimetrically following standard methods (EN ISO 11732: NH_4^+, EN ISO 13359: NO_3^-, NO_2^-; the detection limit for NH_4^+ was 0.03 mg N L^{-1} and detection limit for NO_3^- and NO_2^- was 0.01 mg N L^{-1}) using a SCAN++ system (Skalar Analytical B.V., The Netherlands).

S2 Preprocessing of raw absorbance and fluorescence data and PARAFAC modelling

We measured absorbance from 190 to 800 nm in 0.5 nm steps using a Shimadzu UV-2401 UV/VIS spectrometer (Duisburg, Germany). To produce excitation-emission-matrices (EEMs) we measured the excitation (240 to 450, 2 nm steps), and emission (300 to 600 nm, 5 nm steps), with a slid width of 5 nm for both, using a Perking Elmer LS-50B fluorescence spectrometer (Rodgau, Germany). We corrected the absorbance data for the instrument baseline offset[1] and the fluorescence data for daily variations in instrument stability by applying the Raman correction.[2] Furthermore, we applied inner-filter correction for primary and secondary inner-filter effects.[3] We corrected the emission spectra with the BAM fluorescence calibration kit[4] and normalized it by the area under the Raman peak at 350 nm excitation wavelength[2]. All corrections and the subsequent PARAFAC analysis we performed using Matlab (R2010b, MathWorks, Isamning, Germany), and the DOM Fluor Toolbox (Version 1.7)[5]. To validate the PARAFAC components we used a randomized split half validation.[5]

Figures

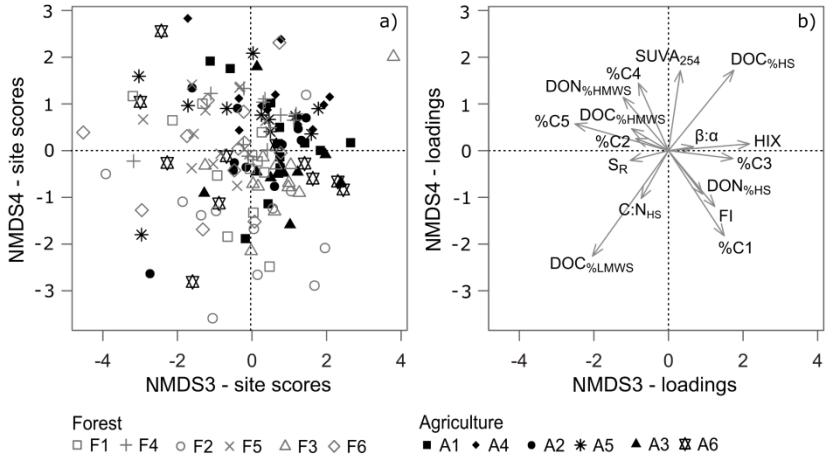

Fig. S1: *Results of the 4 dimensional NMDS ordination. The scores for dimension 3 and 4 are shown in panel a) with agricultural samples marked in black and forest samples in grey. The factor loadings for dimension 3 and 4, as well are shown in the panel b).*

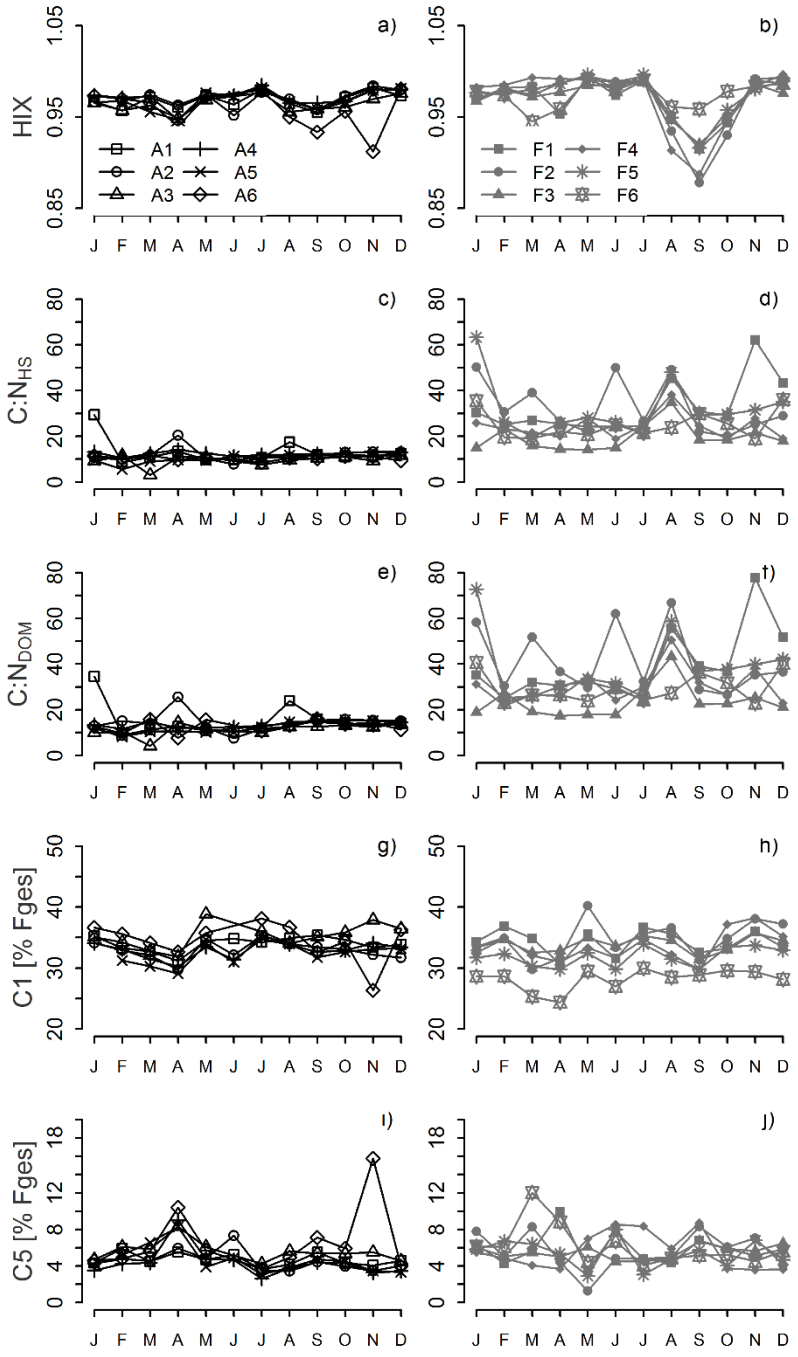

Fig. S2: *Temporal variation of a-b) HIX, c-d) C:N_{HS}, e-f) C:N_{DOM} g-h) %C1 and i-j) %C5 over the one year observation period for agriculture (left) and forest (right) streams.*

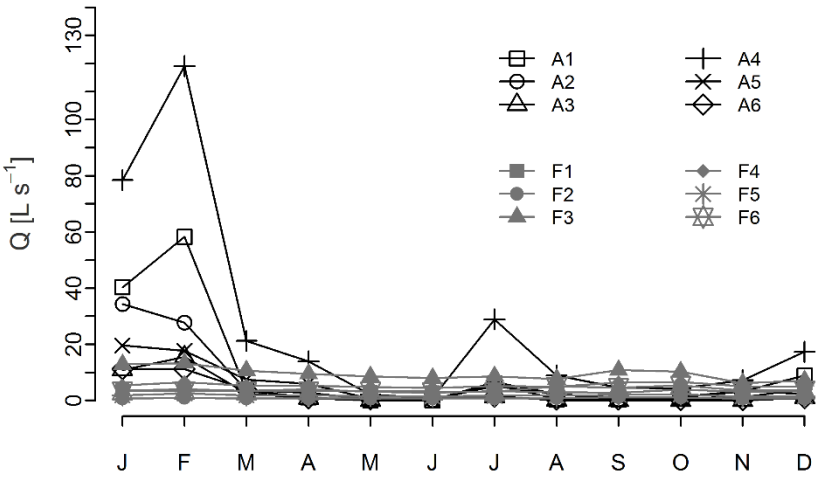

Fig. S3: *Discharge in the individual agricultural (black) and forest (grey) streams during the single sampling dates.*

Tables

Table S1: *Basic characteristics of the investigated forest (F1 - F6) and agricultural (A1 - A6) streams and their catchments. Agriculture refers to arable land with crop-based agriculture and coordinates are given in decimal degrees. For the forest streams additionally to the surface catchment area in parentheses the calculated potential catchment size is given.*

Stream	Lat[a]	Lon[a]	Catchment area [km²]	Mean annual Q [L s^{-1}] (± 1 SD)	Land use (% of catchment area)	Soils (% of catchment area)
A1	52.15	14.11	3.9	10.3 (18.8)	Agriculture (100)	DystricArenosols and HaplicAlbeluvisols (90), GleyicAlbeluvisols and GleyicLuvisols (10)
A2	52.15	14.13	2.1	8.0 (12.4)	Agriculture (100)	DystricArenosols and HaplicAlbeluvisols (88), DystricGleysols (12)
A3	52.34	14.35	3.4	3.5 (5.7)	Agriculture (100)	DystricArenosols and HaplicAlbeluvisols (100)
A4	52.41	14.21	8.8	28.3 (39.4)	Agriculture (83), Forest (9), Pasture (6) Settlement (2)	DystricArenosols and HaplicAlbeluvisols (90), Haplic and GleyicLuvisols (5), Mollic and HisticGleysols (5)
A5	52.44	14.26	4.3	6.1 (7.1)	Agriculture (100)	HaplicAlbeluvisols and HaplicLuvisols (95), FibricHistosols (5)
A6	52.43	14.14	2.5	3.0 (4.5)	Agriculture (98), Settlement (2)	DystricArenosols, HaplicAlbeluvisols and HaplicPodzols (95), GleyicArenosols (5)
F1	52.26	14.07	4.9 (1.2)	3.4 (0.4)	Forest (71), Agriculture (29)	DystricArenosols and HaplicPodzols (90), HaplicAlbeluvisols (10)
F2	52.13	14.47	0.1 (0.3)	0.8 (0.2)	Forest (95), Agriculture (5)	DystricArenosols (100)
F3	52.10	14.49	4.8 (3.4)	9.5 (2.2)	Forest (72), Agriculture (28)	DystricArenosols and HaplicAlbeluvisols (90), HisticGleysols (10)
F4	52.11	14.43	1.7 (1.9)	5.4 (0.7)	Forest (100)	DystricArenosols and HaplicPodzols (100)
F5	52.11	14.43	0.3 (0.7)	1.8 (0.3)	Forest (100)	DystricArenosols and HaplicPodzols (100)
F6	52.58	14.10	0.5 (1.4)	3.8 (0.8)	Forest (76), Agriculture (24)	DystricArenosols and HaplicPodzols (65), HaplicAlbeluvisol (30), Gleyic and Haplic Arenosols (5)

[a] Reference system: WGS84

Table S2: *Tentative characterization of the modelled PARAFAC components. Components 1 to 5 (C1-5) are described by the range and maximum of excitation and emission wavelength, secondary peaks are shown in brackets.*

Component	Excitation	Emission	Characterization according to literature
C1	240-425, 240 (345)	375-545, 430	humic-like fluorophore[6-9]; oxidized quinone-like component[10], terrestrial[6-7, 9]; ubiquitous in natural; common in soils[11]
C2	240-450, 240 (385)	430-600, 510	humic-like fluorophore[6-7]; reduced semiquinone-like component[10]; fulvic acid-like[6-7]; widespread, related to higher plant material and aromatic carbon content[10]; HMW, hydrophob, can sorb to soil[12]
C3	240-380, 240 (315)	350-465, 390	humic-like fluorophore[7-9]; reduced semiquinone-like component[10]; microbial transformed[8], occurs in wastewater and agricultural DOM[6, 9] and soils[11]
C4	240-360, 255 (240)	430-500, 460	humic-like fluorophore[6-7]; oxidized quinone-like component, plant derived (lignin derived)[10]; present in all environments[6]; LMW, recalcitrant[12], occurs in soils[11]
C5	240-340, 240 (275)	320-390, 350	tryptophan-like component[10, 7]; autochthonous[6], amino acids, free or bound in proteins, less degraded material[7], occurs in leachates from fresh plant material and in soils[11]

Table S3: *Mean (± 1SD) specific DOC and DON loads for the forest streams based on the calculated potential catchment size.*

Stream	Specific loads	
	DOC [mg C s^{-1} km^{-2}]	DON [mg N s^{-1} km^{-2}]
F1	8.7 (3.3)	0.22 (0.13)
F2	4.5 (1.9)	0.10 (0.06)
F3	6.9 (3.3)	0.25 (0.10)
F4	5.3 (1.9)	0.16 (0.08)
F5	5.9 (2.6)	0.14 (0.07)
F6	4.9 (1.3)	0.14 (0.03)

Table S4: *DOC, DON, DIN, and TDN concentration, percentage of DON from TDN and discharge Q during the 12 sampling dates for the individual agricultural (A1 – A6) and forest (F1 – F6) streams*.

site		DOC [mg C L^{-1}]	DON [mg C L^{-1}]	DIN [mg N L^{-1}]	TDN [mg N L^{-1}]	DON [%TDN]	Q [L s^{-1}]
A1	January	5.89	0.15	20.04	20.19	0.72	40.3
	February	8.85	0.85	7.63	8.48	10.01	58.3
	March	4.79	0.36	17.31	17.67	2.05	3.5
	April	8.27	0.56	12.69	13.25	4.19	1.2
	May	7.29	0.57	1.32	1.89	30.16	0.0
	June	7.91	0.66	1.30	1.95	33.68	0.1
	July	8.45	0.60	7.01	7.61	7.88	6.0
	August	9.35	0.33	11.05	11.39	2.93	0.5
	September	5.77	0.31	9.55	9.85	3.14	0.4
	October	4.73	0.28	8.59	8.88	3.20	1.2
	November	4.19	0.27	12.89	13.16	2.06	3.2
	December	3.57	0.22	11.34	11.56	1.89	8.9
mean		6.59	0.43	10.06	10.49	8.49	10.3
SD		2.00	0.21	5.56	5.43	11.28	18.8
median		6.59	0.35	10.30	10.62	3.17	2.2
min		3.57	0.15	1.30	1.89	0.72	0.0
max		9.35	0.85	20.04	20.19	33.68	58.3
A2	January	6.77	0.45	19.75	20.20	2.24	34.4
	February	9.89	0.56	12.25	12.81	4.39	27.8
	March	6.35	0.39	15.22	15.60	2.49	3.7
	April	5.21	0.17	13.55	13.73	1.27	2.8
	May	5.97	0.42	6.51	6.92	5.99	0.4
	June	7.60	0.86	1.99	2.85	30.18	0.1
	July	7.04	0.52	11.61	12.13	4.26	6.6
	August	4.16	0.28	9.12	9.40	2.98	2.3
	September	5.13	0.29	7.56	7.85	3.63	0.4
	October	4.11	0.22	4.89	5.11	4.36	1.3
	November	4.56	0.25	6.09	6.34	4.00	3.5
	December	3.73	0.21	7.84	8.05	2.63	2.3
mean		5.88	0.39	9.70	10.08	5.70	7.1
SD		1.78	0.20	4.96	4.94	7.81	11.4
median		5.59	0.34	8.48	8.72	3.82	2.5
min		3.73	0.17	1.99	2.85	1.27	0.1
max		9.89	0.86	19.75	20.20	30.18	34.4

Table S4 continued:

site		DOC [mg C L^{-1}]	DON [mg C L^{-1}]	DIN [mg N L^{-1}]	TDN [mg N L^{-1}]	DON [%TDN]	Q [L s^{-1}]
A3	January	5.60	0.48	20.29	20.77	2.32	10.9
	February	5.52	0.45	13.47	13.92	3.25	15.5
	March	4.68	0.97	11.62	12.59	7.71	2.5
	April	5.38	0.32	15.12	15.44	2.08	0.8
	May	4.86	0.36	9.66	10.02	3.62	0.0
	July	6.57	0.56	10.62	11.18	5.03	1.4
	August	5.77	0.39	5.43	5.82	6.77	0.1
	September	5.72	0.39	1.67	2.06	18.87	0.0
	October	5.34	0.35	0.52	0.87	39.84	0.0
	November	5.72	0.40	0.91	1.30	30.40	0.0
	December	4.42	0.27	13.02	13.29	2.06	1.0
mean		5.42	0.45	9.30	9.75	11.09	2.9
SD		0.59	0.19	6.43	6.47	12.98	5.2
median		5.52	0.39	10.62	11.18	5.03	0.8
min		4.42	0.27	0.52	0.87	2.06	0.0
max		6.57	0.97	20.29	20.77	39.84	15.5
A4	January	9.81	0.63	22.17	22.79	2.74	78.5
	February	8.41	0.62	11.27	11.89	5.20	119.1
	March	8.49	0.49	8.71	9.20	5.35	21.2
	April	9.35	0.67	7.02	7.68	8.71	13.9
	May	3.68	0.26	2.23	2.49	10.44	2.1
	June	10.80	0.74	1.17	1.91	38.54	1.0
	July	9.09	0.60	7.43	8.03	7.46	28.9
	August	8.78	0.53	5.79	6.32	8.44	8.9
	September	8.52	0.50	2.52	3.02	16.54	4.6
	October	7.74	0.47	2.14	2.61	18.00	4.4
	November	8.05	0.52	4.15	4.67	11.08	7.3
	December	6.16	0.37	7.77	8.13	4.51	17.4
mean		8.24	0.53	6.86	7.40	11.42	25.6
SD		1.83	0.13	5.73	5.77	9.71	36.2
median		8.51	0.53	6.40	7.00	8.57	11.4
min		3.68	0.26	1.17	1.91	2.74	1.0
max		10.80	0.74	22.17	22.79	38.54	119.1

Table S4 continued:

site		DOC [mg C L^{-1}]	DON [mg C L^{-1}]	DIN [mg N L^{-1}]	TDN [mg N L^{-1}]	DON [%TDN]	Q [L s^{-1}]
A5	January	4.49	0.32	16.92	17.24	1.86	19.6
	February	9.99	1.04	12.59	13.62	7.60	17.7
	March	8.62	0.71	11.86	12.57	5.65	7.5
	April	10.38	0.84	10.10	10.94	7.64	6.0
	May	7.86	0.66	10.44	11.10	5.97	0.7
	June	8.76	0.63	4.84	5.46	11.46	0.4
	July	10.28	0.71	10.00	10.71	6.61	5.1
	August	7.31	0.43	9.48	9.90	4.31	2.3
	September	7.16	0.42	8.51	8.93	4.68	1.0
	October	6.83	0.38	11.32	11.70	3.21	1.0
	November	6.84	0.39	10.24	10.63	3.62	1.2
	December	5.69	0.35	12.13	12.47	2.77	4.1
mean		7.85	0.57	10.70	11.27	5.45	5.6
SD		1.84	0.23	2.82	2.80	2.65	6.6
median		7.59	0.53	10.34	11.02	5.17	3.2
min		4.49	0.32	4.84	5.46	1.86	0.4
max		10.38	1.04	16.92	17.24	11.46	19.6
A6	January	3.41	0.23	22.14	22.37	1.02	11.2
	February	5.07	0.45	9.22	9.68	4.69	11.2
	March	4.07	0.22	24.50	24.72	0.89	3.9
	April	3.45	0.39	21.53	21.91	1.77	0.8
	May	6.63	0.36	12.98	13.34	2.72	0.4
	July	6.01	0.48	9.49	9.96	4.80	1.4
	August	5.93	0.39	4.57	4.96	7.77	0.4
	September	6.21	0.33	10.88	11.22	2.96	0.4
	October	3.83	0.24	13.52	13.76	1.74	0.2
	November	2.57	0.15	12.34	12.49	1.22	0.2
	December	3.20	0.24	17.88	18.12	1.34	0.8
mean		4.58	0.32	14.46	14.78	2.81	2.8
SD		1.43	0.11	6.26	6.21	2.14	4.3
median		4.07	0.33	12.98	13.34	1.77	0.8
min		2.57	0.15	4.57	4.96	0.89	0.2
max		6.63	0.48	24.50	24.72	7.77	11.2

Table S4 continued:

site		DOC [mg C L^{-1}]	DON [mg C L^{-1}]	DIN [mg N L^{-1}]	TDN [mg N L^{-1}]	DON [%TDN]	Q [L s^{-1}]
F1	January	3.45	0.08	0.12	0.20	42.11	3.6
	February	4.75	0.16	0.27	0.43	37.86	4.0
	March	2.91	0.08	0.14	0.21	36.53	3.5
	April	3.11	0.09	0.14	0.22	39.19	4.0
	May	2.72	0.07	0.10	0.17	42.48	3.1
	June	1.73	0.05	0.14	0.19	26.98	2.8
	July	3.91	0.14	0.11	0.25	56.21	3.3
	August	4.55	0.07	0.13	0.20	35.18	3.0
	September	2.46	0.05	0.02	0.08	70.13	2.8
	October	2.28	0.05	0.06	0.11	47.75	3.9
	November	2.54	0.03	0.02	0.05	53.85	3.1
	December	2.17	0.04	0.10	0.14	26.18	3.4
mean		3.05	0.08	0.11	0.19	42.87	3.4
SD		0.95	0.04	0.06	0.10	12.56	0.4
median		2.82	0.07	0.11	0.19	40.65	3.4
min		1.73	0.03	0.02	0.05	26.18	2.8
max		4.75	0.16	0.27	0.43	70.13	4.0
F2	January	2.92	0.04	1.44	1.48	2.88	0.7
	February	2.94	0.08	1.38	1.46	5.68	0.9
	March	1.21	0.02	1.41	1.43	1.40	0.7
	April	1.37	0.03	1.84	1.87	1.71	0.7
	May	1.28	0.04	1.29	1.33	2.79	0.7
	June	1.23	0.02	1.50	1.52	1.12	0.7
	July	1.55	0.04	1.16	1.20	3.42	0.8
	August	2.34	0.03	1.39	1.42	2.11	0.9
	September	1.28	0.04	1.64	1.68	2.26	1.2
	October	1.15	0.04	1.54	1.58	2.34	1.1
	November	1.36	0.03	1.45	1.48	2.22	0.7
	December	1.11	0.03	1.58	1.61	1.62	0.8
mean		1.65	0.04	1.47	1.51	2.46	0.8
SD		0.68	0.02	0.17	0.17	1.21	0.2
median		1.32	0.04	1.44	1.48	2.24	0.7
min		1.11	0.02	1.16	1.20	1.12	0.7
max		2.94	0.08	1.84	1.87	5.68	1.2

Table S4 continued:

site		DOC [mg C L^{-1}]	DON [mg C L^{-1}]	DIN [mg N L^{-1}]	TDN [mg N L^{-1}]	DON [%TDN]	Q [L s^{-1}]
F3	January	2.27	0.10	0.35	0.45	22.91	12.9
	February	3.78	0.12	0.23	0.35	33.76	13.3
	March	1.69	0.08	0.19	0.27	28.52	10.7
	April	2.42	0.12	0.21	0.33	36.36	9.6
	May	2.33	0.11	0.17	0.28	40.14	8.6
	June	1.50	0.07	0.15	0.23	31.79	8.0
	July	2.46	0.07	0.16	0.23	31.40	8.6
	August	5.09	0.10	0.15	0.25	39.69	7.7
	September	2.04	0.08	0.13	0.20	38.42	10.9
	October	2.01	0.08	0.09	0.17	44.97	10.3
	November	1.95	0.07	0.03	0.09	69.84	6.2
	December	1.89	0.08	0.11	0.18	41.96	7.1
mean		2.45	0.09	0.16	0.25	38.31	9.5
SD		1.01	0.02	0.08	0.09	11.70	2.2
median		2.16	0.08	0.16	0.24	37.39	9.1
min		1.50	0.07	0.03	0.09	22.91	6.2
max		5.09	0.12	0.35	0.45	69.84	13.3
F4	January	2.33	0.06	0.09	0.15	41.69	5.4
	February	2.95	0.12	0.18	0.29	39.46	6.6
	March	1.46	0.05	0.07	0.11	41.59	5.3
	April	1.58	0.05	0.05	0.10	50.00	5.3
	May	1.61	0.04	0.05	0.09	42.33	4.8
	June	1.44	0.05	0.06	0.11	47.22	4.5
	July	1.90	0.05	0.06	0.11	47.37	5.2
	August	2.36	0.04	0.05	0.09	42.55	5.0
	September	1.49	0.04	0.06	0.10	41.03	6.4
	October	1.70	0.06	0.04	0.09	60.44	6.7
	November	1.83	0.04	0.03	0.07	60.29	5.1
	December	1.39	0.05	0.06	0.11	48.37	5.0
mean		1.84	0.05	0.07	0.12	46.86	5.4
SD		0.48	0.02	0.04	0.06	7.13	0.7
median		1.66	0.05	0.06	0.11	44.89	5.3
min		1.39	0.04	0.03	0.07	39.46	4.5
max		2.95	0.12	0.18	0.29	60.44	6.7

Table S4 continued:

site		DOC [mg C L^{-1}]	DON [mg C L^{-1}]	DIN [mg N L^{-1}]	TDN [mg N L^{-1}]	DON [%TDN]	Q [L s^{-1}]
F5	January	4.41	0.05	0.06	0.12	45.22	1.9
	February	2.79	0.10	0.17	0.27	35.58	2.6
	March	1.96	0.07	0.06	0.12	53.06	1.9
	April	1.73	0.05	0.05	0.10	49.00	1.9
	May	1.84	0.05	0.05	0.09	51.09	1.6
	June	1.62	0.04	0.05	0.10	46.07	1.5
	July	2.09	0.07	0.06	0.13	55.12	1.7
	August	2.74	0.04	0.06	0.10	41.67	1.8
	September	1.81	0.04	0.05	0.09	44.92	2.1
	October	1.72	0.04	0.02	0.06	65.00	2.2
	November	2.00	0.04	0.02	0.06	67.72	1.5
	December	1.92	0.04	0.02	0.06	61.42	1.4
mean		2.22	0.05	0.06	0.11	51.32	1.8
SD		0.78	0.02	0.04	0.05	9.66	0.3
median		1.94	0.05	0.05	0.10	50.04	1.8
min		1.62	0.04	0.02	0.06	35.58	1.4
max		4.41	0.10	0.17	0.27	67.72	2.6
F6	January	3.04	0.06	0.83	0.89	7.17	3.4
	February	2.00	0.07	0.84	0.92	8.08	3.6
	March	1.88	0.06	0.78	0.84	7.24	3.4
	April	1.70	0.06	0.84	0.89	6.18	3.4
	May	1.50	0.05	0.81	0.87	6.24	3.4
	June	1.18	0.03	0.80	0.83	4.09	3.2
	July	1.78	0.06	0.44	0.50	12.63	3.1
	August	1.75	0.06	0.77	0.82	6.69	5.0
	September	1.65	0.04	0.83	0.87	4.49	4.7
	October	1.63	0.04	0.81	0.85	5.18	5.5
	November	1.52	0.06	0.78	0.83	6.83	3.5
	December	1.83	0.04	0.85	0.89	4.39	3.8
mean		1.79	0.05	0.78	0.83	6.60	3.8
SD		0.45	0.01	0.11	0.11	2.28	0.8
median		1.73	0.06	0.81	0.86	6.46	3.5
min		1.18	0.03	0.44	0.50	4.09	3.1
max		3.04	0.07	0.85	0.92	12.63	5.5

Table S5: *mean, standard deviation (SD), median, minimum (min) and maximum (max) for DOM composition parameters measured with SEC ($DOC_{\%HMWS}$, $DOC_{\%HS}$, $DON_{\%HMWS}$, $DON_{\%HS}$ given in percent of total DOC and DON concentration; $C:N_{HS}$; $SUVA_{254}$ given in L mg^{-1} m^{-1}) and absorbance and fluorescence spectroscopy (S_R, FI, β:α, contribution of PARAFAC component C1 – C5 given as percentage of total sample fluorescence).*

		$DOC_{\%HMWS}$	$DOC_{\%HS}$	$DON_{\%HMWS}$	$DON_{\%HS}$	$C:N_{HS}$	$SUVA_{254}$
agriculture	mean	5.63	78.83	9.89	90.11	11.29	3.95
	SD	3.64	7.82	9.47	9.47	3.14	0.69
	median	4.65	81.25	8.54	91.46	11.00	3.92
	min	0.80	55.00	0.00	56.14	3.04	2.01
	max	15.70	91.00	43.86	100.00	29.35	6.43
forest	mean	2.13	81.07	2.01	97.81	27.60	3.32
	SD	1.20	4.18	6.28	6.31	10.35	0.98
	median	1.75	82.00	0.00	100.00	25.19	3.25
	min	0.40	71.50	0.00	75.00	14.08	1.26
	max	6.70	89.50	25.93	100.00	63.30	8.02

Table S5 continued:

		S_R	FI	β:α	%C1	%C2	%C3	%C4	%C5
agriculture	mean	0.83	1.55	0.65	33.65	22.66	27.86	10.77	5.06
	SD	0.10	0.05	0.04	2.13	1.59	1.23	2.87	1.92
	median	0.83	1.55	0.64	33.89	22.87	27.78	11.09	4.58
	min	0.44	1.46	0.57	26.34	15.35	25.82	1.76	2.61
	max	1.08	1.67	0.74	38.86	25.41	30.84	15.66	15.78
forest	mean	1.07	1.50	0.60	32.74	24.97	24.61	12.00	5.68
	SD	0.13	0.08	0.06	3.05	3.67	2.05	3.95	1.75
	median	1.03	1.48	0.60	32.94	25.20	24.63	11.43	5.46
	min	0.85	1.35	0.50	24.37	18.31	20.57	4.87	1.28
	max	1.45	1.72	0.74	40.23	30.91	27.64	21.91	12.00

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Supplementary Information - Study 2

Global effects of agriculture on fluvial dissolved organic matter

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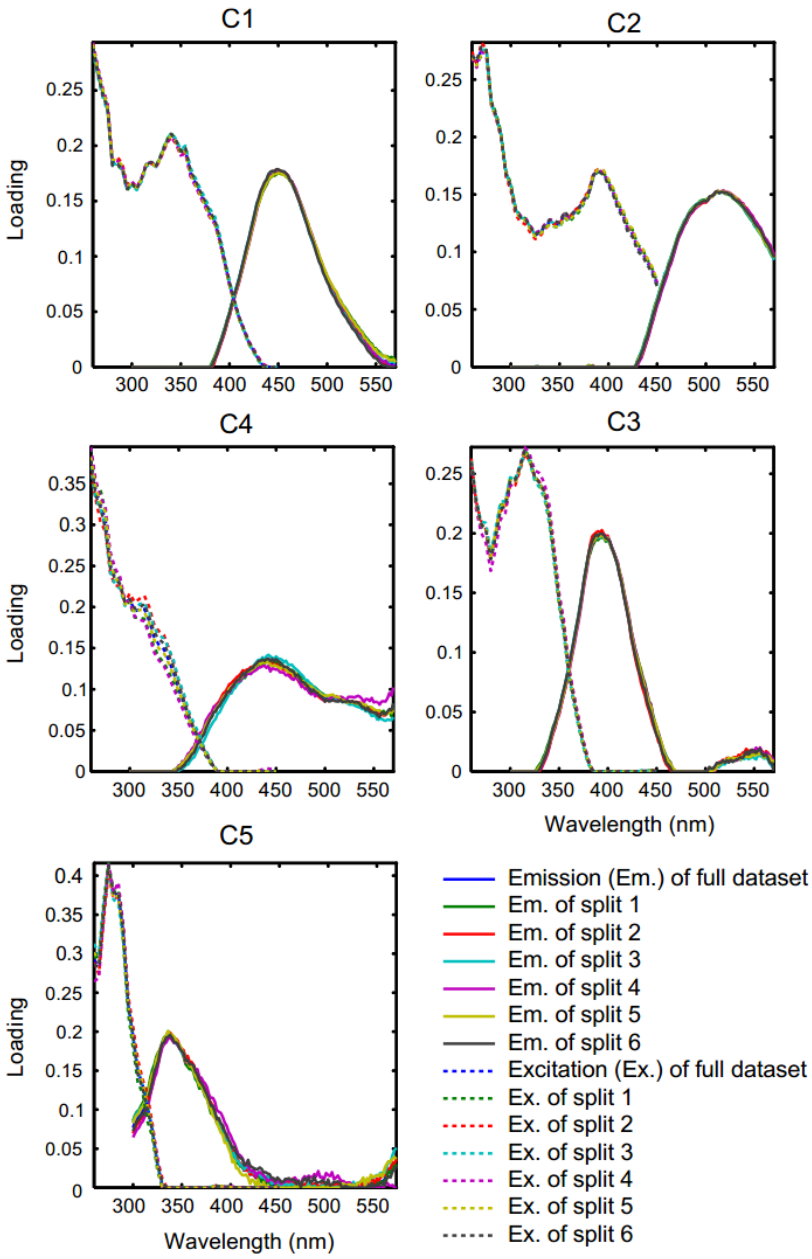
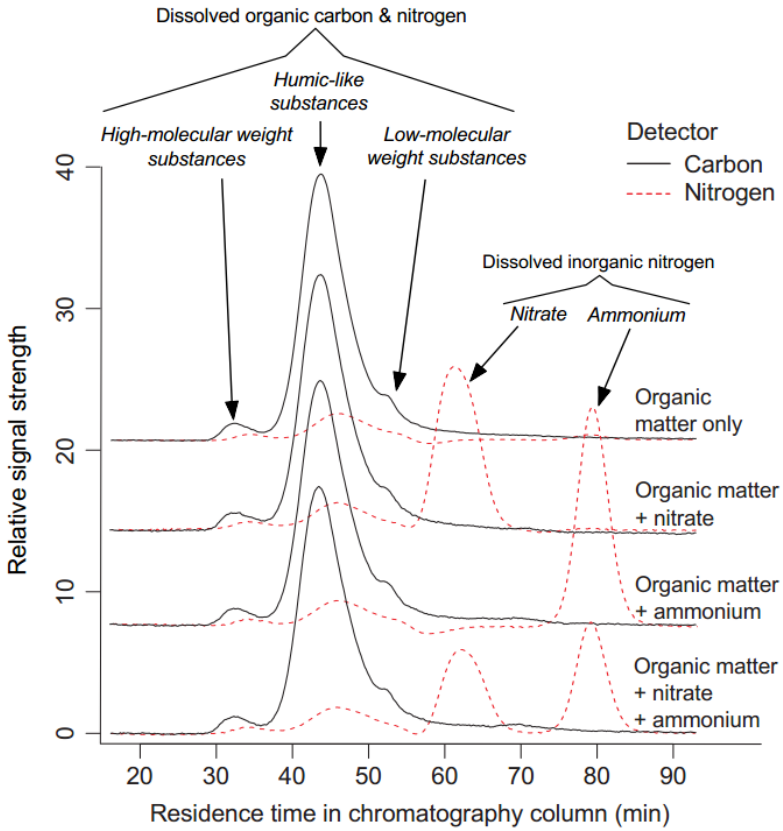


Supplementary Figure S1 | Split-half validation of the PARAFAC model. The models for six halfs generated by split-half validation are shown. When the fits of the splits are similar to each other and the entire model, a high stability of the model and low randomness of the fluorophores is given.



Supplementary Figure S2 | Typical chromatogram of size-exclusion chromatography. The chromatogram shows the distribution of DOM fractions with and without added nitrate and ammonium. The sample for this chromatogram was taken at a wetland outflow (part of the German subdataset of this study) .

Supplementary Catchment Data

The number of sampled catchments ranged between 4 and 19 for each pristine, intensive farming and extensive farming catchments within each season and climate zone (Supplementary Table S2). Excepted from this were tropical catchments, as due to very low DOM concentrations, the DOM composition measurements for some samples exhibited a high uncertainty and thus had to be excluded. Therefore, only two reference catchments could be included for each dry and wet season and only three arable farming catchments could be included for the dry season (Supplementary Table S2). In addition, one stream could only be sampled once due to seasonal stream intermittency (ESP-06). Other streams of the same land use were sampled instead. Moreover, the summer samples for DK-13 and GER-05 and a part of the samples from Chile (7 samples from winter/wet season: CHL-10, CHL-11, CHL-12, CHL-13, CHL-14, CHL-15, CHL-17) and Uruguay (2 samples from wet season: DF-03 and DF-08) were damaged during transport and thus could not be measured. These samples represented only one observation within one of the seasons (Supplementary information Table S2).

Detailed soil and land use characteristics are available for the countries of the northern temperate (Germany, Denmark), Mediterranean (Spain) and southern temperate (Chile) climate zone (Supplementary information Table S3, S4, S5). Here the percentages of agricultural land-use types could be analysed in detail based on the European Corine dataset[1] in Germany and Spain, based on a national mapping in Denmark, and based on Landsat data in Chile an Uruguay. Landsat data was also planned to be used for Brazil, however, the land use classification of the Landsat data were in disagreement with the data from ground-based surveys at the streams. Thus, we used classes based on ground surveys for the land use groupings instead of the Landsat data for Brazil.

References

[1] European Environmental Agency. Clc2006 technical guidelines. Tech. Rep., European Environmental Agency (2007).

Supplementary Table S2 | Site abbreviations, climate zone, country, number of observations, catchment size and GPS coordinates. Each site was sampled during the two main seasons, but due to different reasons, a part of the sites could only be sampled once (see text above for explanations). Pristine, pristine forest and pristine wetland belong to the reference catchments.

Site	Biome	Country	Type	Observations	Catchment size	GPS Y	GPS X
					km2	WGS 84, decimal degrees	
DK-01	Northern temperate	Denmark	intensive farming	2	14.4	55.65	8.53
DK-02	Northern temperate	Denmark	intensive farming	2	9.5	55.49	9.2
DK-03	Northern temperate	Denmark	intensive farming	2	6	56	9.85
DK-04	Northern temperate	Denmark	intensive farming	2	46.4	56.11	9.82
DK-05	Northern temperate	Denmark	intensive farming	2	3.9	56.23	9.76
DK-07	Northern temperate	Denmark	pristine	2	4.6	56.08	9.42
DK-09	Northern temperate	Denmark	pristine	2	0.6	56.25	10.6
DK-10	Northern temperate	Denmark	intensive farming	2	11.2	56.64	9.04
DK-11	Northern temperate	Denmark	pristine	2	5.4	56.47	8.38
DK-12	Northern temperate	Denmark	intensive farming	2	21.9	55.84	9.51
DK-13	Northern temperate	Denmark	pristine	1	0.4	55.11	10.32
DK-14	Northern temperate	Denmark	pristine	2	0.6	55.09	10.54
DK-15	Northern temperate	Denmark	intensive farming	2	4.3	55.12	10.77

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Site	Biome	Country	Type	Observations	Catchment size	GPS Y	GPS X
					km2	WGS 84, decimal degrees	
DK-16	Northern temperate	Denmark	intensive farming	2	5.4	55.8	12.03
DK-17	Northern temperate	Denmark	pristine	2	6.1	55.96	12.35
DK-18	Northern temperate	Denmark	pristine	2	0.6	55.78	12.44
DK-19	Northern temperate	Denmark	intensive farming	2	12.9	55.49	11.77
DK-20	Northern temperate	Denmark	intensive farming	2	15	55.33	11.6
DK-21	Northern temperate	Denmark	pristine	2	0.5	56.12	9.52
GER-02	Northern temperate	Germany	intensive farming	2	4.1	52.18	14.18
GER-03	Northern temperate	Germany	intensive farming	2	3.5	52.34	14.35
GER-04	Northern temperate	Germany	intensive farming	2	2.1	52.15	14.13
GER-05	Northern temperate	Germany	intensive farming	1	3.5	52.17	14.13
GER-06	Northern temperate	Germany	intensive farming	2	3.9	52.15	14.11
GER-07	Northern temperate	Germany	intensive farming	2	4.3	52.44	14.26
GER-08	Northern temperate	Germany	pristine forest	2	5	52.26	14.07
GER-09	Northern temperate	Germany	intensive farming	2	8.9	52.41	14.21
GER-10	Northern temperate	Germany	intensive farming	2	2.5	52.44	14.14
GER-11	Northern temperate	Germany	pristine forest	2	0.1	52.13	14.47

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Site	Biome	Country	Type	Observations	Catchment size	GPS Y	GPS X
					km2	WGS 84, decimal degrees	
GER-12	Northern temperate	Germany	pristine forest	2	1.3	52.1	14.49
GER-13	Northern temperate	Germany	pristine forest	2	0.3	52.11	14.43
GER-14	Northern temperate	Germany	extensive farming	2	20.2	52.76	12.94
GER-15	Northern temperate	Germany	extensive farming	2	8.7	52.78	13.04
GER-17	Northern temperate	Germany	extensive farming	2	3.5	52.29	13.33
GER-18	Northern temperate	Germany	extensive farming	2	4.4	52.29	13.33
GER-20	Northern temperate	Germany	extensive farming	2	24.3	52.78	12.94
GER-21	Northern temperate	Germany	pristine forest	2	1.7	52.11	14.43
GER-22	Northern temperate	Germany	pristine wetland	2	2.4	52.35	14.2
GER-23	Northern temperate	Germany	pristine wetland	2	6.6	52.52	13.84
GER-24	Northern temperate	Germany	pristine wetland	2	0.4	52.47	13.97
GER-25	Northern temperate	Germany	pristine wetland	2	1.2	52.47	13.97
GER-26	Northern temperate	Germany	pristine wetland	2	0.2	52.34	13.8
GER-28	Northern temperate	Germany	pristine wetland	2	6.8	52.16	13.58
ESP-01	Mediterranean	Spain	pristine	2	8.1	41.71	2.61
ESP-02	Mediterranean	Spain	pristine	2	13.2	41.7	2.58

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Site	Biome	Country	Type	Observations	Catchment size	GPS Y	GPS X
					km2	WGS 84, decimal degrees	
ESP-03	Mediterranean	Spain	pristine	2	1.4	41.71	2.56
ESP-04	Mediterranean	Spain	pristine	2	4.7	41.71	2.56
ESP-05	Mediterranean	Spain	pristine	2	0.7	41.66	2.53
ESP-06	Mediterranean	Spain	intensive farming	1	1.4	42.08	2.83
ESP-07	Mediterranean	Spain	intensive farming	2	1.1	42.09	2.84
ESP-08	Mediterranean	Spain	pristine	2	11.5	42.22	2.8
ESP-09	Mediterranean	Spain	intensive farming	2	0.8	42.24	2.86
ESP-10	Mediterranean	Spain	intensive farming	2	1.2	42.24	2.85
ESP-12	Mediterranean	Spain	intensive farming	2	1	42.22	3.06
ESP-13	Mediterranean	Spain	intensive farming	2	1	42.22	3.06
ESP-14	Mediterranean	Spain	pristine	2	19.1	41.83	2.94
BR-01	Tropical	Brazil	pristine	2	2.8	-21.06	-44.19
BR-02	Tropical	Brazil	pristine	1	1.4	-21.11	-44.18
BR-04	Tropical	Brazil	pristine	1	0.1	-21.04	-44.15
BR-11	Tropical	Brazil	intensive farming	1	0.2	-21.02	-44.23
BR-12	Tropical	Brazil	intensive farming	2	4.2	-21.17	-44.05

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Site	Biome	Country	Type	Observations	Catchment size	GPS Y	GPS X
					km2	WGS 84, decimal degrees	
BR-13	Tropical	Brazil	intensive farming	1	1.5	-21.04	-44.22
BR-14	Tropical	Brazil	intensive farming	2	4.2	-21.1	-44.2
BR-15	Tropical	Brazil	intensive farming	2	1.5	-20.99	-44.19
BR-16	Tropical	Brazil	extensive farming	2	2.1	-20.94	-44.06
BR-17	Tropical	Brazil	extensive farming	1	1.1	-21.06	-44.17
BR-18	Tropical	Brazil	extensive farming	1	1.4	-21	-44.19
BR-19	Tropical	Brazil	extensive farming	2	1.9	-21.02	-44.18
BR-20	Tropical	Brazil	extensive farming	2	0.2	-21.05	-44.08
BR-21	Tropical	Brazil	intensive farming	1	0.7	-21.07	-44.16
BR-22	Tropical	Brazil	extensive farming	1	0.5	-21.12	-44.2
CHL-02	Southern temperate	Chile	pristine	1	13.14	-38.55	-72.57
CHL-03	Southern temperate	Chile	extensive farming	2	28.51	-40.53	-72.59
CHL-04	Southern temperate	Chile	pristine	1	17.58	-40.28	-73.29
CHL-05	Southern temperate	Chile	pristine	2	13.61	-40.2	-73.42
CHL-06	Southern temperate	Chile	pristine	1	24.85	-40.23	-73.59
CHL-07	Southern temperate	Chile	pristine	2	9.05	-40.09	-73.37

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Site	Biome	Country	Type	Observations	Catchment size	GPS Y	GPS X
					km2	WGS 84, decimal degrees	
CHL-08	Southern temperate	Chile	pristine	2	24.89	-40.03	-73.03
CHL-09	Southern temperate	Chile	pristine	2	13.15	-39.33	-73.19
CHL-10	Southern temperate	Chile	extensive farming	1	34.37	-38.99	-73.21
CHL-11	Southern temperate	Chile	extensive farming	1	26.63	-38.96	-73.09
CHL-12	Southern temperate	Chile	extensive farming	1	34.27	-38.85	-73.04
CHL-13	Southern temperate	Chile	extensive farming	1	37.13	-39.15	-72.41
CHL-14	Southern temperate	Chile	extensive farming	1	38.78	-39.27	-72.47
CHL-15	Southern temperate	Chile	pristine	1	24.29	-39.41	-72.56
CHL-16	Southern temperate	Chile	extensive farming	2	27.26	-39.02	-72.46
CHL-17	Southern temperate	Chile	extensive farming	1	37.95	-40.34	-72.64
CHL-18	Southern temperate	Chile	extensive farming	2	26.62	-40.23	-72.61
A01	Subtropical	Uruguay	intensive farming	2	9.23	-34.16	-56.68
A12	Subtropical	Uruguay	intensive farming	2	12.31	-32.22	-54.43
A22	Subtropical	Uruguay	intensive farming	2	5.33	-34.06	-58.09
A23	Subtropical	Uruguay	intensive farming	2	4.32	-34.06	-58.06
A37	Subtropical	Uruguay	intensive farming	2	9.1	-33.48	-57.86

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Site	Biome	Country	Type	Observations	Catchment size	GPS Y	GPS X
					km2	WGS 84, decimal degrees	
A39	Subtropical	Uruguay	intensive farming	2	10.12	-33.16	-57.62
A58	Subtropical	Uruguay	intensive farming	2	9.39	-33.17	-58.12
A59	Subtropical	Uruguay	intensive farming	2	10.64	-33.17	-58.25
A60	Subtropical	Uruguay	intensive farming	2	7.69	-33.21	-58.31
A61	Subtropical	Uruguay	intensive farming	2	10	-33.15	-58.27
A63	Subtropical	Uruguay	intensive farming	2	21.7	-33.03	-57.95
A65	Subtropical	Uruguay	intensive farming	2	9.53	-33	-58.03
DF01	Subtropical	Uruguay	extensive farming	2	30.44	-34.06	-56.62
DF02	Subtropical	Uruguay	extensive farming	2	6.48	-34.13	-56.65
DF03	Subtropical	Uruguay	extensive farming	1	8.71	-34.15	-56.7
DF06	Subtropical	Uruguay	extensive farming	2	16.16	-33.22	-57.97
DF07	Subtropical	Uruguay	extensive farming	2	5.63	-32.81	-57.4
DF08	Subtropical	Uruguay	extensive farming	1	8.72	-32.86	-57.39
DF09	Subtropical	Uruguay	extensive farming	2	5.44	-32.8	-57.39
DF10	Subtropical	Uruguay	extensive farming	2	5.56	-32.79	-57.38
DF11	Subtropical	Uruguay	extensive farming	2	17.2	-34.18	-57.83

Continued on next page

Site	Biome	Country	Type	Observations	Catchment size	GPS Y	GPS X
					km2	WGS 84, decimal degrees	
E02	Subtropical	Uruguay	pristine	2	12.72	-33.98	-56.48
E05	Subtropical	Uruguay	pristine	2	20.93	-32.65	-56.54
E07	Subtropical	Uruguay	pristine	2	17.51	-32.33	-56.25
E08	Subtropical	Uruguay	pristine	2	3.06	-32.17	-56.11
E09	Subtropical	Uruguay	pristine	2	5.33	-32.21	-56.08
E30	Subtropical	Uruguay	pristine	2	6.31	-32.53	-56.63
E26	Subtropical	Uruguay	pristine	2	17.82	-32.57	-54.41
EFM	Subtropical	Uruguay	pristine	2	28.54	-31.79	-55.65

Supplementary Table S3 | Site abbreviations and land use in the catchments.

Site	Intensive farming	Extensive farming	Pristine	Other
		%		
DK-01	85.5	0.5	8.2	5.8
DK-02	92.7	0	0.5	6.8
DK-03	75.6	0.1	17.6	6.7
DK-04	86.9	0.3	4.4	8.5
DK-05	93.3	0	2.8	3.9
DK-07	26.1	0.2	70.5	3.2
DK-09	0	0	98.2	1.8
DK-10	88	0	0.4	11.6
DK-11	1.7	0	93.9	4.5
DK-12	88.4	0.3	3.7	7.5
DK-13	0	0	98.8	1.2
DK-14	6.7	0	90.1	3.2
DK-15	88.2	0	1.2	10.5
DK-16	88.1	0	3.5	8.4
DK-17	10.4	0	81	8.6
DK-18	5.5	0	89.8	4.7
DK-19	75.1	0.3	15.7	8.9
DK-20	74.5	0.1	11.6	13.9
DK-21	0.2	0	98	1.8
GER-02	88.5	0	0.5	11
GER-03	99.7	0	0	0.3
GER-04	99.7	0.3	0	0
GER-05	100	0	0	0
GER-06	100	0	0	0

Continued on next page

Site	Intensive farming	Extensive farming	Pristine	Other
		%		
GER-07	100	0	0	0
GER-08	29	0	71	0
GER-09	83.1	5.8	9.6	1.5
GER-10	97.8	0	0	2.2
GER-11	0	0	95.1	4.9
GER-12	27.7	0	72.3	0
GER-13	0	0	100	0
GER-14	15.2	71.9	8.3	4.5
GER-15	8.6	59.8	27.9	3.7
GER-17	17.7	76.9	2	3.4
GER-18	0.4	99.2	0.4	0
GER-20	0	78.5	21.5	0
GER-21	0	0	100	0
GER-22	0	0	100	0
GER-23	3.7	23.6	69	3.7
GER-24	0	0	86.8	13.2
GER-25	0	0	100	0
GER-26	0	0	100	0
GER-28	3.5	3.8	64.4	28.3
ESP-01	0.5	0	92.8	6.7
ESP-02	2.3	0	97.3	0.3
ESP-03	0	0	100	0
ESP-04	0.2	0.7	98.6	0.5
ESP-05	1.4	0	93.9	4.7
ESP-06	77.7	0	15.5	6.8

Continued on next page

Site	Intensive farming	Extensive farming	Pristine	Other
		%		
ESP-07	86.9	0	13.1	0
ESP-08	13.4	0	86.6	0
ESP-09	96.7	0	3.3	0
ESP-10	88.1	0	0	11.9
ESP-12	95	0	0	5
ESP-13	95	0	0	5
ESP-14	4.9	0	91.6	3.5
BR-01	0	0	> 80%	0
BR-02	0	0	> 80%	0
BR-04	0	0	> 80%	0
BR-11	> 50%	0	0	0
BR-12	> 50%	0	0	0
BR-13	> 50%	0	0	0
BR-14	> 50%	0	0	0
BR-15	> 50%	0	0	0
BR-16	0	> 50%	0	0
BR-17	0	> 50%	0	0
BR-18	0	> 50%	0	0
BR-19	0	> 50%	0	0
BR-20	0	> 50%	0	0
BR-21	> 50%	0	0	0
BR-22	0	> 50%	0	0
CHL-02	2	31.6	66.1	0.4
CHL-03	3	86	10.6	0.4
CHL-04	0	2.9	97.1	0

Continued on next page

Site	Intensive farming	Extensive farming	Pristine	Other
		%		
CHL-05	0	0	100	0
CHL-06	0	0	100	0
CHL-07	0	30.7	69.3	0
CHL-08	1.2	5.3	93.5	0
CHL-09	0	13.4	86.6	0
CHL-10	3.4	73.2	23.4	0
CHL-11	4.1	50.1	45.3	0.5
CHL-12	0.5	69.3	30.2	0
CHL-13	0.2	94.4	5.4	0
CHL-14	0	88	12	0
CHL-15	5.6	2.1	92.3	0
CHL-16	5.7	78.9	15	0.4
CHL-17	1.9	90	7.5	0.6
CHL-18	1.7	73.4	24.7	0.2
A01	100	0	0	0
A12	99.4	0	0	0.6
A22	91.1	0	8.3	0.6
A23	100	0	0	0
A37	74.7	0	24.9	0.3
A39	89.7	0	10.3	0.1
A58	>90	0	¡10	0
A59	93.6	0	1.3	5
A60	89.9	0	8.6	1.5
A61	88.8	0	8.7	2.6
A63	>90	0	¡10	0

Continued on next page

Site	Intensive farming	Extensive farming	Pristine	Other
		%		
A65	>90	0	¡10	0
DF01	0	98.6	0	1.4
DF02	0	100	0	0
DF03	0	99.7	0	0.3
DF06	0	99.4	0	0.6
DF07	0	98.8	0	1.2
DF08	0	99.7	0	0.3
DF09	0	98.8	0	1.2
DF10	0	99.9	0	0.1
DF11	0	97.8	1.2	1
E02	22.1	0	72.9	5
E05	0	0	99.4	0.6
E07	24.6	0	74.7	0.7
E08	0	0	100	0
E09	10.7	0	88.7	0.6
E30	8	0	91.9	0.2
E26	21.3	0	97.9	-19.1
EFM	3.7	0	90.6	5.7

Supplementary Table S4 | Site abbreviations and first part of soil types (Albeluvisols – Histosols) in the catchments. Please see Table S5 for first part.

Site	Albeluvisols	Andosols	Arenosols	Cambisols	Ferralsols	Fluvisols	Gleysols	Histosols
DK-01	0	0	0	0	0	0	0	0
DK-02	63.2	0	0	0	0	0	0	0
DK-03	0	0	0	0	0	0	0	0
DK-04	0	0	0	0	0	0	0	0
DK-05	0	0	0	0	0	0	0	0
DK-07	0	0	0	0	0	0	0	0
DK-09	0	0	100	0	0	0	0	0
DK-10	0	0	0	0	0	0	0	0
DK-11	0	0	0	0	0	0	0	0
DK-12	0	0	0	0	0	0	0	0
DK-13	0	0	0	0	0	0	0	0
DK-14	0	0	0	0	0	0	0	0
DK-15	0	0	0	0	0	0	0	0
DK-16	0	0	0	0	0	0	0	0
DK-17	0	0	0	0	0	0	0	0

Continued on next page

Site	Albeluvisols	Andosols	Arenosols	Cambisols	Ferralsols	Fluvisols	Gleysols	Histosols
DK-18	0	0	0	0	0	0	0	0
DK-19	0	0	12.8	87.2	0	0	0	0
DK-20	0	0	0	100	0	0	0	0
DK-21	0	0	0	0	0	0	0	0
GER-02	56.9	0	43.1	0	0	0	0	0
GER-03	96.3	0	3.7	0	0	0	0	0
GER-04	75.7	0	12	0	0	0	11.6	0.7
GER-05	47.6	0	52.4	0	0	0	0	0
GER-06	58.5	0	41.5	0	0	0	0	0
GER-07	94.8	0	0	0	0	0	0	5.2
GER-08	11.4	0	88.6	0	0	0	0	0
GER-09	73.7	0	13.1	0	0	0	6.9	0
GER-10	66.6	0	33.4	0	0	0	0	0
GER-11	0	0	100	0	0	0	0	0
GER-12	0	0	80.2	0	0	0	19.8	0
GER-13	0	0	100	0	0	0	0	0
GER-14	11.4	0	12.2	0	0	0	17.9	58.6

Continued on next page

Site	Albeluvisols	Andosols	Arenosols	Cambisols	Ferralsols	Fluvisols	Gleysols	Histosols
GER-15	0	0	27	0	0	0	9.2	63.7
GER-17	13	0	8.1	0	0	0	26.6	51.9
GER-18	0	0	0.8	0	0	0	0	99.2
GER-20	0	0	0	0	0	0	11.7	88.3
GER-21	0	0	99.8	0	0	0	0.2	0
GER-22	0	0	58.1	0	0	0	30.1	11.8
GER-23	0	0	74.1	0	0	0	0	25.9
GER-24	0	0	81.4	0	0	0	18.6	0
GER-25	0	0	63.2	0	0	0	36.8	0
GER-26	0	0	71.3	0	0	0	0	28.7
GER-28	0	0	67.8	0	0	0	14.3	17.2
ESP-01	0	0	0	0	0	0	0	0
ESP-02	0	0	0	0	0	1.4	0	0
ESP-03	0	0	0	0	0	100	0	0
ESP-04	0	0	0	0	0	66.4	0	0
ESP-05	0	0	0	0	0	0	0	0
ESP-06	0	0	0	49.2	0	0	0	0

Continued on next page

Site	Albeluvisols	Andosols	Arenosols	Cambisols	Ferralsols	Fluvisols	Gleysols	Histosols
ESP-07	0	0	0	100	0	0	0	0
ESP-08	0	0	0	73.2	0	0	0	0
ESP-09	0	0	0	100	0	0	0	0
ESP-10	0	0	0	100	0	0	0	0
ESP-12	0	0	0	0	0	100	0	0
ESP-13	0	0	0	0	0	100	0	0
ESP-14	0	0	0	0	0	73.6	0	0
BR-01	0	0	0	0	100	0	0	0
BR-02	0	0	0	0	100	0	0	0
BR-04	0	0	0	0	100	0	0	0
BR-11	0	0	0	0	100	0	0	0
BR-12	0	0	0	100	0	0	0	0
BR-13	0	0	0	0	100	0	0	0
BR-14	0	0	0	0	100	0	0	0
BR-15	0	0	0	0	100	0	0	0
BR-16	0	0	0	0	100	0	0	0
BR-17	0	0	0	0	100	0	0	0

Continued on next page

Site	Albeluvisols	Andosols	Arenosols	Cambisols	Ferralsols	Fluvisols	Gleysols	Histosols
BR-18	0	0	0	0	100	0	0	0
BR-19	0	0	0	0	100	0	0	0
BR-20	0	0	0	0	100	0	0	0
BR-21	0	0	0	0	100	0	0	0
BR-22	0	0	0	0	100	0	0	0
CHL-02	0	0	0	0	0	0	0	0
CHL-03	0	72.2	0	0	0	0	27.8	0
CHL-04	0	0	0	80.1	0	0	0	0
CHL-05	0	0	0	100	0	0	0	0
CHL-06	0	0	0	100	0	0	0	0
CHL-07	0	0	0	100	0	0	0	0
CHL-08	0	100	0	0	0	0	0	0
CHL-09	0	0	0	100	0	0	0	0
CHL-10	0	0	0	0	0	52.2	0	0
CHL-11	0	2.2	0	0	0	43	0	0
CHL-12	0	74.8	0	0	0	0.5	0	0
CHL-13	0	100	0	0	0	0	0	0

Continued on next page

Site	Albeluvisols	Andosols	Arenosols	Cambisols	Ferralsols	Fluvisols	Gleysols	Histosols
CHL-14	0	100	0	0	0	0	0	0
CHL-15	0	0	0	100	0	0	0	0
CHL-16	0	100	0	0	0	0	0	0
CHL-17	0	0	0	0	0	0	100	0
CHL-18	0	30.7	0	0	0	0	69.3	0
A01	0	0	0	0	0	0	0	0
A12	0	0	0	0	0	0	0	0
A22	0	0	0	0	0	0	0	0
A23	0	0	0	0	0	0	0	0
A37	0	0	0	0	0	0	0	0
A39	0	0	0	0	0	0	0	0
A58	0	0	0	0	0	0	0	0
A59	0	0	0	0	0	0	0	0
A60	0	0	0	0	0	0	0	0
A61	0	0	0	0	0	0	0	0
A63	0	0	0	0	0	0	0	0
A65	0	0	0	0	0	0	0	0

Continued on next page

Site	Albeluvisols	Andosols	Arenosols	Cambisols	Ferralsols	Fluvisols	Gleysols	Histosols
DF01	0	0	0	0	0	0	0	0
DF02	0	0	0	0	0	0	0	0
DF03	0	0	0	0	0	0	0	0
DF06	0	0	0	0	0	0	0	0
DF07	0	0	0	0	0	0	0	0
DF08	0	0	0	0	0	0	0	0
DF09	0	0	0	0	0	0	0	0
DF10	0	0	0	0	0	0	0	0
DF11	0	0	0	0	0	0	0	0
E02	0	0	0	0	0	0	0	0
E05	0	0	0	0	0	0	0	0
E07	0	0	0	0	0	0	0	0
E08	0	0	0	0	0	0	0	0
E09	0	0	0	0	0	0	0	0
E30	0	0	0	0	0	0	0	0
E26	0	0	0	0	0	0	0	0
EFM	0	0	0	0	0	0	0	0

Supplementary Table S5 | Site abbreviations and second part of soil types (Leptosols – Vertisols) in the catchments. Please see Table S4 for first part.

Site	Leptosols	Luvisols	Nitisols	Phaeozems	Planosols	Podzols	Regosols	Vertisols
DK-01	0	0	0	0	0	100	0	0
DK-02	0	0	0	0	0	36.8	0	0
DK-03	0	100	0	0	0	0	0	0
DK-04	0	61.2	0	0	0	38.8	0	0
DK-05	0	100	0	0	0	0	0	0
DK-07	0	0	0	0	0	100	0	0
DK-09	0	0	0	0	0	0	0	0
DK-10	0	100	0	0	0	0	0	0
DK-11	0	0	0	0	0	100	0	0
DK-12	0	92.1	0	0	0	7.9	0	0
DK-13	0	100	0	0	0	0	0	0
DK-14	0	100	0	0	0	0	0	0
DK-15	0	100	0	0	0	0	0	0
DK-16	0	7.3	0	0	0	92.7	0	0
DK-17	0	0	0	0	0	100	0	0

Continued on next page

Site	Leptosols	Luvisols	Nitisols	Phaeozems	Planosols	Podzols	Regosols	Vertisols
DK-18	0	100	0	0	0	0	0	0
DK-19	0	0	0	0	0	0	0	0
DK-20	0	0	0	0	0	0	0	0
DK-21	0	0	0	0	0	100	0	0
GER-02	0	0	0	0	0	0	0	0
GER-03	0	0	0	0	0	0	0	0
GER-04	0	0	0	0	0	0	0	0
GER-05	0	0	0	0	0	0	0	0
GER-06	0	0	0	0	0	0	0	0
GER-07	0	0	0	0	0	0	0	0
GER-08	0	0	0	0	0	0	0	0
GER-09	0	6.3	0	0	0	0	0	0
GER-10	0	0	0	0	0	0	0	0
GER-11	0	0	0	0	0	0	0	0
GER-12	0	0	0	0	0	0	0	0
GER-13	0	0	0	0	0	0	0	0
GER-14	0	0	0	0	0	0	0	0

Continued on next page

Site	Leptosols	Luvisols	Nitisols	Phaeozems	Planosols	Podzols	Regosols	Vertisols
GER-15	0	0	0	0	0	0	0	0
GER-17	0	0	0	0	0	0	0.3	0
GER-18	0	0	0	0	0	0	0	0
GER-20	0	0	0	0	0	0	0	0
GER-21	0	0	0	0	0	0	0	0
GER-22	0	0	0	0	0	0	0	0
GER-23	0	0	0	0	0	0	0	0
GER-24	0	0	0	0	0	0	0	0
GER-25	0	0	0	0	0	0	0	0
GER-26	0	0	0	0	0	0	0	0
GER-28	0	0	0	0	0	0	0.7	0
ESP-01	0	0	0	0	0	0	100	0
ESP-02	0	0	0	0	0	0	98.6	0
ESP-03	0	0	0	0	0	0	0	0
ESP-04	0	0	0	0	0	0	33.6	0
ESP-05	0	0	0	0	0	0	100	0
ESP-06	50.8	0	0	0	0	0	0	0

Continued on next page

Site	Leptosols	Luvisols	Nitisols	Phaeozems	Planosols	Podzols	Regosols	Vertisols
ESP-07	0	0	0	0	0	0	0	0
ESP-08	26.8	0	0	0	0	0	0	0
ESP-09	0	0	0	0	0	0	0	0
ESP-10	0	0	0	0	0	0	0	0
ESP-12	0	0	0	0	0	0	0	0
ESP-13	0	0	0	0	0	0	0	0
ESP-14	0	4.8	0	0	0	0	21.6	0
BR-01	0	0	0	0	0	0	0	0
BR-02	0	0	0	0	0	0	0	0
BR-04	0	0	0	0	0	0	0	0
BR-11	0	0	0	0	0	0	0	0
BR-12	0	0	0	0	0	0	0	0
BR-13	0	0	0	0	0	0	0	0
BR-14	0	0	0	0	0	0	0	0
BR-15	0	0	0	0	0	0	0	0
BR-16	0	0	0	0	0	0	0	0
BR-17	0	0	0	0	0	0	0	0

Continued on next page

Site	Leptosols	Luvisols	Nitisols	Phaeozems	Planosols	Podzols	Regosols	Vertisols
BR-18	0	0	0	0	0	0	0	0
BR-19	0	0	0	0	0	0	0	0
BR-20	0	0	0	0	0	0	0	0
BR-21	0	0	0	0	0	0	0	0
BR-22	0	0	0	0	0	0	0	0
CHL-02	0	0	100	0	0	0	0	0
CHL-03	0	0	0	0	0	0	0	0
CHL-04	0	0	19.9	0	0	0	0	0
CHL-05	100	0	0	0	0	0	0	0
CHL-06	100	0	0	0	0	0	0	0
CHL-07	27.6	0	0	0	0	72.4	0	0
CHL-08	0	0	0	0	0	0	0	0
CHL-09	0	0	0	0	0	0	0	0
CHL-10	0	0	47.8	0	0	0	0	0
CHL-11	0	0	54.8	0	0	0	0	0
CHL-12	0	0	24.7	0	0	0	0	0
CHL-13	0	0	0	0	0	0	0	0

Continued on next page

Site	Leptosols	Luvisols	Nitisols	Phaeozems	Planosols	Podzols	Regosols	Vertisols
CHL-14	0	0	0	0	0	0	0	0
CHL-15	0	0	0	0	0	0	0	0
CHL-16	0	0	0	0	0	0	0	0
CHL-17	0	0	0	0	0	0	0	0
CHL-18	0	0	0	0	0	0	0	0
A01	0	0	0	100	0	0	0	0
A12	0	0	0	100	0	0	0	0
A22	0	0	0	100	0	0	0	0
A23	0	0	0	100	0	0	0	0
A37	0	0	0	100	0	0	0	0
A39	0	0	0	100	0	0	0	0
A58	0	0	0	100	0	0	0	0
A59	0	0	0	100	0	0	0	0
A60	0	0	0	100	0	0	0	0
A61	0	0	0	100	0	0	0	0
A63	0	0	0	100	0	0	0	0
A65	0	0	0	100	0	0	0	0

Continued on next page

Site	Leptosols	Luvisols	Nitisols	Phaeozems	Planosols	Podzols	Regosols	Vertisols
DF01	0	0	0	100	0	0	0	0
DF02	0	0	0	100	0	0	0	0
DF03	0	0	0	100	0	0	0	0
DF06	0	0	0	100	0	0	0	0
DF07	0	0	0	100	0	0	0	0
DF08	0	0	0	100	0	0	0	0
DF09	0	0	0	100	0	0	0	0
DF10	0	0	0	100	0	0	0	0
DF11	0	0	0	100	0	0	0	0
E02	0	0	0	100	0	0	0	0
E05	0	0	0	0	0	0	0	100
E07	0	0	0	0	0	0	0	100
E08	0	0	0	0	0	0	0	100
E09	0	0	0	0	0	0	0	100
E30	0	0	0	0	0	0	0	100
E26	0	0	0	99	1	0	0	0
EFM	0	0	0	100	0	0	0	0

Supplementary Information - Study 3

S1 Preparation of DOM sources and nutrient treatments

Agricultural and forest DOM sources of were prepared by mixing equal parts of stream water from 4 agricultural streams and 4 forest streams located within the catchment of the River Spree (NE Germany) (agricultural streams: A1, A2, A4, A5; forest streams: F1, F2, F4, F5; see Heinz et al. (2015) for further details. We used tangential flow filtration to increase DOM concentrations in order to ensure measurable DOM concentration until the end of the experiment as well as similar DOM concentrations in the agricultural and forest DOM samples. After prefiltration with a 0.45 µm membrane (Hydrosart©, Sartorius, Göttingen, Germany) the composite agricultural and forest DOM samples were ultrafiltrated with a 1 kDa membrane (polyethersulfone, Sartorius, Göttingen, Germany). We diluted the resulting retentate sample of agricultural DOM with purified, distilled (UV) sterilized water to a final DOC concentration of 4 mg C l^{-1} which equaled the concentration of the forest retentate sample. The ultrafiltration and the subsequent dilution of the samples decreased DIN and SRP concentrations, which enabled us to artificially adjust the nutrients to the same level for agricultural DOM and forest DOM. We created a low (AL and FL) and high nutrient treatment (AH and FH) for each of the agricultural and forest DOM samples by adjusting the nutrients to 1 and 10 mg l^{-1} DIN and 0.03 and 0.1 mg l^{-1} SRP, respectively. We added DIN and SRP in the form of KNO_3 and KH_2PO_4 (Merck, Darmstadt).

S2 Laboratory analyses – DIN, SRP and spectral properties

We colometrically determined DIN and comprised of nitrate, nitrite and ammonium following standard methods (EN ISO 11732: NH_4^+, EN ISO 13359: NO_3^-, NO_2^-; the detection limit for NH_4^+ was 0.03 mg N l^{-1} and the detection limit for NO_3^- and NO_2^- was 0.01 mg N L^{-1}) using a SCAN++ system (Skalar Analytical B.V., The Netherlands). SRP was determined photometrically using a Spekol 1500 (Analytic Jena, Jena) at 880 nm after addition of 100 µm Mo-H_2SO_4 and 25 µl ascorbic acid to 2.5 ml of acidified (2N HCl) sample using a Spekol 1500 (Analytic Jena, Jena) at 880 nm.

Absorbance was measured from 190 to 800 nm in 0.5 nm steps using a Shimadzu UV-2401 UV/VIS spectrometer (Duisburg, Germany) and corrected for instrument baseline offset (Green and Blough 1994). Fluorescence measurement was performed using an Aqualog (Horiba, USA). An excitation range from 230 to 600 nm with a 5 nm increment was used. Emission spectra were collected for the wavelength range 214.1 – 619.3 nm with a 1.6 nm increment, using 1 s integration time, a pixel bin of 4 and medium detector gain. Absorbance and fluorescence were measured at room 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 an excitation wavelength of 350 nm (Lawaetz and Stedmon 2009).

S3 – R function for permutational three way ANOVA with interaction

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Details: perm.anova.3() is a function for a 3-way permutational ANOVA with interaction based on F statistics and free permutation based on calculation of permutational ANOVA presented in: Gotelli, N.J., Ellison, A.M., 2004. A primer of ecological statistics. Palgrave Macmillan, Hants, United Kingdom. Error probability (p) is based on the probability that randomly generated F values after permutation of the data are smaller than the true F values. Therefore, if e.g. 95% of the random F values for permuted data are smaller than the true F value for the same data, p is 0.05.

Usage: perm.anova.3(x,…)

Arguments: x = dependent variable, a = Factor 1, b = Factor 2, c = Factor 3, iter = number of iterations, names.vec = names of factors for output

Source code:

```
  # Permutational three way ANOVA function with interaction
~~~~~~~~~~~~~~~~~~~
  perm.anova.3 <- function(x, a, b, c, iter, names.vec) {

    classic <- aov(x ~ a * b * c)
    classic.F <- summary(classic)[[1]]$'F value'[1:7]
    names(classic.F) <- rownames(summary(classic)[[1]])[1:7]

    random.all <- matrix(data = NA, nrow = iter, ncol = 7)
    for (i in 1:iter) {
      x.random <- sample(x = x, size = length(x))
      random.aov <- aov(x.random ~ a * b * c)
      random.F <- summary(random.aov)[[1]]$'F value'[1:7]
      random.all[i,] <- random.F

      print(paste("%done =", i*100/iter, "%"))
    }
    colnames(random.all)                                          <-
rownames(summary(random.aov)[[1]])[1:7]

    test <- matrix(data = NA, nrow = iter, ncol = 7)
    p <- NULL
    for(i in 1:7) {
      test[,i] <- classic.F[i] > random.all[,i]
      p[i] <- 1 - (sum(test[,i]) / iter)
    }
    names(p)   <-   c(names.vec,   paste0(names.vec[1],   ":",
names.vec[2]), paste0(names.vec[1], ":", names.vec[3]),
                  paste0(names.vec[2],   ":",   names.vec[3]),
paste0(names.vec[1], ":", names.vec[2], ":", names.vec[3]))
    return(p)
  }
  #    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~    end    of    function
~~~~~~~~~~~~~~~~~~~~~~~~~~
```

References

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