



## Stable isotopes ( $\delta^{13}\text{C}$ , $\delta^{15}\text{N}$ ) and biomarkers as indicators of the hydrological regime of fens in a European east–west transect



Miriam Groß-Schmolders<sup>a,b,\*</sup>, Kristy Klein<sup>a,b</sup>, Willem-Jan Emsens<sup>c</sup>, Rudy van Diggelen<sup>c</sup>, Camiel J.S. Aggenbach<sup>d</sup>, Yvonne Liczner<sup>c</sup>, Jan Frouz<sup>e</sup>, Jens Leifeld<sup>a,b</sup>, Christine Alewell<sup>a</sup>

<sup>a</sup> Environmental Geosciences, University of Basel, Bernoullistrasse 32, CH-4056 Basel, Switzerland

<sup>b</sup> Agroscope, Climate and Agriculture Group, Reckenholzstraße 191, CH-8046 Zürich, Switzerland

<sup>c</sup> Department of Biology, University of Antwerp, Universiteitsplein 1, BE - 2610 Wilrijk, Belgium

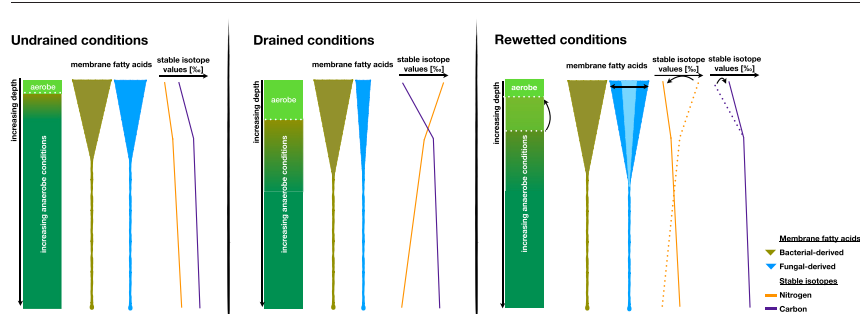
<sup>d</sup> KWR Water cycle Research Institute, Post Box 1072, NL-3430 BB, Nieuwegein, Netherlands

<sup>e</sup> Institute for Environmental Studies, Charles University Benátská 2, CZ-128282801, Prague, Czech Republic

### HIGHLIGHTS

- Stable isotope bulk values, especially  $\delta^{15}\text{N}$ , and microbial-derived mFA quantities differ between hydrological regimes.
- Microbial-derived mFA quantities indicate that stable isotope bulk values were a result of changes in microbial communities.
- $\delta^{15}\text{N}$  values in peat are negatively correlated to the fungal-derived mFA quantities.
- Soil  $\delta^{15}\text{N}$  values are valid markers for microbial metabolic processes and the hydrological regime of fens.
- Time since rewetting was the primary driver for restoration success of the microbial community in the investigated sites.

### GRAPHICAL ABSTRACT



### ARTICLE INFO

Editor: Paola Verlicchi

#### Keywords:

Stable isotopes  
Membrane fatty acids  
Peatland degradation  
Rewetting  
European fens

### ABSTRACT

Peatland degradation is tightly connected to hydrological changes and microbial metabolism. To better understand these metabolism processes, more information is needed on how microbial communities and substrate cycling are affected by changing hydrological regimes. These activities should be imprinted in stable isotope bulk values ( $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ ) due to specific isotopic fractionation by different microbial communities, their metabolic pathways and nutrient sources. We hypothesize that stable isotope values and microbial abundance are correlated and act as indicators of different hydrological regimes. We sampled an East–West transect across European fens in 14 areas and conducted a stable isotope ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) and membrane fatty acid (mFA) analysis. Within each area an undrained, drained and rewetted site was selected. Rewetted sites were separated based on when rewetting occurred. We found differences in the upper layers of all sites in microbial-derived mFAs and stable isotope values corresponding to hydrological regimes. The highest and lowest quantities of microbial-derived mFAs were measured in undrained and drained sites, respectively. Fungal-derived mFAs were especially lower in drained sites. Simultaneously,  $\delta^{15}\text{N}$  stable isotope values were highest in drained sites. In addition, stable isotope values and microbial-derived mFAs showed distinct depth trends. In undrained sites stable isotopes values slightly increased with depth. In drained sites,  $\delta^{15}\text{N}$  values decreased

\* Corresponding author.

E-mail addresses: [Miriam.gross-schmolders@agroscope.admin.ch](mailto:Miriam.gross-schmolders@agroscope.admin.ch) (M. Groß-Schmolders), [kristy.klein@agroscope.admin.ch](mailto:kristy.klein@agroscope.admin.ch) (K. Klein), [willem-jan.emsens@uantwerpen.be](mailto:willem-jan.emsens@uantwerpen.be) (W.-J. Emsens), [ruurd.vandiggelen@uantwerpen.be](mailto:ruurd.vandiggelen@uantwerpen.be) (R. van Diggelen), [Camiel.Aggenbach@kwrwater.nl](mailto:Camiel.Aggenbach@kwrwater.nl) (C.J.S. Aggenbach), [yvonne.liczner@uantwerpen.be](mailto:yvonne.liczner@uantwerpen.be) (Y. Liczner), [jan.frouz@natur.cuni.cz](mailto:jan.frouz@natur.cuni.cz) (J. Frouz), [jens.leifeld@agroscope.admin.ch](mailto:jens.leifeld@agroscope.admin.ch) (J. Leifeld), [christine.alewell@unibas.ch](mailto:christine.alewell@unibas.ch) (C. Alewell).

<http://dx.doi.org/10.1016/j.scitotenv.2022.156603>

Received 31 March 2022; Received in revised form 30 May 2022; Accepted 6 June 2022

Available online 9 June 2022

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downwards, whereas  $\delta^{13}\text{C}$  values increased. Overall microbial-derived mFAs decreased with depth. These patterns presumably result from anoxic conditions and high peat recalcitrance in the deeper layers. In sites with short time of rewetting, the microbial-derived mFAs and stable isotope values were similar to values of drained sites, while with increasing rewetting time values shifted to those of undrained sites. We conclude that biomarkers indicate that stable isotope values reflect specific microbial metabolic processes, which differ with hydrological regimes, and thus could indicate both drainage and rewetting in fens.

## 1. Introduction

Groundwater-fed peatlands (“fens”) in the temperate zones of Europe, North America, and Asia are typically characterized by high biodiversity (Lamers et al., 2015). Because fens are influenced by groundwater and/or surface water flow (Grootjans et al., 2006), they are generally more base-rich and often also nutrient-richer than rainwater-fed bogs (Joosten and Clarke, 2002). In Central and Western Europe, the majority of fens have been affected by drainage and subsequent agricultural use (Grootjans et al., 2006), turning them from sinks to sources of carbon (C; Paul et al., 2021). Recently drained peatlands account for an estimated 5 % of global atmospheric greenhouse gas (GHG) emissions, mostly as  $\text{CO}_2$  (Leifeld and Menichetti, 2018). Furthermore, ecosystem services such as C sequestration are absent in drained fens due to shifts in microbial communities and altered metabolic processes (Bedford and Godwin, 2002; Philippot et al., 2013; Wagg et al., 2014).

In fens, the highest metabolic rates are found in the upper peat layer, on the oxic-anoxic interface (Artz, 2013; Asada et al., 2005; Morris et al., 2011). With increasing depth, the overall decrease in redox potential correlates with decreased metabolic rates, with the lowest rates in the deepest and always waterlogged peat layers (Artz, 2013; Asada et al., 2005; Lin et al., 2014).

Because microbial-induced metabolic processes such as denitrification and nitrification lead to isotopic fractionation, stable isotope values can be used as indicators for peatland hydrological regimes (Alewell et al., 2011; Groß-Schmolders et al., 2020; Krüger et al., 2015). For  $\delta^{13}\text{C}$ , Alewell et al. (2011), Biester et al. (2014), Hobbie et al. (2017), Krüger et al. (2014) and Novák et al. (1999) reported increasing values with depth and enhanced recycling with drainage. In drained sites they reported also a switch to the cycling of other substrates in peat with depth. As Kohl et al. (2015) demonstrated, this distinct  $\delta^{13}\text{C}$  depth pattern is a consequence of changing microbial metabolism. Additionally, a significant negative correlation between the fungal-to-bacterial ratio and  $\delta^{15}\text{N}$  values was reported in nutrient-poor peatlands (Groß-Schmolders et al., 2020). Microbial metabolic processes in fens are often constrained by a limited supply of nitrogen (N) and oxygen (O) (Lin et al., 2014). During microbial incorporation of N and metabolic processing, a fractionation of stable isotopes occurs, as most organisms prefer the lighter and more frequently occurring  $^{14}\text{N}$  (Adam and Grierson, 2001; Asada et al., 2005; Högberg et al., 1996; Kohzu et al., 2003). The preferential incorporation and translocation upwards to stem and foliar of the lighter  $^{14}\text{N}$  by plants result in the enrichment of heavier  $^{15}\text{N}$  in the remaining bulk substrate (Högberg et al., 1996). Additionally, the mycorrhizal uptake of lighter  $^{14}\text{N}$  into plants increases the  $\delta^{15}\text{N}$  values of mycorrhizal biomass and therefore increases the values of the substrate even more (Hobbie and Högberg, 2012). Furthermore, microbial metabolic processes in deeper peat layers increase the  $\delta^{15}\text{N}$  values as long as microbial metabolism of organic substrate (mainly dead plant residues) occurs and lighter  $^{14}\text{N}$  is leached, translocated, or lost via outgassing during denitrification (Damman, 1988; Niemen, 1998; Novák et al., 1999). If microbial activity decreases in the deeper peat layers, enrichment with  $\delta^{15}\text{N}$  stops due to this isotopic fractionation being comparably lower than in a parallel system with high metabolic rates. As microbial abundance is higher in aerobic layers, microbial abundance and stable isotope values can be linked to peatland hydrological regimes (Groß-Schmolders et al., 2020; Groß-Schmolders et al., 2021; Tfaily et al., 2014). Some microbial groups are more active in N cycling than others and, therefore, play a greater role in N isotopic fractionation (Groß-Schmolders et al., 2021;

Tfaily et al., 2014). For example, saprotrophic fungi have a low demand for N, making them unlikely to be a primary driver of increasing  $\delta^{15}\text{N}$  values (Thormann, 2005).

Microbial community composition is, among other factors, driven by substrate quality (Emsens et al., 2020; Thormann, 2005). The C:N ratio, which indicates the degree of decomposition (Kuhry and Vitt, 1996; Malmer and Holm, 1984; Thormann, 2005), can be used as an indicator of substrate quality. With increasing levels of decomposition, a preferential loss of C over N takes place and the C:N ratio decreases.

Sundh et al. (1997) and Torres and Pancost (2016) demonstrated that membrane fatty acids (mFAs) are persistent and largely insoluble compounds of specific cell membranes (microbial communities, living and dead) in peat soils. Because mFAs vary based on their origin (plants, specific microbial groups; Bajerski et al., 2017; Finotti et al., 1993; Piotrowska-Seget and Mroziak, 2003; Reiffarth et al., 2016; Willers et al., 2015), the relative abundance of specific microbial communities can be determined based on an analysis of the occurrence and quantities of mFAs present (Piotrowska-Seget and Mroziak, 2003; Torres and Pancost, 2016).

In our study, we investigated 14 fen regions across Europe, and each region comprised three fen sites that differed in the hydrological regime (undrained, drained, and rewetted sites).

We used stable isotope and two mFA analyses to obtain reliable information on the hydrological regime of the fen sites. We hypothesized that:

- (i) Stable isotope depth trends differ between undrained, drained, and rewetted fen sites,
- (ii) Microbial community composition, as indicated by mFAs, differs between undrained, drained, and rewetted fen sites,
- (iii) Stable isotopic patterns correlate with microbial mFAs.

The verification of these hypotheses would enable us to assess restoration projects with higher precision and, thus, lend support to peatland conservation policies.

## 2. Material and methods

### 2.1. Site description

Peat soil was sampled in 14 lowland fen regions across a gradient from western to eastern Europe (United Kingdom, Belgium, Netherlands, Germany and Poland) in June 2013 (region Cuxhaven) and in May and June 2017 (the remaining 13 regions, as described by (Emsens et al., 2020; Table 1)).

All regions were located in the temperate climatic zone and within the same latitudinal range (N49.65° - N54.35°). Because the regions covered a wide longitudinal gradient (>1500 km), there is a climatological gradient from an oceanic (west, with mild winters and mild summers) to a more continental (east, with cold winters and warm summers) climate.

In each fen region, except for the Cuxhaven region, we collected samples in three fen sites with different hydrological regimes (undrained, drained and rewetted). In Cuxhaven, only samples of drained and undrained sites were available. The classification of the hydrological regime was made by observations in the field (presence of drainage ditches, groundwater table) and an analysis of vegetation composition (Table S1). The undrained sites were covered with typical fen species (e.g., *Carex* spp., brown mosses) and had a groundwater table near the peat surface for most of the year. The groundwater table was below 50 cm (annual average (av.)) in the drainage-affected sites and the vegetation mainly consisted

**Table 1**

Site description of all investigated sites; coordinates [longitude (long.)/latitude (lat.)]; mean annual temperature (MAT) [°C]; precipitation (P) [mm]; time since rewetting (TsR) [years]; (Emsens et al., 2020; CustomWeather, 2020; Krüger, 2016).

Country	Site name	Long/ Lat.	MAT	P	TsR
Belgium	Arlon	5.7/49.7	+9	489	>25
	Zwarte Beek	5.3/51.1	+11	476	>25
Netherlands	Binnenveld	5.6/52.0	+11	569	10–25
	Drentse Aa	6.7/53.0	+11	387	10–25
Germany	Gützkow	13.4/53.9	+10	187	10–25
	Peene mouth	13.7/53.8	+10	187	<10
	Kiel	10.1/54.3	+10	442	<10
	Recknitz	12.6/54.2	+10	439	<10
	Cuxhaven	8.5/53.4	+9	766	–
Poland	Biebrza	23.3/53.7	+7	616	<10
	Rospuda	22.6/53.8	+7	623	>25
	Suwalszczyzna	22.7/54.3	+7	623	<10
	Mazury	21.5/53.7	+9	362	<10
United Kingdom	Anglesey	–4.3/53.3	+10	2280	10–25

of moist or wet grassland species. Rewetting and high levels of groundwater (10–15 cm below surface) was caused either by restoration projects or beaver dam building and had a fen vegetation (e.g. *Carex* spp. and brown mosses). Water table depths in the rewetted sites did not differ from the undrained sites (Emsens et al., 2020; Krüger et al., 2014). All sites had near neutral to slightly acidic pore water (mean pH = 6.45,  $\pm$  0.33; min = 5.6, max = 7.1, measured in the field, WTW Multi 340i, WTW, Weilheim, Germany; Emsens et al., 2020; Krüger et al., 2014).

The rewetted sites were separated in three major “time since rewetting (TsR)” classes, following Emsens et al. (2020; Table 1): (1) <10 years of rewetting, (2) 10 to 25 years of rewetting, and (3) >25 years of rewetting. The classes were used to assess the effect of rewetting time on stable isotope values and mFAs. For each rewetting class an undrained and drained fen were also investigated. This, in the following all three hydrological regimes are assigned to the rewetting time of the respective rewetted fen.

## 2.2. Soil sampling and bulk analysis

At each site three to five subsamples were taken at three depths (0–5 cm; 15–20 cm and 45–50 cm) and mixed into one composite sample per depth. All samples were individually packed in plastic bags and aluminum foil, cooled immediately after collection, and deep-frozen at the end of each day.

Samples were oven-dried at 40 °C for 72 h, and homogenized with a vibrating ball mill (MM400, Retsch, Germany). Stable C and N isotopic values were measured in an elemental analyzer combined with an isotope ratio mass spectrometer (EA-IRMS) (Inegra2, Sercon, Crewe, UK). C isotopic composition ( $\delta^{13}\text{C}$ ) was expressed relative to Vienna Pee-Dee Belemnite (VPDB) standard and reported in delta notation (‰), stable nitrogen isotopes were expressed relative to the atmospheric N standard and reported in delta notation (‰). C:N was determined with the mass relationship of the measured bulk content [mg/g] of C and N.

## 2.3. Fatty acid analyses

The mFA analysis of the soil samples were conducted by using two different methods, A and B (please see below for definition). The mFA at all areas except Cuxhaven were analyzed with method A. Cuxhaven data originated from a different project and samples were analyzed with method B. The reason for this difference is that the analysis was performed by two different laboratories with different analytical standard methods: The Ceske Budejovice Lab used method A and Basel Lab used method B.

### 2.3.1. Phospholipid fatty acid measurement Ceske Budejovice (method A)

Phospholipid fatty acids (PLFAs) were extracted from 0.5 g of lyophilized subsamples. All samples were extracted with a chloroform:methanol:

phosphate buffer mixture (1:2:0.8), after which the extracted lipids were separated using solid-phase extraction cartridges (LiChrolut Si 60, Merck). The samples were eluted in three fractions containing neutral lipids, glycolipids and phospholipids with 2 ml of chloroform, 6 ml of acetone and 2 ml of methanol, respectively (Oravec et al., 2004). The first and third fractions were then subjected to mild alkaline methanolysis (Šnajdr et al., 2008). The free methyl esters of the PLFAs were analyzed by gas chromatography–mass spectrometry (450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA, USA). The GC instrument was equipped with a split/splitless injector, and we used a DB-5MS column for separation (60 m, 0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness). The temperature program began at 60°C and was maintained for 1 min in splitless mode. Next the splitter was opened, and the oven was heated to 160°C at 25°C min<sup>-1</sup>. The second temperature ramp was up to 280°C at 2.5°C min<sup>-1</sup>, which was maintained for 10 min. The solvent delay time was set at 8 min. The transfer line temperature was set at 280°C. Mass spectra were recorded at 1scan s<sup>-1</sup> under electron impact at 70 eV, with mass range 50–350 amu. We identified methylated fatty acids according to their mass spectra using a mixture of chemical standards from Sigma-Aldrich (Prague, Czech Republic) and Matreya LLC (Pleasant Gap, PA, USA).

### 2.3.2. Membrane fatty acid measurement Basel (method B)

We aimed to extract total mFAs to distinguish between mFAs of different bacterial groups, fungi and plants. We processed 0.2–1.1 g of sample for the lipid extraction with a mixture of CH<sub>2</sub>Cl<sub>2</sub>:MeOH (9:1v/v) in an Accelerated Solvent Extractor (Dionex ASE 350). 0.4  $\mu\text{g}/\mu\text{l}$  of an internal standard with nonadecanoic acid was added before processing each sample.

The total lipid extracts (TLE) were saponified by adding 2 ml of KOH dissolved in MeOH (12 %) and putting it in the oven for 3 h at 80 °C.

Following the method of Elvert et al. (2003) TLE was afterwards polarized with 1 ml KCl (0.1 mol) and the neutral fraction was extracted by rinsing three times with hexane. Neutral fraction in the supernatant was separated, dried under a stream of N<sub>2</sub>, and stored in the refrigerator for later analysis. We acidified the rest of the TLE with fuming hydrochloric acid to a pH of 1. The acid fraction was extracted by rinsing again three times with hexane. The acid fraction in the supernatant was separated and hexane dried under a stream of N<sub>2</sub>. Then the acid fraction was methylated by adding 1 ml Boron-Trifluoride (BF<sub>3</sub>) in MeOH (12–14 %) and putting it in the oven for 1 h at 60 °C. Afterwards the mFA fraction was polarized with KCl (0.1 mol) and transferred in 2 ml vials by rinsing three times with hexane. The mFAs were quantified with a Trace Ultra gas chromatograph (GC) equipped with a flame ionization detector (FID) (Thermo Scientific, Waltham, MA, USA). The carrier gas (helium) had a constant flow of 1.2 ml per minute and the GC-FID was set to splitless mode. Detector temperature was 320 °C and the samples (dissolved in hexane) were injected by 300 °C. The starting temperature of the oven was 50 °C. The temperature was increased by 10 °C per minute to 140 °C. The temperature was held for 1 min before it was increased up to 300 °C. This temperature was held for 63 min.

To identify the fungal and bacterial markers, we used the Bacterial Acid Methyl Esters standard (BAME, Supelco Mix). For bacteria, it includes the mFAs C17:0 as general bacterial marker (Willers et al., 2015; Zelles, 1997), a-C-15:0 for gram-positive bacteria (Zelles, 1997; O’Leary and Wilkinson, 1988; Vestal and White, 1989) and C16:1 $\omega$ 9c for gram-negative bacteria (O’Leary and Wilkinson, 1988; Vestal and White, 1989; Zelles, 1997). The membrane fatty acid C18:2 $\omega$ 6c was used as a marker for saprotrophic fungi (Andersen et al., 2010; O’Leary and Wilkinson, 1988; Sundh et al., 1997; Vestal and White, 1989; Zelles, 1997). All these markers are valid for overall membrane fatty acids and can be used to detect different microbial groups in soil (Bajerski et al., 2017; Finotti et al., 1993; Piotrowska-Seget and Mroziak, 2003). Quantification of the mFAs was done using the internal standard, C19:0 mFA, after correcting for the methyl group, added during methylation reaction.

To combine the results of both methods and test whether it was possible to correlate the mFA results of different laboratories, we also tested method B in the Zwarte Beek site. The correlation of both methods showed the same range of absolute quantities for all hydrological regimes and depth

(Table S3). In addition, the ratio of fungal-derived and bacterial-derived mFA (F:B) showed the same pattern (Table S4). These results suggested that the results of both methods could be combined.

#### 2.4. Statistical analysis

We calculated variance and standard deviation (SD) of stable isotope values of the three hydrological regimes. We did a *t*-test to analyze if microbial-derived mFAs are in the drained peat layers of the same population as the values of the undrained and rewetted sites ( $H_0$  – drained, rewetted and undrained quantities are of the same population,  $p \leq 0.05$ ). To test whether stable isotope values and mFAs differed between drained, rewetted and undrained sites and with depth, we used two factor analysis of variance (ANOVA;  $\alpha = 0.05$ ) with hydrological regime and soil depth as fixed factors; separated for the different time classes (Girden, 1992). We calculated the statistical distribution of both stable isotopes, grouped by the rewetting time and separated for each hydrological regime and depth.

In addition, we calculated the Pearson correlation coefficient (*R*) for the nitrogen stable isotopes values and the different groups of microbial-derived mFAs.

All analyses were done using R (version 3.4.1).

### 3. Results

#### 3.1. Carbon-to-nitrogen ratio

The C:N ratios for the deepest sampling depths (45–50 cm) were similar (mean ( $\pm$ SD) =  $17 \pm 3$ ) between the hydrological regimes (Table S5), indicating similar degree of decomposition across the respective fens. The C:N ratio of the 0–5 cm layer was highest in the undrained sites ( $21 \pm 5$ ), intermediate in the rewetted sites ( $17 \pm 4$ ), and lowest in the drained sites ( $13 \pm 2$ ). The C:N ratio of the top layer of the rewetted sites increased from  $14 \pm 3$  to  $22 \pm 2$  with increasing time since rewetting (Table S5).

#### 3.2. Stable nitrogen isotope bulk values

For the deepest sampling depth (45–50 cm),  $\delta^{15}\text{N}$  values were similar ( $1.2 \text{‰} \pm 1.0$  of dry weight) between the hydrological regimes (Fig. 1 (A), Table S2).

However,  $\delta^{15}\text{N}$  values followed different depth trends under different hydrological regimes and were significantly different for the hydrological regimes ( $p < 0.01$ ) as well as for the interaction between depth and the hydrological regime ( $p < 0.01$ , Table 2). In undrained sites, the  $\delta^{15}\text{N}$  values were lowest in the 0–5 cm peat layer ( $-1.2 \text{‰} \pm 0.8$ ) and slightly increased with depth (Fig. 1(A), Table S2). In contrast, in drained sites,  $\delta^{15}\text{N}$  values were highest in the 0–5 cm peat layer ( $2.8 \text{‰} \pm 0.8$ ; Table S1) and decreased with depth (Fig. 1(A)). The  $\delta^{15}\text{N}$  values of rewetted sites were in between the values of undrained and drained sites ( $2.2 \text{‰} \pm 1.0$ ). The  $\delta^{15}\text{N}$  values of rewetted sites increased with depth (Fig. 1(A)).

#### 3.3. Carbon stable isotope bulk values

Similar to the  $\delta^{15}\text{N}$  values,  $\delta^{13}\text{C}$  values showed also very similar values ( $-28.3 \text{‰} \pm 0.6$ ) in the 45–50 cm peat layer (Fig. 1(B), Table S2).

However, and in contrast to the  $\delta^{15}\text{N}$  depth trends, the  $\delta^{13}\text{C}$  values followed a similar trend with depth in all hydrological regimes and were significantly different for different depths ( $p < 0.01$ , Table 2) in all three hydrological regimes.  $\delta^{13}\text{C}$  values were lowest in the 0–5 cm peat layer and increased with depth (Fig. 1(B)). Overall,  $\delta^{13}\text{C}$  values were lowest at the undrained sites ( $-29.6 \text{‰} \pm 0.5$ ) and highest at the drained sites ( $-28.8 \text{‰} \pm 0.8$ ). The  $\delta^{13}\text{C}$  values of the rewetted sites were also in between the values of the drained and undrained sites ( $-29.1 \text{‰} \pm 0.5$ ) and did not differ significantly between hydrological regimes ( $p = 0.09$ ; Table 2).

#### 3.4. Stable isotope bulk values versus time since rewetting

We found a clear link between time since rewetting and stable isotope patterns for  $\delta^{15}\text{N}$  values, with the depth profile of the longest rewetting class nearly similar to the depth profile of undrained sites (Fig. 1(A)). However, the latter was only marginally seen for  $\delta^{13}\text{C}$  (Fig. 1(B)).

The  $\delta^{15}\text{N}$  values at 0–5 cm depth of undrained and drained sites differed significantly from each other between hydrological regimes, depth and for a combination of both ( $p_{\text{hydrological}} < 0.00$ ,  $p_{\text{depth}} = 0.02$ ,  $p_{\text{combination}} = 0.00$ ; Table 2).

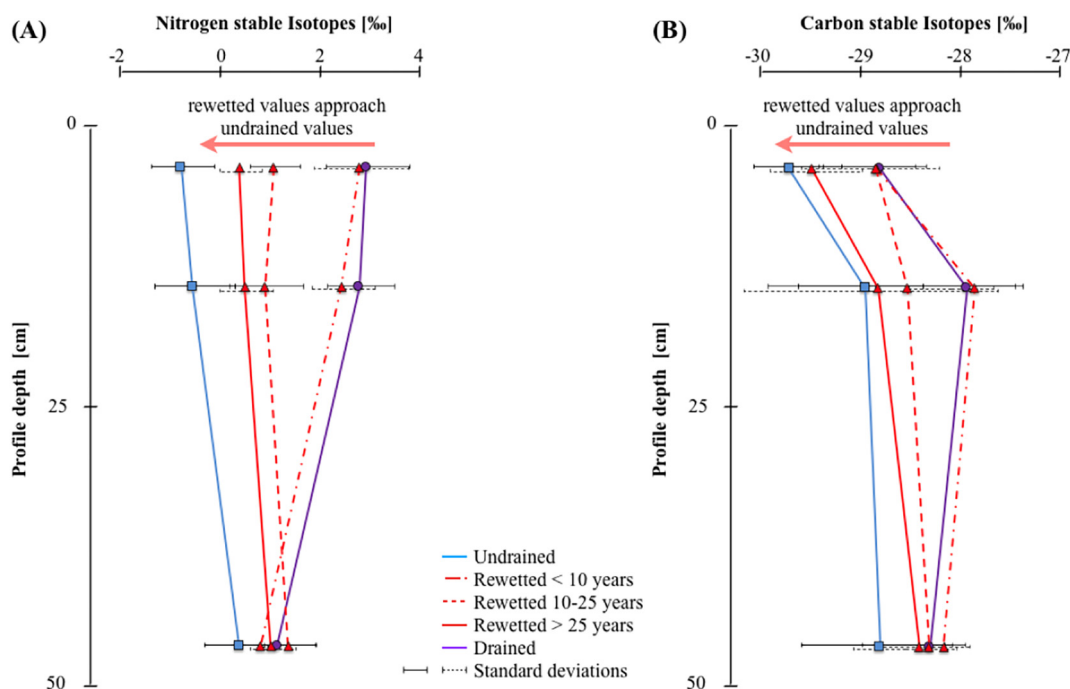


Fig. 1. (A) Nitrogen and (B) carbon stable Isotope values depth pattern (groups of fens are assigned to the time of rewetting of the respective rewetted fen); separated by the hydrology regime (undrained (blue), drained (purple), rewetted (red)).



**Table 2**

F-, and p-values from a two factor ANOVA of all investigated sites for stable isotope ratios (nitrogen, carbon), with hydrological regime and soil depth as the main factors; different time classes (<10, 10–25, > 25 years) were treated as independent; hydrological regime (HR); bold = significance (F - critical < F;  $p \leq 0.05$ ).

Stable Isotope	Factors	<10			10–25			>25			all		
		p	F	F-crit	p	F	F-crit	p	F	F-crit	p	F	F-crit
$\delta^{15}\text{N}$	Depth	<b>0.02</b>	<b>4.2</b>	<b>3.2</b>	0.46	0.8	<b>3.6</b>	<b>0.03</b>	<b>4.3</b>	<b>3.6</b>	0.58	0.5	3.1
	HR	<b>0.00</b>	<b>28.5</b>	<b>3.2</b>	<b>0.00</b>	<b>22.5</b>	<b>3.6</b>	<b>0.00</b>	<b>19.3</b>	<b>3.6</b>	<b>0.00</b>	<b>49.6</b>	<b>3.1</b>
	Interaction of both	<b>0.00</b>	<b>6.7</b>	<b>2.6</b>	<b>0.01</b>	<b>9.2</b>	<b>2.9</b>	<b>0.01</b>	<b>4.7</b>	<b>2.9</b>	<b>0.00</b>	12.0	2.5
$\delta^{13}\text{C}$	Depth	<b>0.00</b>	9.4	3.2	0.11	2.4	3.4	0.14	2.2	3.6	<b>0.00</b>	11.3	3.1
	HR	0.07	2.8	3.2	0.90	0.1	3.4	0.26	1.5	3.6	0.09	2.5	3.1
	Interaction of both	0.98	0.1	2.6	0.93	0.2	2.7	0.97	0.1	2.9	0.99	0.7	2.5

In the class up to 10 years of rewetting, the  $\delta^{15}\text{N}$  values of rewetted sites ( $2.6\text{‰} \pm 1.3$ ) were between the  $\delta^{15}\text{N}$  values of drained ( $3.4\text{‰} \pm 0.7$ ) and undrained sites ( $-0.6\text{‰} \pm 1.2$ ; Fig. 1, Tables 2 and S2). The  $\delta^{13}\text{C}$  values were similar at this depth across hydrological regimes ( $-29.2\text{‰} \pm 0.6$ ;  $p = 0.07$ ; Fig. 1(B), Tables 2 and S2).

In the class of 10–25 years of rewetting, the stable isotope values in 0–5 cm depth of rewetted sites ( $\delta^{15}\text{N} = 0.9\text{‰} \pm 0.5$ ;  $\delta^{13}\text{C} = -29.0\text{‰} \pm 0.3$ ) shifted towards those of undrained sites ( $\delta^{15}\text{N} = -1.8\text{‰} \pm 0.5$ ;  $\delta^{13}\text{C} = -29.3\text{‰} \pm 0.7$ ; Fig. 1, Table S2).

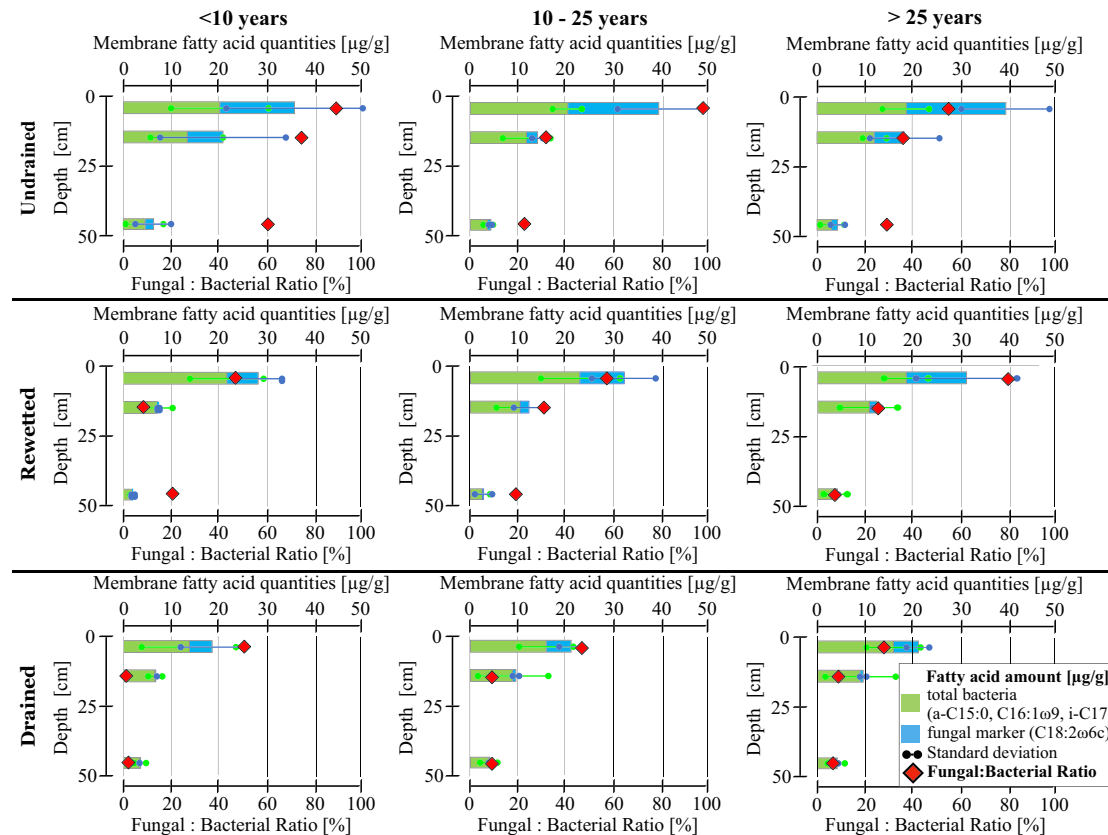
For the class of >25 years of rewetting, the  $\delta^{15}\text{N}$  values of rewetted sites were lower ( $-0.3 \pm 0.9$ ) than those of drained sites ( $2.6\text{‰} \pm 0.7$ ) and were in the same range of the values of the undrained sites ( $-1.7\text{‰} \pm 0.5$ ; Fig. 1). The detected differences were significantly different for  $\delta^{15}\text{N}$  values, dependent on depth ( $p = 0.03$ ) and the hydrological regimes ( $p < 0.01$ ). Additionally, the  $\delta^{13}\text{C}$  values of rewetted sites ( $-29.5\text{‰} \pm 0.5$ ) were equal to those of undrained sites ( $-29.5\text{‰} \pm 0.3$ ; Fig. 1(B)). The  $\delta^{13}\text{C}$  values were not significant different for depth nor for the hydrological regime (Table 2).

### 3.5. Microbial-derived mFA quantities

Across all sites total microbial-derived mFAs decreased from  $30.97 \mu\text{g g}^{-1} \pm 8.7$  in the 0–5 cm peat layer to  $3.91 \mu\text{g g}^{-1} \pm 2.3$  in the 45–50 cm peat layer, with similar patterns across hydrological regimes (Fig. 2, Table S6). This corresponds to a reduction of 87%. The mFA quantities differed significantly between different hydrological regimes ( $p < 0.01$ ; Table 3).

Fungal-derived mFAs decreased significantly ( $p < 0.01$ ; Table 3) with depth, from  $11.62 \pm 10.5 \mu\text{g g}^{-1}$  to  $0.55 \mu\text{g g}^{-1} \pm 1.7$  (Table S5). This corresponds to a reduction of 95%. Bacterial-derived mFAs were significantly ( $p < 0.01$ , Table 3) reduced by 83% with increasing depth. Quantities ranged from  $19.35 \mu\text{g g}^{-1} \pm 8.7$  in the 0–5 cm peat layer to  $3.36 \mu\text{g g}^{-1} \pm 2.3$  in the 45–50 cm peat layer (Table S6).

With respect to different hydrological regimes, undrained sites had the highest quantities of microbial mFAs  $41.33 \mu\text{g g}^{-1} \pm 9.0$  in the 0–5 cm peat layer. In contrast, drained sites had the lowest microbial-derived mFAs ( $21.71 \mu\text{g g}^{-1} \pm 5.6$ ) in the 0–5 cm peat layer (Table S6). The mFAs of



**Fig. 2.** Membrane fatty acid quantities (fungal- (blue) and bacterial- (green)) and Fungal:Bacterial Ratio (red diamonds), separated by the hydrological regime (undrained, drained, rewetted) and time since rewetting (<10 years ( $n = 6$ ), 10–25 years ( $n = 4$ ), >25 years ( $n = 3$ )).

**Table 3**

F-, and p-values from a two factor ANOVA of all investigated sites for membrane fatty acids (bacteria, fungi), with the hydrological regime and soil depth as the main factors; different time classes were treated as independent; hydrological regime (HR); bold = significance (F - critical < F; p ≤ 0.05).

mFAs	Factors	<10			10–25			>25			all		
		p	F	F-crit	p	F	F-crit	p	F	F-crit	p	F	F-crit
Bacteria	Depth	<b>0.00</b>	<b>51.7</b>	<b>3.2</b>	<b>0.00</b>	<b>32.9</b>	<b>3.4</b>	<b>0.00</b>	<b>52.8</b>	<b>3.6</b>	<b>0.00</b>	<b>137.8</b>	<b>3.1</b>
	HR	<b>0.02</b>	<b>4.8</b>	<b>3.2</b>	0.40	0.9	3.4	<b>0.02</b>	<b>4.98</b>	<b>3.6</b>	<b>0.00</b>	<b>8.6</b>	<b>3.1</b>
	Interaction of both	0.30	1.3	2.6	0.54	0.8	2.7	0.28	1.38	2.9	<b>0.04</b>	<b>2.67</b>	<b>2.5</b>
Fungi	Depth	<b>0.00</b>	<b>7.5</b>	<b>3.2</b>	<b>0.00</b>	<b>24.2</b>	<b>3.4</b>	<b>0.00</b>	<b>38.7</b>	<b>3.6</b>	<b>0.00</b>	<b>42.2</b>	<b>3.1</b>
	HR	<b>0.00</b>	<b>6.6</b>	<b>3.2</b>	0.01	6.5	3.4	<b>0.00</b>	<b>15.6</b>	<b>3.6</b>	<b>0.00</b>	<b>20.8</b>	<b>3.1</b>
	Interaction of both	0.33	1.2	2.6	0.02	3.3	2.7	<b>0.00</b>	<b>6.8</b>	<b>2.9</b>	<b>0.00</b>	<b>5.8</b>	<b>2.5</b>
Microbes	Depth	<b>0.00</b>	<b>26.1</b>	<b>3.2</b>	<b>0.00</b>	<b>39.2</b>	<b>3.4</b>	<b>0.00</b>	<b>67.7</b>	<b>3.6</b>	<b>0.00</b>	<b>101.9</b>	<b>3.1</b>
	HR	<b>0.00</b>	<b>7.0</b>	<b>3.2</b>	<b>0.03</b>	<b>4.1</b>	<b>3.4</b>	<b>0.00</b>	<b>15.2</b>	<b>3.6</b>	<b>0.00</b>	<b>19.7</b>	<b>3.1</b>
	Interaction of both	0.44	1.0	2.6	0.12	2.0	2.7	<b>0.00</b>	<b>5.7</b>	<b>2.9</b>	<b>0.00</b>	<b>4.8</b>	<b>2.5</b>

rewetted sites ranged between those of drained and undrained sites ( $29.86 \mu\text{g g}^{-1} \pm 6.9$ ; Table S6).

With respect to hydrological regimes, fungal-derived mFAs showed the largest differences in their concentrations between the different water saturation regimes, especially in the 0–5 cm peat layer (Fig. 2). At this depth, fungal-derived mFAs were highest in the undrained sites ( $20.27 \mu\text{g g}^{-1} \pm 11.5$ ) and lowest at the drained sites ( $5.61 \mu\text{g g}^{-1} \pm 4.0$ ; Table S6). This means a significant reduction of 74 % in fungal-derived mFAs between drained and undrained sites ( $p < 0.01$ , Table S9). Additionally, bacterial-derived mFAs in the 0–5 cm peat layer were significantly higher in undrained ( $21.06 \mu\text{g g}^{-1} \pm 6.5$ ) sites than in drained sites ( $16.43 \mu\text{g g}^{-1} \pm 7.2$ ; Table S6).

With increasing time since rewetting, mFAs in rewetted sites approached the abundance found in undrained sites in the 0–5 cm peat layer (Fig. 2). With <10 years of rewetting, microbial-derived mFAs ( $28.93 \mu\text{g g}^{-1} \pm 6.4$ ; Table S7) were not significantly higher than those of drained sites ( $20.06 \mu\text{g g}^{-1} \pm 8.4$ ,  $p = 0.98$ ; Tables S7 and S9) nor significantly lower than in undrained sites ( $36.03 \mu\text{g g}^{-1} \pm 12.3$ ,  $p = 0.06$ ; Tables S7 and S9). With >25 years of rewetting, the quantities of microbial-derived mFAs ( $43.24 \mu\text{g g}^{-1} \pm 6.7$  Table S7) were almost as high as those of undrained sites ( $48.25 \mu\text{g g}^{-1} \pm 6.1$ ; Fig. 4, Table S7). In this class, quantities were significantly different from drained ( $p = 0.15$ ) but not from undrained sites ( $p = 0.04$ ; Table S9).

Our results showed a higher F:B of the upper peat layers in undrained than drained sites. Whereas in the 0–5 cm peat layer the F:B was 49 at undrained sites, and only 24 at drained sites and 31 at rewetted sites (Table S8). With increasing rewetting time, fungal-derived mFAs shifted towards the quantities of undrained sites. In the class of <10 years of rewetting, F:B accounted for 23 in the 0–5 cm peat layer (Table S8). This increased to 40 with >25 years of rewetting (Table S8).

We found that fungal-derived mFAs decreased and  $\delta^{15}\text{N}$  values increased from undrained to rewetted to drained in the 0–5 cm peat layer. Fungal-derived mFAs and  $\delta^{15}\text{N}$  values were significant negative correlated ( $r = -0.7$ ; Fig. 4).

## 4. Discussion

### 4.1. Carbon-to-nitrogen ratios across hydrological regimes

The C:N ratios of the undrained fen sites were similar to ratios reported by Bridgman et al. (1998). The C:N ratio is higher in the top layer of undrained fens as a consequence of low N values in fen vegetation and low rates of decomposition (Malmer and Holm, 1984). The values for drained sites showed lower C:N ratios, most likely due to higher decomposition rates and as a consequence of preferential C loss compared to N (Malmer and Holm, 1984). For rewetted sites, the ratio increased depending on the time since rewetting, which could be linked to the decreasing decomposition rates of plant residues (Malmer and Holm, 1984).

### 4.2. Stable isotope bulk values across hydrological regimes

The  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of the 45–50 cm peat layers were in a similar range across hydrological regimes. This result indicates a relatively undisturbed deeper peat layer with low metabolic rates for all investigated sites (Artz, 2013; Asada et al., 2005).

In contrast,  $\delta^{15}\text{N}$  values of the upper peat layers (0–5 cm and 15–20 cm) differed significantly between the hydrological regimes. Especially in the uppermost layer, the values were lowest in the undrained sites and significantly higher ( $p < 0.01$ ) in drained sites. These results are in line with those from Denk et al. (2017) and Groß-Schmolders et al. (2020). In bogs, Groß-Schmolders et al. (2020) found no depth trends in the water-saturated peat layers of undrained and rewetted sites, while, at drained sites,  $\delta^{15}\text{N}$  values changed with depth. The depth patterns in ombrotrophic peatlands contrast with the drained and rewetted sites of the groundwater fed fens investigated here. For ombrotrophic drained sites,  $\delta^{15}\text{N}$  values increased with depth down to a  $\delta^{15}\text{N}$  turning point and decreased below that point down to the onset of the anaerobic peat layer. In fens studied here,  $\delta^{15}\text{N}$  decreased at the drained sites and showed only slightly increasing trends at water-saturated sites (undrained and rewetted). Such differences in ombrotrophic and minerotrophic sites could be driven by the different microbial abundances (see below in Section 4.3).

For  $\delta^{13}\text{C}$ , we found no difference in depth trends between three hydrological regimes, as the  $\delta^{13}\text{C}$  values increased with depth for all hydrological regimes. This observation is in line with the findings of other studies that also reported increasing  $\delta^{13}\text{C}$  values with depth in peatland soils (Krüger et al., 2015; Nadelhoffer and Fry, 1988). We saw the highest  $\delta^{13}\text{C}$  values in drained sites and the lowest in undrained sites. With increasing drainage, recalcitrant and  $\delta^{13}\text{C}$ -depleted substrates such as lignin are also processed, which leads to increased mobilization of lighter  $^{12}\text{C}$  and further increasing  $\delta^{13}\text{C}$  values in the remaining bulk soil with depth (Lerch et al., 2011). Furthermore, Boström et al. (2007) assumed that the  $^{13}\text{C}$  enrichment in drained peat soils with depth is a result of the increased contribution of microbial-derived C with depth (see below in Section 4.3).

### 4.3. Microbial-derived membrane fatty acid quantities and composition dependent on hydrological regime

Overall, the highest quantities of total microbial-derived and fungi-derived mFAs were found in the 0–5 cm peat layers of the undrained sites. This is in line with the findings of Fisk et al. (2003), who found highest microbial abundance in sites with highest water saturation. A plausible explanation is the higher quantity and quality (lower degree of decomposition) of bulk C in combination with low N supply in the undrained sites, which favors higher fungal metabolic rates (Fisk et al., 2003; Scanlon and Moore, 2000).

While bacterial-derived mFA quantities decrease less for different hydrological regimes in the 0–5 cm peat layer, fungal-derived mFAs were significantly lower in drained sites compared to undrained sites. This is presumably related to the different ecological niches and adaptability to

changing conditions of fungi vs. bacteria (Gilbert et al., 1998; Winsborough and Basiliko, 2010). Fungal metabolic processes may be more important than bacterial metabolic processes in the uppermost part of undrained minerotrophic fens (Thormann, 2005). Their abundance is closely linked to environmental conditions, such as moisture, O and N availability. Therefore, fungi are far more sensitive to hydrological changes in the 0–5 cm peat layers than bacteria, mainly because of the nitrogen supply (Thormann, 2005). With a decreasing C:N ratio in drained sites, fungal abundance is known to decrease, whereby bacteria have a competitive advantage over fungi in these areas (Thormann et al., 1999). Hence, fungal metabolism and abundance decreased with increasing drainage corresponding to a decreased C:N ratio in the 0–5 cm peat layers in drained minerotrophic fens (Gilbert et al., 1998; Winsborough and Basiliko, 2010).

In the rewetted sites, the water table increased again. Therefore, with changing environmental conditions (increasing C:N ratio, moss growth), bacteria have a decreased competitive advantage over fungi and fungal abundance and metabolism could rise in the 0–5 cm peat layer (Fig. 3; Fenner et al., 2005; Thormann, 2005; Winsborough and Basiliko, 2010).

#### 4.4. Stable isotopes reflect microbial abundance – fungi make the difference

We found parallel changes between microbial-derived mFAs and stable isotope values, especially for  $\delta^{15}\text{N}$  values. With lower water tables,  $\delta^{15}\text{N}$  values increased, and microbial-derived mFAs decreased in the upper peat layers. The reason for such a parallel trend is that isotope fractionation is shaped by microbial metabolic processes in the soil, which are more present in aerobic conditions (Dijkstra et al., 2006; Dröllinger et al., 2019; Kohl et al., 2015).

For bogs, previous studies have shown that microbial metabolism is a driver of stable isotope values. For  $\delta^{13}\text{C}$ , Krüger et al. (2015) stated that microbial metabolic processes increase  $\delta^{13}\text{C}$  values with depth in drained peatlands. Kohl et al. (2015) reported the same trend and linked it to a shift in the dominant microbial groups. Each microbial group focuses on a specific metabolic process and uses specific substrates, which leads to a typical fractionation rate for each microbial group. Hence, a shift from fungal metabolism to bacterial metabolism will also lead to changes in stable isotope values (Kohl et al., 2015).

For  $\delta^{15}\text{N}$  values, a correlation between increasing  $\delta^{15}\text{N}$  values with drainage was reported to be due to changes in microbial metabolism (Carrell et al., 2019). Moreover, Gundale et al. (2011) hypothesized that increasing  $\delta^{15}\text{N}$  values in drained peatland sites were the result of higher recycling rates of N and less incorporation of  $^{14}\text{N}$  by microorganisms (and thus preferential release of the lighter  $^{14}\text{N}$ ). Hereby, the investigated significant differences of  $\delta^{15}\text{N}$  values for a combination of depth and hydrological regime can be explained. The decreased water saturation in the upper layers of drained sites and therefore induced higher N cycling processes lead to higher  $\delta^{15}\text{N}$  values in the upper layers (Gundale et al., 2011). In contrast in the deeper layers less transformation processes are ongoing and the  $\delta^{15}\text{N}$  values are low and in the same range than in the undrained sites. Hence also the depth trend must show significant differences regarding the hydrological regime.

We found evidence that a changing F:B could also be responsible for the specific  $\delta^{15}\text{N}$  values in our study sites. The pattern of simultaneously changing  $\delta^{15}\text{N}$  values and microbial abundance was also observed in fens studied by Preston and Basiliko (2015). They reported that the  $\delta^{15}\text{N}$  pattern was linked more to the microbial community composition (e.g., the relative abundance of different microbial groups) than to overall microbial activity. This observation fits with the correlation between a specific  $\delta^{15}\text{N}$  depth trend and a changing F:B in drained bogs (Groß-Schmolders et al., 2020).

Therefore, we suggest that the influence of fungi on cycling processes is highest under undrained, wet conditions in our study. Fungi are a prominent microbial group in the 0–5 cm peat layers of undrained fens as decomposers of primary plant material under aerobic conditions (Tfaily et al., 2014; Thormann, 2005). The combination of naturally low  $\delta^{15}\text{N}$  values in primary plant material and low demand (and, therefore, low recycling rate) of N for fungal-induced recycling processes leads to a low enrichment of  $\delta^{15}\text{N}$  in fungal-dominated layers (Strickland and Rousk, 2010; Thormann et al., 2004; Wallander et al., 2009). Fungi are known to be especially vulnerable to changing substrate quality and, therefore, show clearer reactions to changing environmental conditions than other microbial groups (Peltoniemi et al., 2009; Preston and Basiliko, 2015), especially at higher N levels (Carrell et al., 2019). Brunner et al. (2013) reported that bacterial-forced cycling increases with drainage in fens. Bacterial metabolism is generally faster than fungal metabolism and requires more available N (Brunner et al., 2013; Tunlid and White, 1992). Hence, fungi lose their

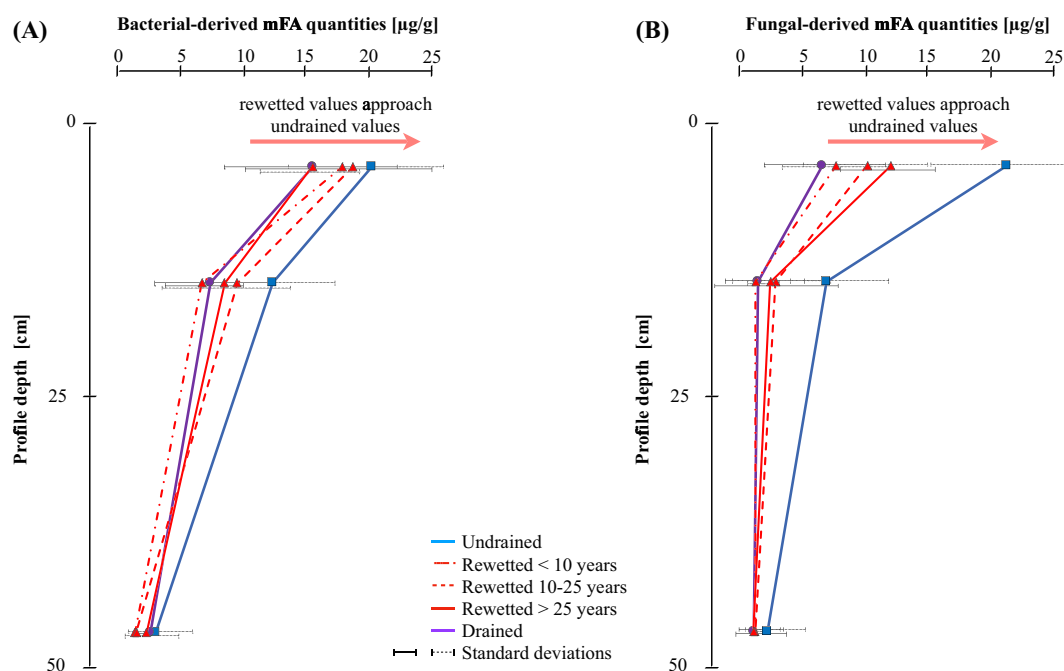


Fig. 3. (A) Bacterial-derived and (B) fungal-derived membrane fatty acid (mFA) quantity depth pattern (groups of fens are assigned to the time of rewetting of the respective rewetted fen); separated by the hydrology regime (undrained (blue), drained (purple), rewetted (red)).

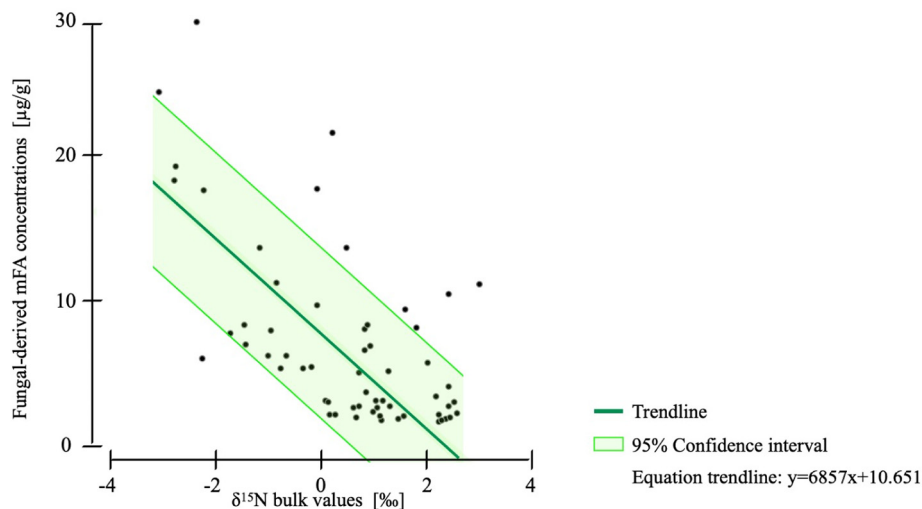


Fig. 4. Correlation between nitrogen stable isotopes values and fungal-derived membrane fatty acids (mFA) of all sites and all hydrological regime.

competitive advantage with drainage and lower C:N ratio, and a shift to bacterial-induced cycling occurs, resulting in increased  $\delta^{15}\text{N}$  values in the upper peat layer with drainage (Rousk and Bååth, 2007; Winsborough and Basiliko, 2010).

#### 4.5. Reestablishment of undrained conditions with time

With increased rewetting time, microbial-derived mFAs and stable isotope values of rewetted sites shifted towards the values of undrained sites. This result is in line with the findings of Urbanová and Barta (2020), who also reported a reestablishment of microbial communities after a longer period of rewetting.

With increased time since rewetting, fungal mFAs and, with them,  $\delta^{15}\text{N}$  values in rewetted sites were no longer significantly different from those of undrained sites but differed significantly from those of drained sites. Hence, with increasing rewetting time, the microbial community structure and dominant metabolic processes changed and shifted towards the community composition of undrained sites, indicating a recovery of ecosystem functioning (Urbanová and Barta, 2020).

However, we observed not at all rewetted sites a reestablishment of the stable isotope values and microbial-derived mFAs towards those of undrained sites. Differences in soil organic matter quantity and quality, as discussed by Emsens et al. (2020) and Fenner et al. (2005), could explain this. A major change in soil organic matter quality with drainage could impede the reestablishment of microbial communities that are typically found in undrained sites (Peltoniemi et al., 2009). Because stable isotope values depend on specific microbial metabolic processes, the absence of certain microbial groups and a low overall microbial community size will lead to values different from those of undrained sites, even after decades of rewetting (Emsens et al., 2020).

## 5. Conclusion

By combining microbial-derived mFA and stable isotope values ( $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ ) analyses, we were able to differentiate between the hydrological regimes (undrained, drained, and rewetted) of fens. With increasing time since rewetting, the stable isotope values of rewetted fens approached those of undrained sites.

The combination of stable isotopes and microbial mFAs indicated that changing  $\delta^{15}\text{N}$  values, in particular, were a result of a change in microbial communities.

In summary, our findings support our hypotheses

- (i) that stable isotope values, especially  $\delta^{15}\text{N}$ , differ between different hydrological regimes,

- (ii) that mFA profiles change with the hydrological regime in parallel to  $\delta^{15}\text{N}$  values, and
- (iii) that  $\delta^{15}\text{N}$  values in peat are negatively correlated to the fungal-derived mFA quantities.

Thus, we conclude that soil  $\delta^{15}\text{N}$  values are valid markers for microbial metabolic processes and the hydrological regime of fens. Furthermore, we found that time since rewetting was the primary driver for restoration success of the microbial community in the investigated sites, and thus biomarkers and bulk stable isotope values are suitable indicators of rewetting success.

#### Credit authorship contribution statement

MG-S, CJS and YL conducted the sampling and measurements. MG-S conducted the evaluation and analysis of the data and cowrote the paper with CA. KK, W-JE, RvD, CJS, and JF added to the discussion. JL and CA had the project idea, supervised and added to the discussion.

#### Declaration of competing interest

The authors declare that they have no conflict of interest.

#### Acknowledgement

We are really grateful to all members of the REPEAT team for assistance and the opportunity to connect our data. This research has been supported by the Swiss National Science Foundation (SNF; grant no. 169556).

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2022.156603>.

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