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# Inferring northern peatland methane emissions from testate amoebae: A proof of concept study

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## SUMMARY

Peatlands are efficient carbon sinks due to waterlogged soils causing oxygen depletion and slowing organic matter decomposition, leading to peat accumulation. However, peatlands are also a natural source of methane (CH<sub>4</sub>), a powerful greenhouse gas, to the atmosphere. Methane production (by methanogens) and oxidation (by methanotrophs) are controlled by water table depth, soil temperature and hydrochemistry. Measuring CH<sub>4</sub> emissions is resource demanding. Several measurements method are used, which introduces potential bias for comparisons among studies. Thus, a simple and reliable indicator tool would be desirable for both researchers and managers. Currently, such a tool does not exist. Testate amoebae (TA), an abundant and diverse group of shelled protists occurring in peatlands, are well-established proxies of present water table depth (WTD). As their shells are well preserved in peat, they are commonly used to infer past hydrological changes using predictive mathematical models called transfer functions. As CH<sub>4</sub> emissions are also tightly linked to WTD, and although TA are not directly involved in CH<sub>4</sub> production or consumption, we hypothesised that CH<sub>4</sub> emissions would be significantly correlated to TA community composition and could therefore be inferred from TA communities living in peatland mosses. We tested this hypothesis using compilations of CH<sub>4</sub> plot emissions measurements from European and North American bogs and fens, and TA data from moss samples collected from the same plots. Testate amoeba communities were significantly correlated to CH<sub>4</sub> fluxes. As our models were based on several independent studies for both flux measurements and TA communities, methodological differences among studies (e.g., CH<sub>4</sub> emission measurements, TA taxonomy) may potentially cause bias in the model. Nevertheless, the results are promising, and this proof-of-concept study suggests that past and present peatland CH<sub>4</sub> emissions could be inferred from TA shells preserved in peat over centuries and in mosses growing at the surfaces of peatlands.

**KEY WORDS:** bioindication, bog, fen, greenhouse gases, protist, soil

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## INTRODUCTION

Peatlands cover only about 3 % of the world's land area but are key ecosystems in the global carbon (C) cycle (Yu *et al.* 2011). Northern peatlands store about 500 ± 100 Gt of C in their soil (Yu 2012, Loisel *et al.* 2014) or even up to 1000 Gt of C (Nichols & Petee 2019), which represents about one quarter (or more if the latter figure is considered) of the world soil C

stock (Armstrong *et al.* 2015). The key to C sequestration is water-saturation of peatland soils, which causes anoxic conditions that strongly reduce organic matter decomposition (Freeman *et al.* 2001), leading to its accumulation as peat (Mitsch & Gosselink 2000).

However, wetlands, including peatlands, are also the largest natural source of methane (CH<sub>4</sub>) to the atmosphere (Rinne *et al.* 2007, Bridgman *et al.*

2013). Despite its lower atmospheric concentration as compared to CO<sub>2</sub>, CH<sub>4</sub> is an important greenhouse gas because of its stronger radiative activity (Krumholz *et al.* 1995). Global warming is especially marked at high latitudes (IPCC 2014), which causes permafrost melting and changes in wetland biogeochemistry, possibly leading to increased methane emissions (Bridgham *et al.* 2013). Therefore, being able to easily monitor CH<sub>4</sub> emissions and predict how they may change in response to climate change or peatland disturbance or restoration is crucial to understanding the mechanisms behind climate change, as well as its effects (Belyea & Baird 2006).

Both biotic and abiotic factors interact to regulate the C cycle of peatlands. Methane is produced by methanogenic archaea below the water table, under anoxic conditions (Krumholz *et al.* 1995). Methane diffuses through the peat up to the surface, and in this process a large portion can be oxidised to CO<sub>2</sub> by methanotrophic bacteria in the aerobic peat layer before it is released to the atmosphere (Sundh *et al.* 1995). Water table depth (WTD) is, therefore, one of the main factors controlling CH<sub>4</sub> emissions. Vascular plants stimulate CH<sub>4</sub> production by providing labile C sources but can also enhance fluxes by easing transport to the surface thus partly bypassing oxidation (Rinne *et al.* 2007, Korrensalo *et al.* 2018). Therefore, vegetation type and, especially, the abundance of vascular plants such as sedges also determine emission rates (Bubier *et al.* 1995a). Methane production is also driven by peat temperature, the optimal production occurring around 22 °C (Krumholz *et al.* 1995).

To determine the C exchange of a given peatland and the atmosphere, two main approaches are used. Where large homogenous surfaces exist, the eddy covariance method provides flux data integrated over space (Rinne *et al.* 2007). This method measures net CO<sub>2</sub> and CH<sub>4</sub> fluxes directly above the peatland at high frequency. If peatlands are small or heterogeneous, as is often the case in developed areas with long histories of human influence such as western Europe, chamber measurements are more suitable (Bubier *et al.* 2005). To estimate the CO<sub>2</sub> and CH<sub>4</sub> balance over a year (or growing season), numerous measurements must be taken to cover the broadest range of conditions, especially surface and peat temperature and WTD. Such an effort is highly resource demanding especially if several distant sites, each with numerous plots, are studied.

An alternative approach could be to estimate fluxes from proxies such as biotic communities (e.g., vegetation or soil organisms), microtopography, or direct (ideally automated) measurements of key

driving factors such as WTD or soil temperature. The rationale for the bioindicator approach is that biotic communities respond to environmental factors over time and, therefore, one-off measurements may be used to infer ecosystem functioning over a long period (e.g., one growing season or one year). The choice of a bioindicator depends on the variable of interest for inference (Havlicek 2012).

Bioindicators are organisms that are sensitive to environmental conditions or human disturbances (soil moisture, pollution, pH, etc.) which are used in ecological studies as indicators of environmental states (Markert *et al.* 2003). Protists are especially useful bioindicators because they are, *inter alia*, particularly sensitive to environmental changes, due to their broad distribution and their short generation times (Payne 2013). A bioindicator may become a palaeoecological proxy if its preserved remains can be used to reconstruct past climate or environmental changes.

Testate amoebae (TA), a polyphyletic group of protists living in shells that they produce, are well-established bioindicators of WTD and pH in peatlands (Charman *et al.* 2007, Mitchell *et al.* 2013, Amesbury *et al.* 2016). TA are ubiquitous and can be found in almost all habitats, but are especially abundant and diverse in *Sphagnum* mosses (Todorov & Bankov 2019). The morphology and composition of the shell are the main taxonomic features and are usually species-specific (Figure 1; Todorov & Bankov 2019). TA shells are decay resistant and well preserved in peat, making it possible to determine past changes in TA communities and, from these, to infer past environmental changes (Beyens 1985).

In community ecology studies of TA, species–environment interactions are usually assessed using multivariate analyses such as principal component analysis (PCA), redundancy analysis (RDA) or the weighted averaging partial least squares (WA-PLS) method, which allow identification of the variables which best explain the community patterns across environmental gradients (Booth *et al.* 2008, Lamentowicz *et al.* 2010). The variable that best explains the community patterns is a candidate for development of a transfer function (TF) which can be used to infer that variable based on the present or past (fossil) community composition (Fritz *et al.* 1991). The use of TA-based TFs to infer palaeohydrological conditions in peatlands is now common in peatland palaeoecology (Beyens 1985, Charman 2001, Booth 2002, Marcisz *et al.* 2015, Swindles *et al.* 2019).

While transfer functions based on TA communities are routinely used to infer WTD in peatlands, their direct use as bioindicators of CH<sub>4</sub> emissions has not yet been explored. Davies *et al.*

*Diffflugia bacilifera**Planocarina carinata*

Figure 1. Two species of testate amoebae with very specific shells that are known to be good bioindicators of wet conditions in *Sphagnum*-dominated peatlands (Amesbury *et al.* 2016). The length of the shell is shown in each frame. Photos: Alicia Frésard.

(2021) inferred CH<sub>4</sub> emissions from WTD which was, in turn, inferred from TA communities, thereby accumulating the errors associated with each model and thus potentially reducing the reliability of inference. So far, no TF exists to infer past or present methane emissions directly from TA communities. The link between TA and CH<sub>4</sub> emissions is tenuous but lies in the shared control of WTD on both CH<sub>4</sub> emissions (production minus oxidation) and TA community composition in peatlands. Both CH<sub>4</sub> emissions (Dise 1993) and WTD (Booth *et al.* 2005) change rapidly during the season in response to weather events but TA communities are more stable; although some seasonal patterns can be observed in natural peatlands (Warner *et al.* 2007) and even more in response to experimental manipulations (Marcisz *et al.* 2014, Koenig *et al.* 2017, Koenig *et al.* 2018). Therefore, in this study we hypothesised that TA species or morphotypes could be good proxies for the reconstruction of CH<sub>4</sub> emissions.

We aimed to develop a proof of concept for such models. To reach this goal, and to base our model on the maximum number of sites, we compiled data from published and unpublished studies along with original CH<sub>4</sub> emissions and TA community data from several peatlands in Europe and Canada. The studied habitats cover a gradient from fen to bog. The use of such a compilation of data comes with two main risks of bias: 1) the taxonomical bias inherent to the fact that different analysts identified the testate amoebae communities; and 2) the gas flux measurement bias inherent to the fact that several different methods

were used to measure the CH<sub>4</sub> fluxes. This results in a trade-off where the increase in model performance due to larger sample number may be partly or entirely compensated by the concomitant increase in biases and errors. We used two different approaches, 1) high TA taxonomical resolution, 2) lower TA taxonomical resolution, and ran our models with three different datasets. We expected that the model with best performance would be the one based on: high taxonomical resolution, as previously shown by Mitchell *et al.* (2014); TA identifications made by the same observer, which limits the risk of taxonomic confusion (Payne *et al.* 2011); and a dataset containing CH<sub>4</sub> values from geographically close plots.

## METHODS

### Study sites

We obtained data from a total of 12 peatlands in Europe and Canada (Figure 2). At each study site we selected several plots, aiming to cover the broadest available range of habitats from bog (ombrotrophic nutrient-poor acidic peatland) to fen (minerotrophic, less acidic, with mineral input from groundwater or surface runoff) (Rydin & Jeglum 2013) and, within each main habitat type, different microhabitats (hollows, lawns and hummocks). WTD and CH<sub>4</sub> fluxes were measured at each plot. Vegetation cover was assessed in detail for the Swiss plots (Bois-des-Lattes and Le Cachot). The list of study sites, general



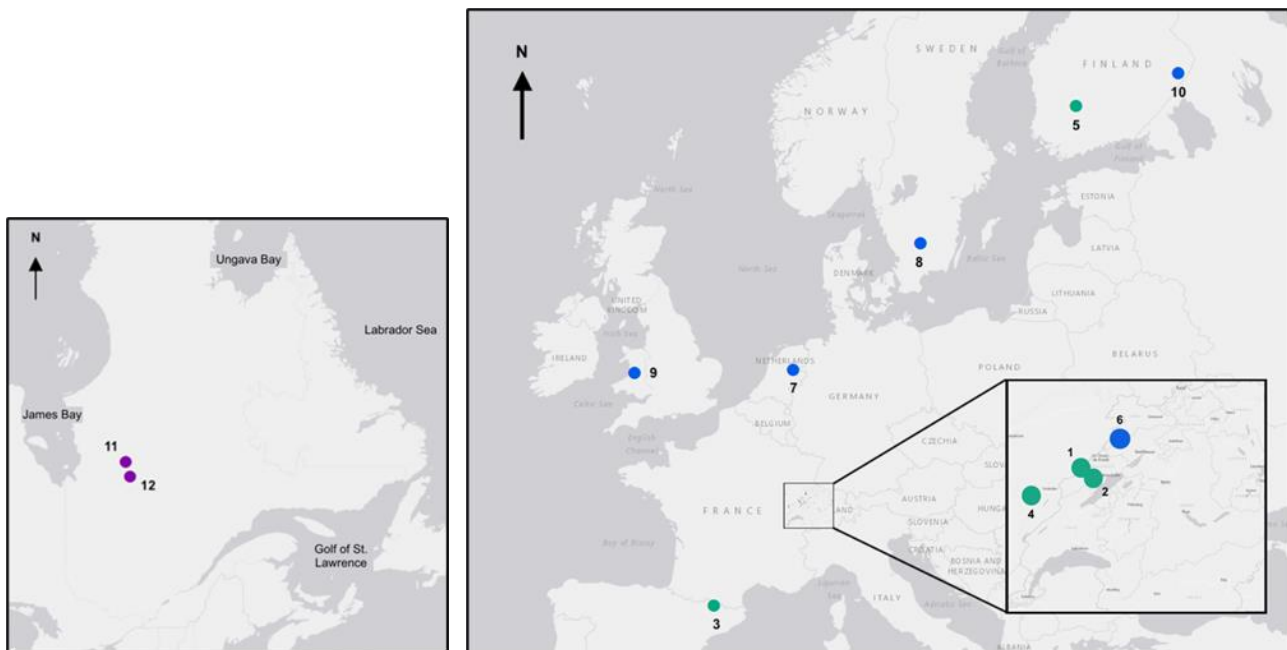


Figure 2. Maps showing the locations of the sites sampled for analysis of testate amoeba communities and methane fluxes. In green: sites for Dataset 1, sampled in 2020 and 2021. In blue: samples added to Dataset 1 to create Dataset 2. In purple (North America): samples added to Dataset 2 to create Dataset 3. 1: Le Cachot; 2: le Bois-des-Lattes; 3: Bernadouze; 4: le Forbonnet; 5: Siikaneva; 6: la Chaux-des-Breuleux; 7: Dwingeloo; 8: Koparrås; 9: Morecambe; 10: Salmisuo; 11: Lac le Caron; 12: Mosaik.

characteristics and the summary information about all plots are given in the Supplement (Excel file).

### Datasets

We compiled our data into three nested datasets, named Dataset 1, Dataset 2 and Dataset 3, with incremental numbers of sites. Dataset 1 contained data from 49 European sites and the TA communities were analysed by a single observer (Alicia Frésard). Four plots were removed due to missing or outlying CH<sub>4</sub> fluxes (originally 53 plots in Dataset 1, see Supplement for complete outliers list). Dataset 2 contained Dataset 1 and the data from a published study on the effect of elevated CO<sub>2</sub> and nitrogen (N) deposition on CH<sub>4</sub> emissions from peatlands in Finland, Sweden, Switzerland, the UK and The Netherlands (Silvola *et al.* 2003), along with the corresponding TA communities from 100 plots in total identified by Edward Mitchell (Mitchell *et al.* 2000). Dataset 2 contained 147 entries in total because two plots were removed as outliers. The third dataset (Dataset 3) contained Dataset 1, Dataset 2 and a dataset containing the CH<sub>4</sub> values and corresponding TA communities of 16 plots from an unpublished study by L. Pelletier and colleagues on CH<sub>4</sub> emissions in Canadian peatlands (163 entries for Dataset 3). The TA communities of Dataset 3 were identified by Julie Loisel (Table 1).

Our choice of creating three different datasets was motivated by the fact that a different observer analysed the TA for each dataset. The three observers used different identification keys and worked independently, which can be a potential source of bias or identification errors. Continent-wide analyses show broad consistency in TA ecology when morphologically similar species are pooled to limit the potential identification bias among datasets (Amesbury *et al.* 2016, Amesbury *et al.* 2018, Qin *et al.* 2021). However, using TA data from one region to create a transfer function which is then applied to another region could yield partly erroneous results, at least in terms of amplitude of the signal (Turner *et al.* 2013). It is therefore useful to compare the performance of individual or combined datasets when developing a new transfer function.

We used two different approaches to build our models (Table 1). To counter the effects of inconsistency and bias in the TA identification, it is possible to adopt a low-resolution approach, for example by grouping taxa into morphotypes. However, a lower taxonomical resolution might reduce model performance (Mitchell *et al.* 2014), illustrating a trade-off between comparability among data and precision of inference. Approach 1 involved identifying the TA species at highest taxonomical resolution using various identification guides. Using

Table 1. Summary of the three datasets and the two approaches used for our analysis.

Sampling campaigns	Frésard (this study) 2020–2021	Silvola <i>et al.</i> (2003)	Pelletier, Loisel, Booth, Garneau (unpublished) 2005–2007	
Number of samples	49	98	16	Total entries for the models based on all datasets # 163
Included in Dataset(s)	1, 2, 3	2, 3	3	
TA identification	Alicia Frésard	Edward Mitchell	Julie Loisel	
Approach 1: no. of taxa #	57	44	26	Total no. of taxa # 66
Approach 2: no. of morphotypes #	36	35	23	Total no. of morphotypes # 41

this method, 66 species were identified. In Approach 2 we grouped the species into 41 morphotypes, following Amesbury *et al.* (2016) (Table 2).

### ***Sphagnum* sampling and TA community analysis for Dataset 1**

The *Sphagnum* moss samples for the TA community analyses were taken close to the CH<sub>4</sub> measurement plots. We recorded WTD using dipwells installed next to the CH<sub>4</sub> measurement collars.

Testate amoebae occupy the whole vertical gradient from the top of the moss to the young peat (Heal 1962, Roe *et al.* 2017). Therefore, we tried to collect the whole shoot (total length 3–5 cm) including the living and recently dead parts of the moss. As TA communities can vary over short horizontal distances (~15 cm), even within apparently homogeneous surfaces in relation to micro-environmental gradients (Mitchell *et al.* 2000), it is best to collect several sub-samples which are representative for the plot. Therefore, we collected a composite sample of about ten individual *Sphagnum* shoots from different spots, aiming to cover the range of microtopography present in each plot. We used tweezers and rinsed them with alcohol between plots.

To extract TA, the samples were cut into small pieces, placed in a bottle with water, shaken for about one minute, and filtered at 300 µm to remove coarse particles. The filtrate was left to settle for several hours after which the supernatant was carefully poured off. The concentrated filtrate was then fixed with ethanol (final concentration 90 % ethanol). Each sample was analysed with an Olympus IX 81 inverted microscope. We identified 150 individuals per

sample (Woodland *et al.* 1998). For Dataset 1, we based our identification on several resources including Todorov & Bankov (2019) and the identification website “Microworld” (Siemensma 2022). For Datasets 2 and 3 we used existing counts with updated taxonomic nomenclature. We tried to match the three TA identifications by comparing the identification keys or by communication.

For Approach 2 (grouped by morphotypes), we grouped the 66 identified taxa of Approach 1 into 41 morphotypes (Table 2) based on Amesbury *et al.* (2016). Five species identified for Approach 1 did not match the groups of Amesbury *et al.* (2016). *Hyalosphenia insecta* and *H. elegans* - type 2, only observed in Finnish samples, were added to the group HYA ELE. *Centropyxis plagiostoma* was added to the group CEN AER. We created one group (HYA MIN) for *Hyalosphenia minuta*, and one (HAB ANG) for the rotifer *Habrotrocha angusticolis*, which is frequently identified and counted in testate amoeba ecological and palaeoecological studies (Warner & Chengalath 1988).

### **Methane flux measurements**

The seasonal CH<sub>4</sub> fluxes were measured at different frequencies and times of year in the individual studies. As a result, we did not have annual flux data for all sites. Therefore, we chose to use summer values as these can be assumed to correspond to peak fluxes (Dise 1993, Frohling & Crill 1994). The values are means of multiple measurements per plot, usually over several months. More information about the numbers of measurements averaged, as well as the durations of sampling, can be found in the

Table 2. List of the TA species identified in Approach 1, grouped into the 41 morphotypes (based on Amesbury *et al.* 2016) used for Approach 2.

Morphotype groups for Approach 2	Species	Morphotype groups for Approach 2	Species
ALA MIL	<i>Alabasta militaris</i>		<i>Euglypha compressa</i> -type
			<i>Euglypha ciliata</i> -type
GAL ARE	<i>Galeripora artocrea</i> <i>Galeripora catinus</i>	EUC CIL	<i>Euglypha cristata</i> <i>Euglypha dolioformis</i> <i>Euglypha strigosa</i> -type
GAL DIS	<i>Galeripora discoides</i>		<i>Euglypha laevis</i>
ARC HEM	<i>Arcella rotundata</i>	EUG ROT	<i>Euglypha rotunda</i> -type <i>Euglypha simplex</i>
AMP WRI	<i>Amphitrema wrightianum</i>	HAB ANG	<i>Habrotrocha angusticollis</i> (rotifer)
ARC FLA	<i>Archerella flavum</i>	HEL ROS	<i>Heleopera rosea</i>
ARG DEN	<i>Argynnia dentistoma</i>	HEL SYL	<i>Heleopera sylvatica</i>
ASS MUS	<i>Assulina muscorum</i>	HEL PET	<i>Heleopera sphagni</i>
ASS SEM	<i>Assulina scandinavica</i> <i>Assulina seminulum</i>		<i>Hyalosphenia elegans</i>
BUL IND	<i>Bullinularia indica</i>	HYA HEL	<i>Hyalosphenia elegans</i> -type 2 <i>Hyalosphenia insecta</i>
CEN ACU	<i>Centropyxis aculeata</i>	HYA MIN	<i>Hyalosphenia minuta</i>
CEN AER	<i>Centropyxis aerophila</i> <i>Centropyxis orbicularis</i> <i>Centropyxis plagiostoma</i>	HYA PAP	<i>Hyalosphenia papilio</i>
CEN ECO	<i>Centropyxis laevigata</i>	HYA SUB	<i>Hyalosphenia subflava</i>
COR TRI	<i>Corythion dubium</i> <i>Trinema complanatum</i> <i>Trinema lineare</i> -type	LES SPI	<i>Lesquereusia spiralis</i>
CRY OVI	<i>Cryptodifflugia oviformis</i>	NEB PEN	<i>Nebela penardiana</i> <i>Longinebela tubulosa</i>
CYC ARC	<i>Cyclopyxis eurystoma</i> <i>Difflugia globulosa</i> <i>Phryganella acropodia</i>	NEB COL	<i>Nebela collaris</i> -type
DIF ACU	<i>Difflugia acuminata</i> <i>Difflugia bacilliarium</i>	NEB FLA	<i>Nebela flabellulum</i>
DIF OBL	<i>Difflugia bacillifera</i> <i>Difflugia oblonga</i> (pyriformis)		<i>Nebela guttata</i>
DIF LEI	<i>Difflugia leidy</i>	NEB TIN	<i>Nebela pechorensis</i> <i>Nebela tincta</i> -type
DIF LUC	<i>Difflugia lucida</i> <i>Difflugia pristis</i>	PHY GRI	<i>Physochila griseola</i>
		PLA SPI	<i>Placocista spinosa</i>
		PLA CAR	<i>Planocarina carinata</i> <i>Planocarina marginata</i>
		QUA SYM	<i>Quadrullella symmetrica</i>
		SPHENO	<i>Sphenoderia fissirostris</i>
		TRI ARC	<i>Trigonopyxis arcula</i> <i>Trigonopyxis minuta</i>

Supplement. All CH<sub>4</sub> fluxes were converted to  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The fluxes were calculated using the following formula:

$$F = \frac{\Delta\text{CH}_4}{\Delta t} * \frac{V}{S} * \frac{Pa}{R*T} \quad [1]$$

where  $F$  is the flux in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $\Delta\text{CH}_4$  is the difference in concentration in ppm,  $\Delta t$  is the duration of the measurement in seconds,  $V$  and  $S$  are the volume and surface area of the chamber in  $\text{m}^3$  and  $\text{m}^2$ , respectively,  $Pa$  is the atmospheric pressure in Pascal,  $R$  is the gas constant ( $8.314 \text{ J mol}^{-1} \text{ K}^{-1}$ ) and  $T$  is the temperature inside the chamber in Kelvin. We kept all CH<sub>4</sub> values between 0.4 and  $-0.3 \mu\text{mol m}^{-2} \text{s}^{-1}$  and excluded all other values (five values, see Supplement) as outliers. Outliers can substantially reduce the accuracy of a transfer function and are, therefore, usually removed from studies using TA (Markel *et al.* 2010). They probably result from measurement errors or, if not, may correspond to specific microsites that are poorly represented in the dataset and are thus impossible to model correctly in a transfer function. Especially high chamber fluxes can be attributed to the process of ebullition (Pelletier *at al.* 2007), by which methane is released directly to the surface. Removal of these high values means that the process of ebullition may not have been captured.

All fluxes and the method used to derive seasonal fluxes of CH<sub>4</sub> are given in the Supplement.

### Numerical analysis

All numerical analyses were carried out using R statistical software (R Development Core Team 2015). We inferred the WTD of ten plots (four from Bernadouze and six from Le Forbonnet) from the TA communities using the European transfer function developed by Amesbury *et al.* (2016), as no WTD data were available for these locations or the dipwells were too far from the study plots. Approaches 1 and 2 were both tested with all three datasets. Therefore, we tested six different models (Table 1). We used the R packages Rioja version 0.9-26 (Juggins 2020) and Vegan version 2.5-7 (Oksanen *et al.* 2020) to develop palaeoecological transfer functions, applying the Weighted Averaging Partial Least Squares model (WAPLS) (scripts are provided in the Appendix). WAPLS regression provides a simple yet robust method for the reconstruction of environmental values from species assemblages while efficiently dealing with multicollinearity (ter Braak & Juggins 1993). We cross-validated all models with the bootstrap method and assessed their performance using the root mean square prediction and  $R^2$ .

## RESULTS

### Environmental variables

Our study sites spanned a broad range of environmental conditions. WTD for Dataset 1 ranged from 5 cm (SN\_B\_HO\_02) to 42.5 cm (BDLB1). In Le Cachot peatland, two hollows (CAG05 and CAG09) were submerged by water during several months of the year, even though the mean seasonal WTD values were 5.6 cm and 10.4 cm respectively. In the full dataset (Dataset 3), the WTD range spanned almost 60 cm (-1 to 57 cm). Our plots ranged through the whole fen to bog gradient and different microhabitat types were sampled (hollows, lawns, hummocks, heaths; see Supplement).

### Testate amoeba community composition

The fifty samples of Dataset 1 were analysed in 2020 to identify the TA communities, reaching a count of more than 150 TA per sample. We identified 66 taxa using Approach 1. The most abundant species identified varied among datasets. The most abundant species in Dataset 1 were *Assulina muscorum* (present in 14 % of the samples of Dataset1), *Archerella flavum* (12.5 %), *Hyalosphenia papilio* (9 %) and *H. elegans* (6.8 %). The most abundant species in Dataset 2 were *H. elegans* (present in 10 % of the samples of Dataset 2), *Habrotricha angusticolis* (10 %), *Nebela tincta* (8.3 %) and *Corythion dubium* (8 %). The most abundant species in all our samples (Dataset 3) were *H. elegans* (9.5 %), *A. muscorum* (9 %), *N. tincta* (6.9 %), *H. angusticolis* (6.7 %), *H. papilio* (6.6 %) and *A. flavum* (6.5 %) (Table 3).

### Methane flux data

Measured CH<sub>4</sub> fluxes varied considerably among the studied sites, ranging between  $0.0015 \mu\text{mol m}^{-2} \text{s}^{-1}$  (fen hummock) and  $0.38 \mu\text{mol m}^{-2} \text{s}^{-1}$  (bog hollow) (Figure 3, Figure 4 and Supplement). Fluxes varied substantially within each microhabitat type. The differences in fluxes between microhabitats were in general as expected, with the highest CH<sub>4</sub> fluxes in bog hollows (SN\_B\_HO\_02 & SN\_B\_HO\_05,  $0.38 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) (Figure 4). Heath, and bog and fen hummock plots, had small CH<sub>4</sub> fluxes ( $<0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), except for one fen hummock plot (SN\_F\_HU\_03) which had a large CH<sub>4</sub> flux ( $0.33 \mu\text{mol m}^{-2} \text{s}^{-1}$ , WTD = 22 cm).

Within-site variability was lowest in Bernadouze ( $0.0187\text{--}0.2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and highest in Siikaneva ( $0.0015\text{--}0.3788 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Indeed, methane fluxes Siikaneva were the highest under wet conditions (WTD around 10 cm) and the lowest



Table 3. List of TA species, total count of each species in all samples, and the proportion (%) of each species within Dataset 3. The species shown in **bold** type are usually the most common in peatlands. The species highlighted were the most abundant in our samples.

Taxon	Total count	Proportion of Dataset 3 (%)	Taxon	Total count	Proportion of Dataset 3 (%)
<b><i>Alabasta militaris</i></b>	259	0.909	<i>Galeripora artocrea</i>	62	0.214
<i>Amphitrema wrightianum</i>	383	1.344	<i>Galeripora catinus</i>	572	2.007
<i>Arcella rotunda</i>	101	0.354	<i>Galeripora discoides</i>	92	0.323
<b><i>Archerella flavum</i></b>	1849	6.486	<b><i>Habrotrocha angusticollis</i> (rotifer)</b>	1926	6.757
<i>Argynnia dentistoma</i>	4	0.014	<i>Heleopera rosea</i>	139	0.488
<b><i>Assulina muscorum</i></b>	2584	9.065	<b><i>Heleopera sphagni</i></b>	880	3.087
<i>Assulina scandinavica</i>	17	0.060	<i>Heleopera sylvatica</i>	109	0.382
<b><i>Assulina seminulum</i></b>	873	3.063	<b><i>Hyalosphenia elegans</i></b>	2702	9.479
<i>Bullinularia indica</i>	383	1.344	<i>Hyalosphenia elegans</i> type2	108	0.379
<i>Centropyxis aculeata</i>	33	0.116	<i>Hyalosphenia insecta</i>	1	0.004
<i>Centropyxis aerophila</i>	55	0.193	<i>Hyalosphenia minuta</i>	217	0.761
<i>Centropyxis laevigata</i>	18	0.063	<b><i>Hyalosphenia papilio</i></b>	1877	6.585
<i>Centropyxis orbicularis</i>	103	0.361	<i>Hyalosphenia subflava</i>	6	0.021
<i>Centropyxis plagiostoma</i>	8	0.028	<i>Lesquereusia spiralis</i>	1	0.004
<b><i>Corythion dubium</i></b>	1799	6.311	<i>Longinebela tubulosa</i>	9	0.032
<i>Cryptodifflugia oviformis</i>	22	0.077	<i>Microcorycia radiata</i>	27	0.095
<i>Cyclopyxis eurystoma</i>	154	0.540	<b><i>Nebela collaris</i>-type</b>	1658	5.816
<i>Difflugia acuminata</i>	3	0.011	<i>Nebela flabellulum</i>	197	0.691
<i>Difflugia bacillarium</i>	29	0.102	<i>Nebela guttata</i>	34	0.119
<i>Difflugia bacillifera</i>	44	0.154	<i>Nebela pechorensis</i>	2	0.007
<i>Difflugia globulosa</i>	46	0.161	<i>Nebela penardiana</i>	2	0.007
<i>Difflugia leidyi</i>	588	2.063	<b><i>Nebela tinctoria</i>-type</b>	1984	6.960
<i>Difflugia lucida</i>	4	0.014	<b><i>Phryganella acropodia</i></b>	783	2.747
<i>Difflugia oblonga</i> (pyriformis)	1	0.004	<b><i>Physochila griseola</i></b>	1213	4.255
<i>Difflugia pristin</i>	3	0.011	<i>Placocista spinosa</i>	153	0.537
<b><i>Euglypha compressa</i>-type</b>	1461	5.125	<i>Planocarina carinata</i>	86	0.302
<b><i>Euglypha ciliata</i>-type</b>	353	1.238	<i>Planocarina marginata</i>	29	0.102
<b><i>Euglypha cristata</i></b>	16	0.056	<i>Quadrullella symmetrica</i>	2	0.007
<i>Euglypha dolioformis</i>	2	0.007	<i>Sphenoderia fissirostris</i>	16	0.056
<i>Euglypha laevis</i>	408	1.431	<b><i>Trigonopyxis arcuata</i></b>	130	0.456
<b><i>Euglypha rotunda</i>-type</b>	111	0.389	<b><i>Trigonopyxis minuta</i></b>	8	0.028
<i>Euglypha simplex</i>	4	0.014	<i>Trinema complanatum</i>	50	0.175
<b><i>Euglypha strigosa</i>-type</b>	1503	5.273	<i>Trinema lineare</i> -type	211	0.740

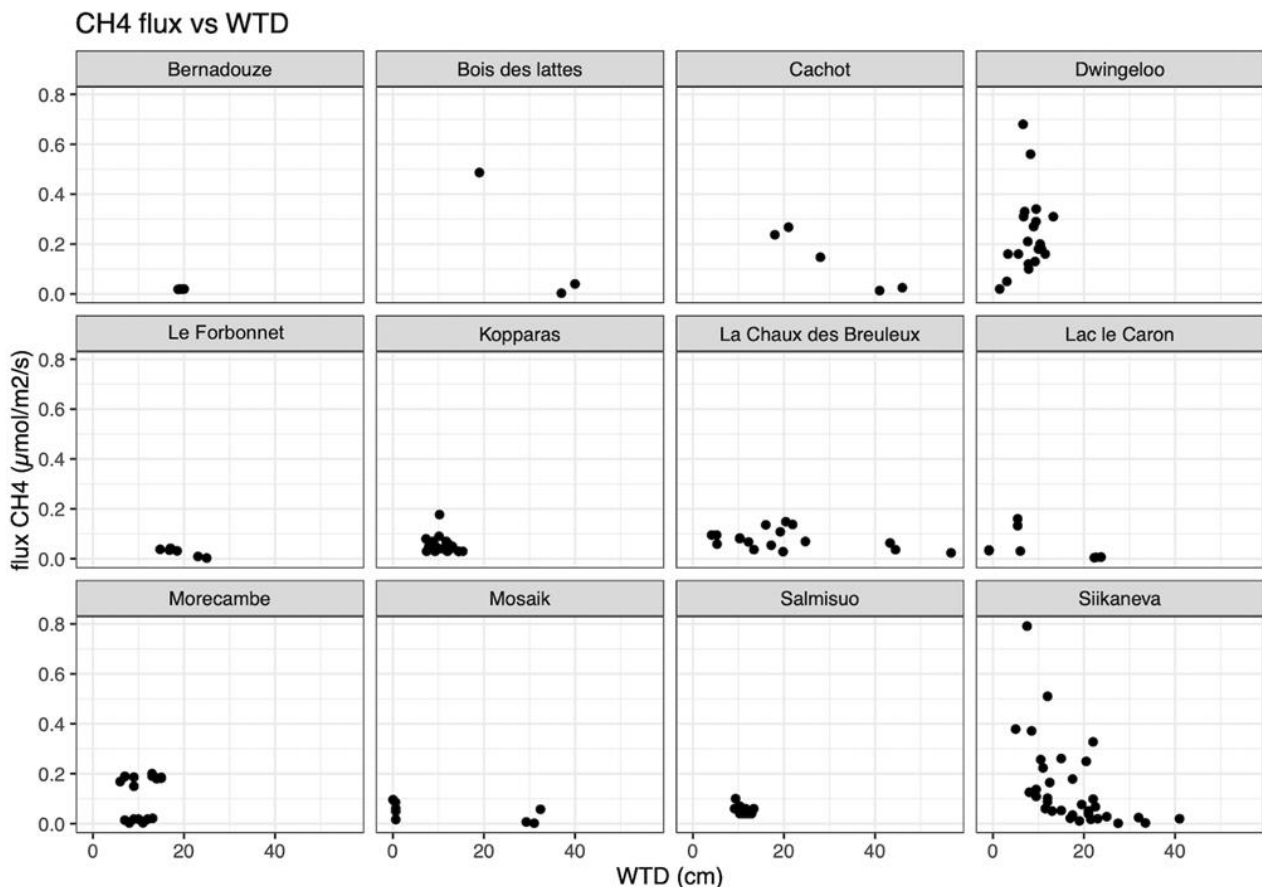


Figure 3. Methane fluxes (in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) in relation to water table depth (WTD) for the 12 studied peatlands. WTD typically determines the thickness of the methane-consuming (aerobic) layer of peat, leading to a gradient of decreasing  $\text{CH}_4$  flux with increasing WTD. Some studies suggest that the maximum  $\text{CH}_4$  fluxes are found at intermediate WTD values (around 10 cm) (Turetsky *et al.* 2014).

under dry conditions (WTD around 40 cm) (Figure 3 and Figure 4). This tendency has been described previously and is in line with the methanogenesis process in peatland (Rinne *et al.* 2007).

### Performance of transfer functions

For all approaches, the best model performance based on the bootstrap cross-validation method (boot) was the one built with Dataset 1, i.e., based on the dataset with the same observer for TA identification and the same year of sampling (Table 4). The performance was lower with Dataset 2 ( $r^2$  (boot) = 0.304 for Approach 1,  $r^2$  (boot) = 0.246 for Approach 2), and the least performant models were always the ones combining all data (Dataset 3) ( $r^2$  (boot) = 0.262 for Approach 1,  $r^2$  = 0.192 for Approach 2; Figure 5). Overall, the model with the best performance ( $r^2$  (boot) = 0.378, RMSE = 0.084) was the one based on Approach 2 and Dataset 1, i.e., with a reduced taxonomic resolution (morphotypes), one observer and all sites sampled over two years. This result differed from our original hypothesis. However, the

performance of the model with Dataset 1 and Approach 1 was very similar ( $r^2$  (boot) = 0.370, RMSE = 0.085).

## DISCUSSION

### Factors influencing methane emissions

Water table depth is a key factor controlling  $\text{CH}_4$  emissions in peatlands, together with soil temperature and vegetation type (Turetsky *et al.* 2014). Maximum  $\text{CH}_4$  fluxes usually occur in fens, due to the (often) higher water table and greater abundance of vascular plants. The WTD for which  $\text{CH}_4$  emissions are highest is usually between -10 cm (flooded) and 0.1 cm in fens, and around 20 cm in bogs (Turetsky *et al.* 2014). The expected negative correlation between  $\text{CH}_4$  flux and WTD was observed at Siikaneva, which was also the site with the highest diversity among plots and the most  $\text{CH}_4$  measurements (Figure 3). At this site, the highest  $\text{CH}_4$  fluxes were measured at a WTD of 10 cm, and

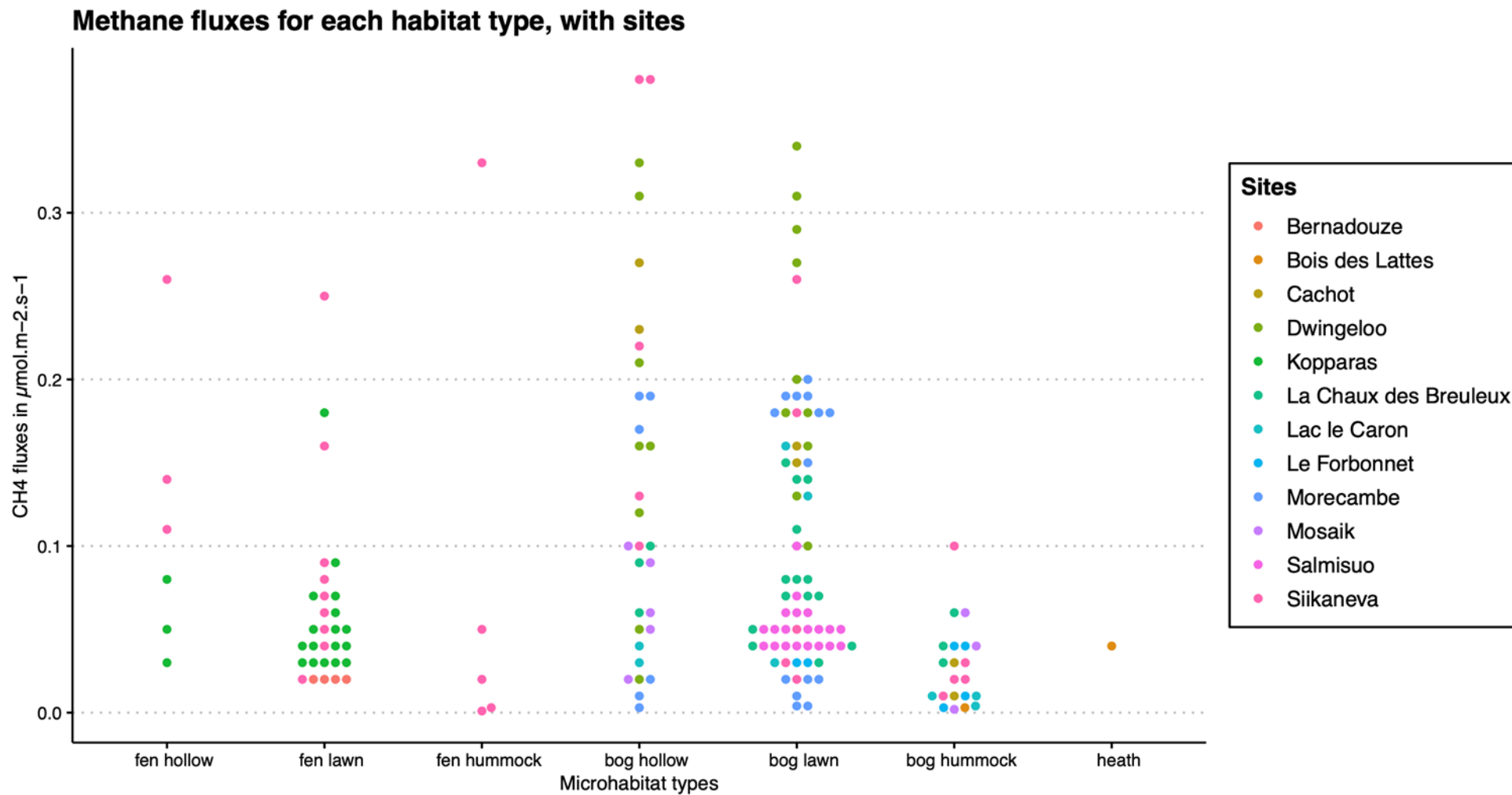


Figure 4. Methane fluxes measured for the seven microhabitat types (fen hollow, fen hummock, fen lawn, bog hollow, bog hummock, bog lawn and heath). Colours represent the twelve sampled sites.



Table 4. Performance statistics for all transfer function models for inferring peatland CH<sub>4</sub> emissions based on testate amoebae, based on bootstrap cross-validation method (boot). The best models (in **bold**) are those with the highest R<sup>2</sup> and lowest root mean squared error (RMSE) values.

Approach	Dataset	R <sup>2</sup>	RMSE	Selected/best model
<b>1</b>	<b>1</b>	<b>0.370</b>	<b>0.085</b>	<b>1 component</b>
1	2	0.304	0.089	5 components
1	3	0.262	0.088	5 components
<b>2</b>	<b>1</b>	<b>0.378</b>	<b>0.084</b>	<b>1 component</b>
2	2	0.246	0.080	2 components
2	3	0.192	0.081	2 components

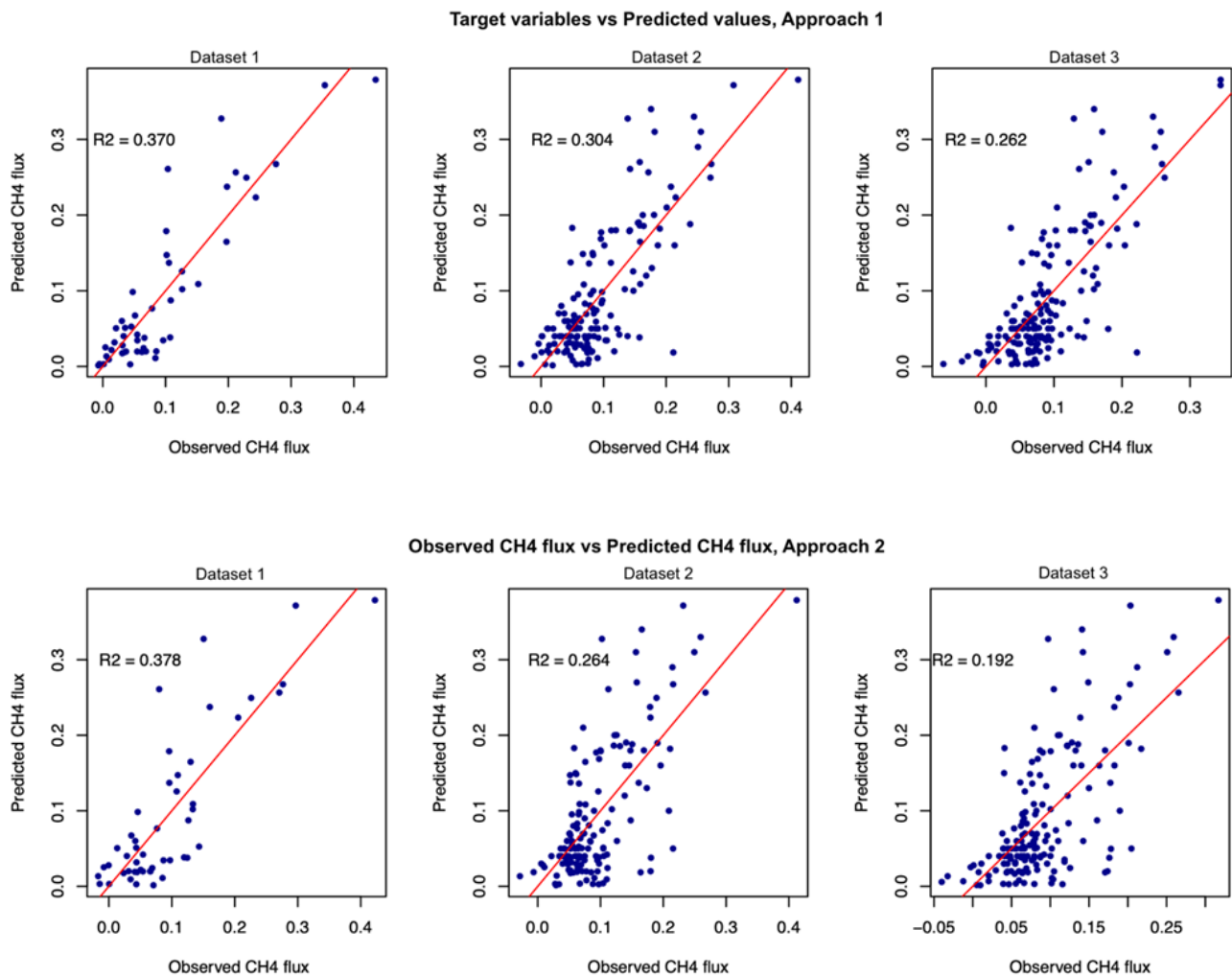


Figure 5. Biplots of CH<sub>4</sub> fluxes (in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) measured on site, versus fluxes inferred using testate amoeba (TA) based transfer functions. Approaches 1 and 2, with (a) Dataset 1, (b) Dataset 2 and (c) Dataset 3.



the lowest at a WTD of 40 cm. However, this tendency was not clear for other sites. This could be explained by a small range of CH<sub>4</sub> flux values, or by sample plots being in general too similar (shorter gradient). In our study, the highest CH<sub>4</sub> fluxes were found in bogs (SN\_B\_HO\_02 and SN\_B\_HO\_05, with fluxes of 0.38  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), but taking into account the outliers which were removed for better TF model performance, the highest fluxes were found in fens (SN\_F\_HO\_03 with a flux of 0.79  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , WTD = 7.5 cm (see Figure 3 and Supplement).

In addition to WTD, the vegetation type has a strong predictive power for CH<sub>4</sub> emissions (Bubier *et al.* 1995b, Levy *et al.* 2012, Gray *et al.* 2013, Korrensalo *et al.* 2018), because plants provide a pool of labile carbon for methanogenic microorganisms. Furthermore, plants with aerenchymatous roots, such as sedges, offer CH<sub>4</sub> a bypass route from peat to the atmosphere, enhancing CH<sub>4</sub> release compared to diffusive CH<sub>4</sub> fluxes. In our case, the vegetation cover of the chamber area was assessed in detail only for the Swiss plots Le Bois-des-Lattes and Le Cachot. The type of vegetation for the other plots is indicated by the microhabitat types. Due to the potential effect of vegetation on CH<sub>4</sub> emissions to the atmosphere, a detailed record of vegetation cover of all sampled plots is recommended for future studies.

Temperature is the third main factor affecting CH<sub>4</sub> emissions in peatlands, but we did not have soil temperature measurements for all plots. In our study, we sampled a wide latitudinal gradient of sites, in different regions with different climates. As in the case of vegetation, we recommend measuring soil temperature for future studies.

### Temporal variations in CH<sub>4</sub> fluxes and TA communities

For each individual study, the seasonal CH<sub>4</sub> fluxes were measured at different times of the year and at different frequencies. For example, the Swiss sites sampled in 2021 (Le Cachot and Le Bois-des-Lattes) and Le Forbonnet had short sampling campaigns extending only over one month. As CH<sub>4</sub> emissions can vary over the course of a day, a season or between years (Dise 1993, Frolking & Crill 1994, Bubier *et al.* 2005, Korriakoski *et al.* 2017), the performance of our models was probably affected by the differences in the length or timing of CH<sub>4</sub> sampling period. A short sampling period will not support assessment of effects on the annual mean CH<sub>4</sub> flux of large seasonal variations or episodes like flooding or drought. In comparison, the sampling campaigns for the added sites of Dataset 2 and 3 had much longer sampling campaigns (four months or more), the longest being the one for the Salmisuo site in Finland, with 90

campaigns over six months. To build a more robust model it will, therefore, be necessary to conduct CH<sub>4</sub> flux measurement campaigns over an annual cycle and harmonise measurement protocols across all sites. Furthermore, our study focused on annual mean methane fluxes. It would be interesting to assess how CH<sub>4</sub> fluxes vary over a period of several years and how changes in TA communities match this variability.

### Transfer function model performance

#### *Building datasets*

We hypothesised that testate amoeba communities would be good proxies for the reconstruction of CH<sub>4</sub> emissions from *Sphagnum*-dominated peatlands and that the best model would be obtained using the highest taxonomic resolution and communities analysed by a single observer. The best model for each approach was indeed the one with the dataset based on observations made by the same observer, with samples from European countries only, all sampled during summer in 2020 and 2021 (Dataset 1). The performance of the models decreased when using a dataset based on several TA analysers and samples from different time periods. The model performances ( $R^2$  (boot)) for the first approach were 0.370, 0.304 and 0.262 for Datasets 1, 2 and 3, respectively, and for the second approach they were 0.378, 0.246 and 0.192. This result is consistent with our expectations.

Applying a transfer function developed in one region to a different region may lower the quality of results (Amesbury *et al.* 2016) due to differences in TA ecology (in particular, variations in sensitivity among microsites), the still debated degree of cosmopolitanism in TA (Heger *et al.* 2009) and, when applying transfer functions to palaeoecology, the possible lack of modern analogues from one site to another (Turner *et al.* 2013). Furthermore, the techniques or precautions used in measuring CH<sub>4</sub> fluxes might vary between sites and through time (Amesbury *et al.* 2016, Markel *et al.* 2010). Regarding the fact that our third dataset contained plots separated by long geographical distances, from European to Canadian peatlands, and our second and third datasets had CH<sub>4</sub> values measured during three different periods (between 1996 and 1998, then between 2005 and 2007, and finally between 2020 and 2021), it is not surprising that our three models had different performances. However, in practice, TFs based on WTD data from various regions give consistent results on relative magnitude and direction of shifts even if absolute values vary among the models (Charman *et al.* 2007, Turner *et al.* 2013, Amesbury *et al.* 2016). Therefore, a TF based on a

large dataset with different origins is useful to show the direction of the shift in CH<sub>4</sub> emissions in (palaeo)ecological studies.

In addition to the spatial and temporal variations, the differences in TA identification (conflicting names or taxonomical resolution) among observers is a known caveat (Payne *et al.* 2011). To increase the size of the dataset we involved a higher number of observers and, with this, the risk of identification bias also increased. We tried to harmonise the TA taxonomy across all three datasets, but some taxa lacked a contemporary analogue due to changes in taxonomy or errors. The three observers used different identification keys, the standard identification guide at the beginning of the 2000s usually being Charman *et al.* (2000) although that guide was often adapted in various ways (Amesbury *et al.* 2016). Such identification biases are common or even normal in TA taxonomical studies, and using a lower taxonomical resolution approach to build models is an option for overcoming this problem.

#### *Taxonomic challenges and how to deal with them*

Identification errors are a known source of bias in testate amoeba ecology (Mitchell *et al.* 2008) and the way this is usually dealt with when several datasets are combined is to lump species together into morphotypes (Amesbury *et al.* 2018). In contrast to our initial hypothesis, the model based on morphotypes (Approach 2) performed slightly better with Dataset 1 ( $R^2$  (boot): 0.378 and  $R^2$  (boot): 0.370) than the model based on high taxonomical resolution (Table 4). Even if the two model performances were similar, this finding is not consistent with previous studies, which showed that reducing the taxonomic resolution lowers the model statistical performance (Mitchell *et al.* 2014). However, the best performances on Datasets 2 and 3 were always with Approach 1. The reduced quality of prediction does not mean that our low taxonomical resolution models had no predictive power at all. A model based on low taxonomical resolution might be strong enough to give information on the direction of the CH<sub>4</sub> emission shift in palaeoecological studies. The predictive loss between Approaches 1 and 2 for the same dataset is also smaller than the predictive loss between datasets for the same approach, suggesting that taxonomic bias is less of a problem than discrepancies in CH<sub>4</sub> flux measurements due to differences in the methods used. Furthermore, the use of high taxonomical resolution is questionable because TA taxonomy still lacks precision due to the scarcity of molecular data and the cryptic diversity that exists within this paraphyletic group of organisms (Heger *et al.* 2009, Kosakyan *et al.* 2016).

When using a broad taxonomical resolution with TA for the development of training sets for transfer functions, one should follow some recommendations to limit bias. For example, when creating the training set, different observers should work together and communicate during counting or by comparing pictures (Payne *et al.* 2011). Moreover, publishing an identification key and the identification criteria is primordial, and the difficult taxa should be excluded or grouped. In our study we tried to communicate with the different observers, but the long period of time between identifications complicated the process and samples were not available to proof the identifications. To be as consistent as possible during the identification process, we created an illustrated key with our own identifications and pictures. Despite these precautions, some species have probably been grouped, not on purpose but due to their close morphological traits; for example, *Euglypha compressa* and *Euglypha ciliata*, or taxa from the genera *Diffflugia* and *Centropyxis*, which are known to be complicated genera (Payne *et al.* 2011).

The fact that small taxonomical errors can lead to large reconstruction errors (Payne *et al.* 2011) can also advocate for the use of taxonomical groups, and it is important not to neglect this almost inevitable issue. Grouping eases the process for new or inexperienced observers and shortens the observation time. Therefore, some studies suggest meeting halfway by grouping the easily confused taxa together while still trying to identify the other species as accurately as possible (Mitchell *et al.* 2014). We also favour the “meet-halfway” approach - with groups only for easily mistaken taxa - in order to counteract the taxonomical inconsistencies while still achieving good model performance.

An approach that was not tested during this study is to select a reduced number of easily identifiable species with well-defined ecological optima. This approach was tested in Swiss peatlands using 10 TA taxa and gave good results (Koenig *et al.* 2015). Thus, it may be possible to select a small number of easily identifiable TA species based on their CH<sub>4</sub> optima. This approach would have the advantage to ease the identification process for non-specialists while reducing the risk of confusion. This is indeed crucial as our goal is to develop a user-friendly tool to monitor fluxes of CH<sub>4</sub> (and possibly other GHGs such as CO<sub>2</sub> and N<sub>2</sub>O) at the surfaces of peatlands. A potential limitation of such an approach is that some species complexes could include species with different CH<sub>4</sub> optima, which would reduce the explanatory power of our models. Two known examples in northern peatlands are the *Hyalosphenia papilio* complex, which includes at least 13

molecular species with contrasting biogeographical distributions (Heger *et al.* 2013, Singer *et al.* 2019), and the *Nebela tinctoria-collaris* complex which includes at least eight pseudo-cryptic species with contrasting ecological preferences in the Swiss Jura Mountains and Alps (Kosakyan *et al.* 2013, Singer *et al.* 2015, Singer *et al.* 2018).

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## AUTHOR CONTRIBUTIONS

Conceptualisation: AF, EADM, MM; supervision of the research: EADM; fieldwork: all co-authors; testate amoebae identifications: AF, EADM, JL; data analysis: AF, MM; first draft of the manuscript: AF. All co-authors helped to re-write the manuscript and agreed to the published version.

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**Appendix:** R scripts**WAPLS**

```

library(readxl)
library(rioja)
library(vegan)
library(dplyr)
library(tidyr)

# load data -----
amibes <-
  read_excel(
    "Data_name",
    sheet = "Feuil1"
  )
methane <- read_excel("Data_name2")

# set up -----
methane_clean <- methane %>%
  select("Site name", "flux CH4 (µmol/m2/s)", "WTD (cm)") %>%
  rename(Ech = "Site name",
         flux = "flux CH4 (µmol/m2/s)",
         WTD = "WTD (cm)") %>%
  distinct()

amibes <- amibes %>%
  rename(Ech = "Echantillon:") %>%
  select(- starts_with("TOTAL")) %>%

data_full1 <- right_join(methane_clean, amibes) %>%
  drop_na()

# TF -----

Spec1 <- data_full1 %>%
  filter(flux < 0.4 & flux > -0.3) %>%
  select(-c(Ech, flux, WTD))

Spec1 <- Spec1[,which(colSums(Spec1)>0)]
Spec1 <- decostand(Spec1, "total")

CH41 <- data_full1 %>%
  filter(flux < 0.4 & flux > -0.3) %>%
  select(flux) %>%
  pull()

WTD1 <- data_full1 %>%
  filter(flux < 0.4 & flux > -0.3) %>%
  select(WTD) %>%
  pull()

CH4_model1 <- WAPLS(Spec1, CH41, tolDW = TRUE)
fit.xv1 <- crossval(CH4_model1, cv.method="boot", nboot=1000)
rand.t.test(fit.xv1)

```