Influence of temperature on the $\delta^{13}$C values and distribution of methanotroph-related hopanoids in Sphagnum-dominated peat bogs

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Abstract

Methane emissions from peat bogs are mitigated by methanotrophs, which live in symbiosis with peat moss (e.g. Sphagnum). Here, we investigate the influence of temperature and resultant changes in methane fluxes on Sphagnum and methanotroph-related biomarkers, evaluating their potential as proxies in ancient bogs. A pulse-chase experiment using $^{13}$C-labelled methane in the field clearly showed label uptake in diploptene, a biomarker for methanotrophs, demonstrating in situ methanotrophic activity in Sphagnum under natural conditions. Peat cores containing live Sphagnum were incubated at 5, 10, 15, 20 and 25°C for two months, causing differences in net methane fluxes. The natural $\delta^{13}$C values of diploptene extracted from Sphagnum showed a strong correlation with temperature and methane production. The $\delta^{13}$C values ranged from −34‰ at 5°C to −41‰ at 25°C. These results are best explained by enhanced expression of the methanotrophic enzymatic isotope effect at higher methane concentrations. Hence, $\delta^{13}$C values of diploptene, or its diagenetic products, potentially provide a useful tool to assess methanotrophic activity in past environments. Increased methane fluxes towards Sphagnum did not affect $\delta^{13}$C values of bulk Sphagnum and its specific marker, the C$_{23}$ n-alkane. The concentration of methanotroph-specific bacteriohopanepolys (BHPs), aminobacteriohopanetetrol (aminotetrol, characteristic for type II and to a lesser extent type I methanotrophs) and aminobacteriohopanepentol (aminopentol, a marker for type I methanotrophs) showed a non-linear response to increased methane fluxes, with relatively high abundances at 25°C compared to those at 20°C or below. Aminotetrol was more abundant than aminopentol, in contrast to similar abundances of aminotetrol and aminopentol in fresh Sphagnum. This probably indicates that type II methanotrophs became prevalent under the experimental conditions relative to type I methanotrophs. Even though BHP concentrations may not directly reflect bacterial activity, they may provide insight into the presence of different types of methanotrophs.
Peat bogs play an important role in the global carbon cycle since they sequester one-third of the Earth’s terrestrial carbon (Smith et al., 2004). Peat bogs are responsible for approximately 10% of the total methane flux to the atmosphere, having a major impact on atmospheric methane concentrations (Gorham, 1991). High latitudes are especially vulnerable to climate change, and it is, therefore, important to understand the effect of changing environmental conditions on carbon and net methane flux rates in peat bogs.

Aerobic methane oxidation by bacteria (methanotrophs) is a significant terrestrial methane sink and hence plays an important role in the global methane cycle (Dean et al., 2018; Hanson & Hanson, 1996). In peat bogs, methanotrophs live in symbiosis with peat moss (Sphagnum), living inside the hyaline cells of Sphagnum and on the stems. There they reduce methane emissions and provide CO2 and fixed nitrogen to Sphagnum when growing under submerged conditions (Kip et al., 2010; Larmola et al., 2010, 2014; Raghoebarsing et al., 2005). In order to predict the consequences of perturbations in the global carbon cycle, a better understanding of the influence of environmental factors on methanotrophs and the methane cycle in peat bogs is essential. Mesocosm experiments have revealed that both methane production and oxidation increase with increasing temperature, albeit that the extent of methane retention by the Sphagnum-methanotroph consortium strongly decreases with increasing temperature (van Winden, Reichart, McNamara, Benthien, & Sinninghe Damsté, 2012). This confirmed the hypothesis that peat bogs may act as a positive feedback to global warming. Assessing the long-term impact of changing environmental conditions on methanotrophs and the methane cycle in peat bogs, however, also relies on reconstructions of methane fluxes in ancient peats.

Ombrotrophic peat bogs receive their nutrients solely via precipitation, which makes them relatively nutrient poor. The low pH buffering capacity, cation exchange and humic acid release by Sphagnum mosses result in a strongly acidic environment (Soudzilovskaia et al., 2010, and references therein). Due to the low pH of the bog water, the reservoir of exchangeable inorganic carbon is low and the level of diffusion of atmospheric CO2 into the bog water is also little, which makes submerged-growing Sphagnum mosses CO2-limited and to a large extent dependent on CO2 derived from organic matter degradation (Smolders, Tomassen, Pijnappel, Lamers, & Roelofs, 2001). Methanotrophs supply methane-derived CO2, which may account up to 35% of the carbon uptake by Sphagnum (Kip et al., 2010; Larmola et al., 2010).

The knowledge on environmental controls, including temperature, on methanotrophy is still limited. If palaeoclimate archives could be used to study the controls on methane fluxes in peat bogs, including temperature and humidity, this would open up additional sources of information. While advanced microbial techniques exist to study current microbial community structures (see Yates, Nakatsu, Miller, & Pillai, 2016), the limited longevity of genetic material, however, limits the use of advanced microbial tools to reconstruct changes through time. Changes in past methanotrophic activity in peat bogs can potentially be studied using hopanoids. These bacterial markers are common lipids of methanotroph, also those occurring in Sphagnum peats (van Winden, Talbot, Kip, et al., 2012). However, they are not exclusive to methanotrophs since many other bacteria produce them as well (Belin et al., 2018; Farrimond et al., 1998; Rohmer, Bouvier-Nave, & Ourisson, 1984; Sinninghe Damsté, Rijpstra, Dedysy, Foesel, & Villanueva, 2017; Sinninghe Damsté et al., 2004). Bacteriohopanepolysols (BHPs) have a polyfunctionalized side chain attached to the pentacyclic carbon skeleton, to which a variety of polar groups can be attached (Talbot & Farrimond, 2007). Differences in these polar groups hold species-specific information (Ourisson, Rohmer, & Poralla, 1987; Rohmer et al., 1984; Rush et al., 2016), and therefore, BHPs may provide insight into methanotroph community structures (Coolen et al., 2008; van Winden, Talbot, Kip, et al., 2012). Methanotrophs occurring in peat bogs can be generally divided into two distinct phylogenetic groups: γ-proteobacteria (type I) and α-proteobacteria (type II; Esson et al., 2016; Hanson & Hanson, 1996). Characteristic biomarkers for type I methanotrophs are aminobacteriohopanepentols (aminopentol), while aminobacteriohopanetetrol (aminotetrol) is primarily produced by type II methanotrophs, but also to a minor extent by type I methanotrophs and some sulphate-reducing bacteria (Blumenberg et al., 2006; Coolen et al., 2008; Cvejic, Bodrossy, Kovács, & Rohmer, 2000; Neunlist & Rohmer, 1985a, 1985b; Osborne et al., 2017; Rohmer et al., 1984; Rush et al., 2016; Talbot, McClymont, Inglis, Evershed, & Pancost, 2016; Talbot, Watson, Murell, Carter, & Farrimond, 2001; van Winden, Talbot, Kip, et al., 2012).

Methanotroph lipids are typically severely depleted in 13C since biogenic methane has relatively depleted δ13C values, ranging from approximately −45% to −65% (Hornibrook, Longstaffe, & Fyfe, 2000), and this methane acts as a carbon source. Therefore, these lipids serve as an indicator for methanotrophy as shown for lakes and marine settings (Collister, Summons, Lichtfouse, & Hayes, 1992; Coolen et al., 2008; Elvert, Whiticar, & Suess, 2001; Freeman, Hayes, Trendel, & Albrecht, 1990; Pancost & Sinninghe Damsté, 2003; Spooner et al., 1994). However, hopanoids in Sphagnum and other peats often show only a limited depletion in 13C (see Inglis, Naafs, Zheng, Schellekens, and Pancost (2019) for a comprehensive overview). For example, van Winden et al. (2010) reported δ13C values ranging from −31% to −38% for hopenes (hop-17(21)-ene + 2-methylhop-17(21)-ene) and −34% to −37% for 17β,21β-(H)-bishomohopanediol (derived from tetrafuctionalized hopanoids) in Sphagnum moss species from contemporary peatlands.

Pancost, van Geel, Baas, and Sinninghe Damsté (2000) also reported
2.3 | Temperature experiments

Transparent cores (height 50 cm, diameter 7 cm), containing approximately 30 cm of peat with on top living S. cuspidatum, were taken from the field site in July 2009, when temperatures ranged between 10 and 20°C. These peat cores were incubated at 5, 10, 15, 20 and 25°C in triplicate at the Alfred Wegener Institute (AWI) in Bremerhaven for two months. The lower 30 cm of the cores, consisting of decomposed peat below the living Sphagnum, were covered with aluminium foil and sealed with rubber stoppers. Since peat has a very large redox buffering capacity, no amendments were done to make the water anaerobic or acidic. Eriophorum plants were not included in the incubation. Water levels were kept at the level of the top of the Sphagnum capitula, which increased in height with growth. Water level was kept constantly high by addition of bog water originating from the field site on a regular basis (several times per week) to ensure the Sphagnum remained fully submerged.

Sphagnum is a very basic plant, growing from the top and decaying at the bottom. After two months, when the photosynthetic capitulum was newly grown, the whole Sphagnum plant was harvested and sectioned into top parts (capitulum) and lower parts. Top parts of Sphagnum mosses were analysed for the δ13C of Sphagnum-derived C23 n-alkane to capture only lipids grown under the experimental conditions. The lower parts, where methanotrophs reside (Raghoebarsing et al., 2005; van Winden, Reichart, et al., 2012; van Winden, Talbot, Kip, et al., 2012), were analysed for BHPs and δ13C values of a bacterial lipid (diolopetene). The bulk carbon isotopic composition was measured of both the top and lower parts of the Sphagnum mosses.

2.4 | Extraction and derivatization of lipids

Total extracts of freeze-dried Sphagnum mosses (whole plant) derived from the field experiment were obtained with an Accelerated Solvent Extractor (Dionex), using a mixture of DCM:MeOH (9:1, v/v). Top parts and lower parts of freeze-dried Sphagnum mosses obtained from the temperature experiments were extracted using a modified Bligh and Dyer extraction procedure, to enable BHP
analyses (Talbot, Rohmer, & Farrimond, 2007). An aliquot of the extract was separated into an apolar fraction and a polar fraction over an activated Al$_2$O$_3$ column using hexane:DCM (9:1 v/v) and DCM:MeOH (1:1 v/v). To purify samples for hopene analyses (lower Sphagnum parts), apolar fractions were treated by urea addition. Apolar fractions were dissolved in 200 μl urea-saturated methanol, 200 μl acetone and 200 μl hexane and shaken. After 30 min at −20°C, solvents were evaporated under a stream of nitrogen. The urea crystals, containing the adductable normal and iso alkanes, were washed with hexane three times. To obtain the non-adductable branched and cyclic hydrocarbons (including the hopanes), the urea crystals were washed with hexane three times. The wash solvents were collected, dried under nitrogen and dissolved in hexane prior to GC analyses.

For BHP analyses, another aliquot of the total extract of the lower Sphagnum parts was acetylated using acetic anhydride and pyridine (1:1) at 50°C for 1 hr and left at room temperature overnight, after addition of the internal standard pregnanediol. The acetylated extract was dried at 50°C under a continuous nitrogen flow and dissolved in MeOH/propane-2-ol (60:40 v/v), prior to LC/MS analyses.

2.5 | Gas chromatography (GC), GC/mass spectrometry (GC/MS) and GC-isotope ratio monitoring mass spectrometry (IRMS), Elemental Analyzer (EA)-IRMS

Hopene-containing fractions were analysed on a gas chromatograph (HP 6890) equipped with a flame ionization detector (FID) set at constant pressure (100 kPa). A fused silica column (30 m × 0.32 mm i.d., film thickness 0.1 μm) coated with CP Sil-5CB was used with helium as a carrier gas. Extracts were injected on-column at 70°C. The temperature increased with 20°C/min to 130°C and 4°C/min to 320°C, followed by an isothermal hold for 20 min. Components were identified using gas chromatography–mass spectrometry (Thermo Trace GC Ultra), using the same column and temperature programme as for GC analyses.

Compound-specific δ$^{13}$C values were determined by isotope ratio monitoring–gas chromatography mass spectrometry (GC-IRMS, Thermo Fisher Delta V), using the same column and temperature programme as for GC analyses. Carbon isotopic values are reported in the delta notation relative to the VPDB standard.

Bulk carbon isotopes were analysed on homogenized subsamples from the lower and upper parts of the Sphagna. To minimize the impact of isotopic differences between different plant parts about ten times, the required amount of carbon was weighed in tin sample cups. The CO$_2$ produced was subsequently diluted with He before being introduced online into the mass spectrometer (Finnigan Delta plus). Accuracy and precision based on replicate analyses of samples and an in-house standard (calibrated to VPDB using international standards) were <0.1‰.

![Figure 1](image1.png)  
**Figure 1** In situ pulse-chase experiment showing $^{13}$C incorporation (in atomic %) into the bacterial lipid diploptene through time, after injection of $^{13}$C-labelled methane into the peat. Compound-specific carbon isotopes were measured on the lower parts of the pool-derived Sphagnum, where potential methane oxidation rates were highest (van Winden, Reichart, et al., 2012)

![Figure 2](image2.png)  
**Figure 2** Response of potential methanotrophy proxies on temperature (°C) in the two-month peat mesocosm incubation study. (a) Diploptene concentrations (μg/g dry weight) in the lower parts of Sphagnum mosses, with error bars representing ± standard errors. (b) The δ$^{13}$C values of diploptene (% vs. VPDB) in the lower parts of Sphagnum mosses. (c) Bulk δ$^{13}$C values (% vs. VPDB) of newly grown top parts and of lower parts of Sphagnum sp. and compound-specific δ$^{13}$C values of the C$_{23}$ n-alkane measured for newly grown top parts of Sphagnum sp. All δ$^{13}$C values represent the means of three replicate incubations and the standard deviation is indicated.
2.6 | High-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS)

Acetylated total extracts were analysed for BHPs by reversed-phase high-performance liquid chromatography (HPLC). The derivatization and analytical procedure have been described previously (Cooke, Talbot, & Farrimond, 2008). In short, reversed-phase HPLC analysis was carried out using a Surveyor HPLC system (ThermoFinnigan) fitted with a Gemini (Phenomenex) C18 5 μm HPLC column (150 mm, 3.0 mm i.d.) and a security guard column of the same material. Separation was achieved at a flow rate of 0.5 ml/min at 30°C with the following gradient profile: 90% A and 10% B (0 min); 59% A, 1% B and 40% C (at 25 min), then isocratic (to 40 min) and returning to the starting conditions over 5 min and finally stabilizing for 15 min before the next injection, where A = MeOH, B = water and C = propan-2-ol (all Fisher Scientific HPLC grade). LC/MS was performed using a ThermoFinnigan LCQ ion trap mass spectrometer equipped with an APCI source operated in positive ion mode. LC/MS analysis was carried out in data-dependent mode with three scan events: SCAN 1—full mass spectrum, range m/z 300–1,300; SCAN 2—data-dependent MS² spectrum of most abundant ion from SCAN 1; and SCAN 3—data-dependent MS³ spectrum of most abundant ion from SCAN 2. Quantification was performed using m/z traces targeting the characteristic base peak ions and calculated using averaged response factors for a limited suite of nitrogen and non-nitrogen containing BHP standards where N-containing BHPs give an averaged response 12 times that of the internal standard and those with no nitrogen 8 times that of the standard (Cooke et al., 2008). Accuracy of the quantification was ±20%.

3 | RESULTS

To establish in situ methane uptake by methanotrophs and verify methanotroph-derived lipids, an isotopic pulse-chase experiment using 13C-labelled methane was performed in the field. Within 14 days, a shift of 0.015% in 13C, or 15% in δ13C, was observed in diploptene, the most abundant hopene in the lower parts of the pool-derived Sphagnum (Figure 1). The magnitude and evolution of the shift in δ13C indicate uptake of the 13C-labelled methane and, hence, evidence for diploptene as marker for methanotrophs.

To study potential methanotrophy proxies, intact peat cores containing living Sphagnum spp. obtained from the field were incubated at 5, 10, 15, 20 and 25°C for two months. We have previously demonstrated that with increasing temperature, methane production and consumption (presumably through methanotrophy) increases (van Winden, Reichart, et al., 2012). Therefore, this set of experiments is useful to evaluate biomarker proxies for methanotrophy. The concentrations of diploptene were highest in the lower parts of the Sphagnum mosses obtained from the mesocosm temperature experiments but did not vary significantly (p > .01) with increasing incubation temperature (Figure 2a). However, the δ13C values of diploptene showed a strong negative relation with temperature (p < .01, R² = .84, Figure 2b), from −33.9‰ (at 5°C) to −40.7‰ (at 25°C). A strong correlation between methane production and diploptene δ13C values (p > .01, R² = .84; Figure 3) was also observed.

The summed BHP concentration in Sphagnum showed an increase with increasing temperature upon incubation (Figure 4a). The concentrations of bacteriohopanetetrol (BHT) showed consistently increasing concentrations with increasing temperature from 10°C onwards (Figure 4b), while those of aminotriol did not increase until 25°C (Figure 4c), where a sudden fourfold increase was noted. Concentrations of aminopentol and aminotetrol, both biomarkers for methanotrophs (van Winden, Talbot, Kip, et al., 2012, and references therein), also showed this strong non-linear response at 25°C (Figure 4e–d). Aminotetrol concentrations were almost five times higher than those of aminopentol (Figure 4). Concentrations of summed BHPs in Sphagnum cores incubated at 5, 10 and 15°C were lower compared to natural field-derived Sphagnum samples, while those incubated at 20 and 25°C showed higher concentrations (Figure 4a). All reported individual BHP concentrations at 25°C were elevated compared to natural samples (Figure 4b–d). Aminotriol and aminopentol abundances in the natural Sphagnum samples were higher than those measured in Sphagnum cores incubated at 5–20°C, while BHT and aminotetrol abundances of natural samples were more in line with observations under experimental conditions in the range 5–20°C.

Both top and lower parts of Sphagnum yielded bulk δ13C values of around ~24‰ to ~26‰ that did not vary with incubation temperature (Figure 2c). Also, the C23 n-alkane, extracted from newly grown top parts of Sphagnum, exhibited no significant variation in δ13C values with temperature; they ranged from −34‰ to −36‰ (Figure 2c).

FIGURE 3 Compound-specific δ13C values of diploptene (in ‰ vs. VPDB) versus the rate of methane production (μg cm⁻² day⁻¹). The lines show an increase in methane production with increasing temperature (R² = .84).
4 | DISCUSSION

4.1 | BHP distributions as potential proxies for methanotrophy in ancient peats

The increase in the summed BHP concentration in *Sphagnum* moss with increasing temperature (Figure 4a) indicates that bacterial BHP production may have had a strong response to the increased temperature and associated increase of the methane flux and anticipated level of methanotrophy (cf. van Winden, Reichart, et al., 2012). Aminopentol and aminotetrol, quite specific BHPs for methanotrophs (van Winden, Talbot, Kip, et al., 2012 and references therein), showed a strong non-linear response to the increase in temperature/methane flux, only displaying relatively high abundances at 25°C (Figure 4d,e), suggesting that their abundances do not linearly correlate with the anticipated methanotrophic activity. Osborne et al. (2017) also showed a non-linear 10-fold increase in the aminopentol concentration with increasing temperature (from 4 to 40°C) in methanane-amended aerobic river-sediment incubations with only marginal increases in aminotriol and aminotetrol concentrations. These changes were most likely the effect of temperature since Sherry, Osborne, Sidgwick, Gray, and Talbot (2016) showed that changes in methane concentrations alone did not result in changes of the microbial community. This suggests that BHP production may have increased to ensure membrane integrity in the incubations at 25°C, or these methanotrophs were better able to adapt to the perturbation. Still, some caution should be taken with this interpretation, as Osborne et al. (2017) and the results of our study demonstrate that the bacterial BHP production in response to environmental conditions is non-linear. Furthermore, Osborne et al. (2017) found that aminotriol production stabilized after 10 days, while aminotetrol and aminopentol concentrations still increased with time, even after 28 days. Our incubation experiment lasted for 2 months, but it is possible that the duration of the experiment may have been insufficient for the methanotrophic community to fully adapt and express the effect of temperature on bacterial growth and BHP production.

Abundances of aminotetrol and aminopentol were small relative to the summed BHP abundance (Figure 4). This suggests that methanotrophs represent only a minor fraction of the BHP-producing bacterial community. In fact, the most abundant BHPs in *Sphagnum* mosses are unsaturated BHT pentose, BHT pentose, BHT cyclitol ether and adenosylhopanes, which are not produced by methanotrophs (van Winden, Talbot, Kip, et al., 2012). Not all type II methanotrophs produce aminotetrol, and this trait appears to be confined to type II methanotrophs belonging to the *Methylocystaceae* family. *Methylocella*-like type II methanotrophs only produce BHT and aminotriol (van Winden, Talbot, Kip, et al., 2012; Talbot et al., unpublished data). Aminotriol and BHT are significantly more abundant in *Sphagnum* compared to aminotetrol and aminopentol (Figure 4), and while the BHT concentration follows the same pattern as the summed BHP concentration, the aminotriol abundance shows a close resemblance to the patterns of aminotetrol and aminopentol (Figure 4c–e), suggesting that it is also produced by *Methylocella*-like type II methanotrophs. Hence, the abundance of methanotrophs may be considerably higher than what would be estimated based on aminotetrol and aminopentol abundances alone. Still, as only aminotetrol and aminopentol are relatively specific for methanotrophs, we will have to rely on these for palaeoenvironmental reconstructions on ancient peats.

Analyses of compound-specific isotopes of intact BHPs would enable a tremendous step forward in the understanding of their sources. Recently, a novel method was proposed to measure the $^{13}$C composition of BHPs, using semi-preparative ultrahigh pressure liquid chromatography followed by high-temperature gas chromatography–isotope ratio mass spectrometry (Hemingway et al., 2018). This method, or high-temperature gas chromatography, as recently developed by Lengger et al. (2018), would allow this and may provide more insight into the use of methanotroph-derived BHPs.

The methanotroph BHP markers aminotetrol and aminopentol have been detected in peat cores from Misten Bog, Belgium (van Winden, Talbot, De Vleeschouwer, Reichart, & Singninghe Damsté, 2012) and in a peat core from Bissendorffer Moor, Germany (Talbot, McClymont, et al., 2016), where in both cases aminotetrol was more abundant than aminopentol, in agreement with our results (Figure 4). Aminotetrol and aminopentol were even present in the 55 Ma old Cobham lignite (Talbot, Bischoff, Inglis, Collinson, & Pancost, 2016), indicating that these BHPs can be used to trace back methanotrophy in ancient peats. Their occurrence reveals the past presence of methanotrophic bacteria of type I and/or II, where changes in the relative distributions point towards changes in methanotrophic communities. Hence, they potentially provide useful information to assess methanotrophic community structures in past environments,
especially in the absence of other information sources such as DNA and unsaturated fatty acids.

### 4.2 Diploptene $\delta^{13}$C as a potential proxy for methanotrophy in ancient peat

The field pulse-chase experiment using $^{13}$C-labelled methane clearly showed label incorporation into diploptene by the magnitude of the isotopic shift of 0.015% $^{13}$C or 15‰ $\delta^{13}$C (Figure 1). Therefore, these results confirm the in situ methanotrophic activity in *Sphagnum* under natural conditions (Kip et al., 2010; Raghoebarsing et al., 2005) and make it likely this also occurs in the mesocosm experiments performed in this study. The amended $^{13}$C-labelled methane represented only a fraction of the total available methane and is also not homogenously transported through the contained *Sphagnum* and peat. It is, therefore, not surprising that the isotopic shift in the $\delta^{13}$C of diploptene is not larger than the 15‰ observed. This label uptake in diploptene, which is in line with previous laboratory studies (van Winden et al., 2010), confirms its, at least partial, methanotrophic origin (Rohmer et al., 1984). Moreover, these results show a direct and fast response of the $\delta^{13}$C of diploptene to changes in the $\delta^{13}$C of the methane. In the studied *Sphagnum* moss, diploptene (hop-22(29)-ene) was the most abundant C$_{30}$ hopene, while in other *Sphagnum* mosses or peats, its precursor lipid, diplopterol (Raghoebarsing et al., 2005) or hop-17(21)-ene (van Winden et al., 2010), was to be the most abundant C$_{30}$ hopanoids. Probably, hop-17(21)-ene is the isomerization product of diploptene, which might be readily formed in the acidic peat environment (van Winden, Talbot, De Vleeschouwer, et al., 2012).

The concentrations of diploptene in the *Sphagnum* moss from the mesocosm experiments did not show significant changes with increasing temperature (Figure 2a), suggesting no major increase in biomass of the associated methanotrophs. In contrast, diploptene $\delta^{13}$C values showed a strong correlation with temperature (Figure 2b). Consequently, there is also a strong correlation of diploptene $\delta^{13}$C values with methane production (Figure 3), which has previously been established by measurement of the methane flux from the *Sphagnum* cores when the methanotroph-containing
Sphagnum layer was removed (van Winden, Reichart, et al., 2012). If diploptene δ¹³C values would be controlled by temperature directly, one would expect an opposite trend than observed (Figure 2b); at higher temperatures, chemical reactions are faster and isotopic discrimination is expected to decrease with increasing temperature (Hayes, 2001). This was, for example, observed by Jahnke, Summons, Hope, and Des Marais (1999) in their study of the effect of growth temperature on isotopic fractionation in methanotroph cell cultures. Therefore, the decreasing δ¹³C values of diploptene could be interpreted as being caused by the enhanced methane flux related to the increase in temperature. The decreasing δ¹³C values of diploptene could, thus, be caused by an increased contribution of ¹³C-depleted methanotroph-derived diploptene to the total bacterial diploptene pool. The trend of more depleted δ¹³C values with increasing methanotrophic activity is, however, not supported by diploptene concentrations, which did not change significantly with temperature (Figure 2a). Indeed, the concentrations of methanotroph-specific BHPs also did not show a clear temperature response (Figure 4c–e), except at 25°C. Furthermore, the methanotrophs represented only a small proportion of methanotroph-derived BHPs does not necessarily directly translate for the proportion of methanotroph-derived diploptene. BHPs and diploptene may have different roles within the cell, and/or diploptene could be an intermediate product, which could be the reason for a lack of build-up (Bradley, Pearson, Sáenz, & Marx, 2010). In addition, as discussed, aminopentol and aminotetrol abundances may significantly underestimate the size of the methanotrophic community.

Despite these considerations, an alternative explanation should be considered. It may well be that substrate availability influenced the extent of fractionation and, thereby, diploptene δ¹³C values. Jahnke et al. (1999) showed that δ¹³C values of methanotroph lipids strongly depend on methane concentrations, with much stronger isotopic fractionation at higher methane concentrations. An increase from 3% to 37% methane in the headspace of their culture experiments resulted in up to 22‰ more negative δ¹³C values of total methanotroph lipids. Therefore, the decrease in diploptene δ¹³C values with increasing methane availability (Figure 3) can also be explained through enhanced expression of the enzymatic isotope effect associated with methanotrophy, which was suppressed by substrate or mass transport limitations at lower temperatures or lower methane concentrations (Hayes, 2001).

Unfortunately, we did not analyse the isotopic composition of methane and carbon dioxide during the mesocosm experiments, which could have further assisted in the interpretation of the results. In addition, it would be recommended to perform a set of mesocosm experiments with Sphagnum moss where the methane concentration is varied, while the temperature is kept constant, to decouple the temperature effect from the methane concentration effect, similar to the experiment performed by Sherry et al. (2016) and Osborne et al. (2017).

Diploptene and hopane δ¹³C values have been used previously to trace methanotrophy in past peatlands. In a Carex-dominated Holocene peat deposit from the Tibetan Plateau, diploptene δ¹³C values as negative as −50‰ have been reported (Zheng et al., 2014). In a recent study by Huang et al. (2018) in a peatland in Central China, the δ¹³C values of 17β,21β(H)-norhopane ranged between −28‰ and −40‰ and the more negative values were interpreted to be caused by enhanced methanotrophy. In both studies, the more negative hopanoid δ¹³C values corresponded to dryer conditions, which were explained to be the result of aeration of deeper peat deposits below the rooted zone, enhancing methanotrophy benefitting from an enhanced diffusive methane flux at the oxic-anoxic interface (Huang et al., 2018; Zheng et al., 2014). Extending the geological record, Inglis et al. (2015) observed no change in hopane δ¹³C values in a Paleogene peatland (Schönningen, Germany), although there was a rise in the water table. In contrast, in the Cobham lignite, a Paleoene wetland deposit, negative δ¹³C values, extending to −42‰ and −76‰ for the C₂₅ and C₂₉ hopanes, respectively, were observed (Pancost et al., 2007). In the context of our data from experiments with extant peats, these depleted hopane δ¹³C values in the geological record suggest an intensified methane cycle during these key periods of Earth’s past.

The environmental variability in peat bogs is much higher than represented in our limited set of experiments where we singled out diffusion-dominated waterlogged conditions with S. cuspidatum, all optimal for methanotrophy. Transport pathways largely govern in situ methane concentrations, which complicate the interpretation of the diploptene δ¹³C values as palaeoproxies. In turn, temperature also plays a key role on the transport efficiency of methane. Still, diploptene (or hopane) δ¹³C values of ancient peats would represent a median where the variability is averaged out. The observed relationship of decreasing diploptene δ¹³C values with enhanced methane flux rates (Figure 3) is indirect and not unequivocal, still it indicates that a record of δ¹³C values of diploptene, or its diagenetic products, could be a useful proxy to assess at least relative shifts in past levels of methanotrophy.

4.3 | Sphagnum bulk and compound-specific δ¹³C values as potential proxies for methanotrophy in ancient peat

No significant variation was observed in the δ¹³C values of bulk Sphagnum moss and the C₂₅ n-alkane, the most abundant n-alkane in S. cuspidatum and other Sphagnum sp. (Baas, Pancost, van Geel, & Sinninghe Damsté, 2000; Nott et al., 2000), with incubation temperature (Figure 2c) and, hence, with changes in methane production. This lack of variation in Sphagnum δ¹³C values suggests that Sphagnum does not take up more methane-derived CO₂ relative to
CO$_2$ from other sources at increased levels of methane production and anticipated methanotrophy. This is somewhat surprising since it is thought that *Sphagnum* also uses the CO$_2$ produced by methanotrophs for photosynthesis (Kip et al., 2010; Raghoebarsing et al., 2005). Increased levels of methanotrophy would thus expected to be reflected in a more negative $\delta^{13}C$ signature of both specific lipids produced by *Sphagnum* or even in its bulk value.

Increasing temperatures could have stimulated organic matter degradation; hence, the increased methane flux at higher temperatures may be offset by a corresponding increase in the CO$_2$ flux from enhanced organic matter degradation. The isotopic composition of the combined recycled carbon source for *Sphagnum* would thereby remain the same. In addition, the isotopic fractionation between CO$_2$ and methane during methanogenesis may be balanced, with methane becoming more enriched in $\delta^{13}C$ when more is produced, while CO$_2$ becomes less enriched in $\delta^{13}C$ when less CO$_2$ is turned into methane. Such an inverse relationship between the carbon isotopic values of CO$_2$ and methane has been observed in peat bogs by Hornibrook et al. (2000). This way, changes in the isotopic composition of methane and CO$_2$ would cancel each other out. In any case, the lack of a clear temperature response of the stable carbon isotope values in both bulk *Sphagnum* and *Sphagnum*-derived biomarkers implies that reconstruction of methane oxidation and methane (re)cycling in peat bogs will have to rely on biomarkers specific for methanotrophs, such as $\delta^{13}C$ values of hopanes and specific BHPs.

## 5 | CONCLUSIONS

A field pulse-chase experiment using $^{13}C$-labelled methane clearly demonstrated $^{13}C$ incorporation into diploptene and hence support in situ methanotrophic activity in *Sphagnum* under natural conditions. Mesocosm experiments with *Sphagnum* peat cores incubated at different temperatures resulted in large variations in the $\delta^{13}C$ values of diploptene extracted from *Sphagnum*, with values of −34‰ at 5°C and −41‰ at 25°C.

The diploptene $\delta^{13}C$ values showed a strong correlation with temperature and methane production, while concentrations of diploptene did not vary significantly. The diploptene $\delta^{13}C$ values can be explained by elevated methane availability at higher temperatures, resulting in enhanced expression of the enzymatic isotope effect. The $\delta^{13}C$ values of bulk *Sphagnum* or *Sphagnum*-derived C$_{23}$ n-alkane did not show any significant variation with temperature. The lack of any trend implies that reconstruction of methane oxidation and methane (re)cycling in peat bogs will have to rely on biomarkers specific for methanotrophs, such as $\delta^{13}C$ values of hopanes and specific BHPs. Methanotroph-specific BHPs, aminotetrol and aminopentol, showed a non-linear response to temperature. Aminotetrol was more abundant compared to aminopentol, which indicates that type II methanotrophs became prevalent during the experiment. Relative BHP concentrations may therefore provide insight into the presence of different types of methanotrophs in ancient peats. Finally, the $\delta^{13}C$ values of diploptene, or its diagenetic products, potentially provide a useful tool to assess methanotrophic activity in past environments.

## ACKNOWLEDGMENTS

The associate editor, David Naafs, and 3 anonymous reviewers are thanked for constructive comments on earlier versions of this paper. We acknowledge Michiel Kienhuis, Jort Ossebaar and Stefan Schouten, NIOZ Texel, for technical assistance with GC-IRMS measurements. We thank Rieke Wagner, Utrecht University, for use of the peat coring equipment and Thony van der Gon Netscher for making instruments for field experiments. This research was funded by a grant of the Darwin Center for Biogeosciences to GJR and JSSD. H.M.T. thanks the Science Research Infrastructure Fund from HEFCE for funding the Thermo Finnigan LCQ ion trap mass spectrometer. N.P.M. was funded by the Natural Environment Research Council award number NE/R016429/1 as part of the UK-SCAPE programme delivering National Capability. We acknowledge the UK-SCAPE Flux tower network. JSSD received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement no. 694569—MICROLIPIDS). This work was carried out under the programme of the Netherlands Earth System Science Centre (NESSC) and financially supported by the Dutch Ministry of Education, Culture and Science (OCW).

## CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interests.

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How to cite this article: van Winden JF, Talbot HM, Reichart G-J, McNamara NP, Benthiem A, Sinninghe Damsté JS. Influence of temperature on the $\delta^{13}C$ values and distribution of methanotroph-related hopanoids in *Sphagnum*-dominated peat bogs. *Geobiology*. 2020;18:497–507. https://doi.org/10.1111/gbi.12389