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Distinct Anaerobic Bacterial Consumers of Cellobiose-Derived Carbon in Boreal Fens with Different CO_2/CH_4 Production Ratios

Heli Juottonen,[a,b,c]* Alexander Eiler,[b] Christina Biasi,[d] Eeva-Stiina Tuittila,[e] Kim Yrjälä,[a] Hannu Fritze[c]

Department of Biosciences, General Microbiology, University of Helsinki, Helsinki, Finland[a]; Department of Ecology and Genetics, Limnology, Uppsala University, Uppsala, Sweden[b]; Natural Resources Institute Finland, Helsinki, Finland[c]; Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland[d]; School of Forest Sciences, University of Eastern Finland, Joensuu, Finland[e]

ABSTRACT Northern peatlands in general have high methane (CH_4) emissions, but individual peatlands show considerable variation as CH_4 sources. Particularly in nutrient-poor peatlands, CH_4 production can be low and exceeded by carbon dioxide (CO_2) production from unresolved anaerobic processes. To clarify the role anaerobic bacterial degraders play in this variation, we compared consumers of cellobiose-derived carbon in two fens differing in nutrient status and the ratio of CO_2 to CH_4 produced. After [^{13}C]cellobiose amendment, the mesotrophic fen produced equal amounts of CH_4 and CO_2. The oligotrophic fen had lower CH_4 production but produced 3 to 59 times more CO_2 than CH_4. RNA stable-isotope probing revealed that in the mesotrophic fen with higher CH_4 production, cellobiose-derived carbon was mainly assimilated by various recognized fermenters of *Firmicutes* and by *Proteobacteria*. The oligotrophic peat with excess CO_2 production revealed a wider variety of cellobiose-C consumers, including *Firmicutes* and *Proteobacteria*, but also more unconventional degraders, such as *Telmatobacter*-related *Acidobacteria* and subphylum 3 of *Verrucomicrobia*. Prominent and potentially fermentative *Planctomycetes* and *Chloroflexi* did not appear to process cellobiose-C. Our results show that anaerobic degradation resulting in different levels of CH_4 production can involve distinct sets of bacterial degraders. By distinguishing cellobiose degraders from the total community, this study contributes to defining anaerobic bacteria that process cellulose-derived carbon in peat. Several of the identified degraders, particularly fermenters and potential Fe(III) or humic substance reducers in the oligotrophic peat, represent promising candidates for resolving the origin of excess CO_2 production in peatlands.

IMPORTANCE Peatlands are major sources of the greenhouse gas methane (CH_4), yet in many peatlands, CO_2 production from unresolved anaerobic processes exceeds CH_4 production. Anaerobic degradation produces the precursors of CH_4 production but also represents competing processes. We show that anaerobic degradation leading to high or low CH_4 production involved distinct sets of bacteria. Well-known fermenters dominated in a peatland with high CH_4 production, while novel and unconventional degraders could be identified in a site where CO_2 production greatly exceeds CH_4 production. Our results help identify and assign functions to uncharacterized bacteria that promote or inhibit CH_4 production and reveal bacteria potentially producing the excess CO_2 in acidic peat. This study contributes to understanding the microbiological basis for different levels of CH_4 emission from peatlands.

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Address correspondence to Heli Juottonen, heli.juottonen@alumni.helsinki.fi.

* Present address: Heli Juottonen, Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland.

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Methane (CH_4) emission rates from peatlands, which together with other wetlands form the largest natural source of CH_4 (1), vary considerably with water table level, temperature, vegetation, and peatland type (2, 3). These factors have been shown to affect the activity and community composition of CH_4-producing archaea and in some cases the pathway of CH_4 production (4–11). It is much less clear how the environmental variation of peatland ecosystems influences the anaerobic bacterial degraders, which produce the precursors of methanogenesis and, on the other hand, compete with methanogens for substrates.

Peat is partially decomposed plant material, the main components being cellulose, hemicellulose, and recalcitrant compounds, such as lignin and humic substances (12). In anoxic peat below the water level, decomposition of plant-derived polysaccharides requires a cooperation of functional microbial groups: primary fermenters hydrolyzing polymers and fermenting the monomers, such as sugars, secondary fermenters turning the resulting organic acids into the methanogenic substrates acetate and H_2 plus CO_2, and finally, methanogens producing CH_4 (13). Polysaccharide hydrolysis, and thus the activity of nonmethanogenic anaerobic bacteria, has been proposed to be the rate-limiting step for wetland CH_4 production (5, 14–16).

The complete anaerobic degradation of carbohydrates under methanogenic conditions, when both acetate and H_2 plus CO_2 are precursors of methanogenesis, should theoretically produce equal amounts of CH_4 and CO_2 (17). In contrast, a large excess of anaerobic CO_2 production is often observed in peat, particularly in low-pH and nutrient-poor peatlands (18–22). These sites in particular, but northern peatlands in general, have low levels of Fe(III), nitrate, and sulfate, which are considered to limit CO_2 production resulting from the degradation of organic matter with the reduction of these inorganic electron acceptors (18, 19). Small sulfate pools in peat may have unexpectedly high turnover rates (23, 24), but currently, the main proposed sources for the excess CO_2 in nutrient-poor peat are fermentation or anaerobic respiration with phenolic and quinone-containing compounds of organic residues, commonly referred to as humic substances, as electron acceptors (13, 19, 21, 25). Identification of the anaerobic bacterial degraders active in peatlands with contrasting CH_4 versus CO_2 production could provide insights into the processes producing the excess CO_2.

The current information on peat microbes involved in the intermediary anaerobic processes, leading to methanogenesis or excess CO_2 production, is still restricted to relatively few studies and sites. Work on a temperate spruce fen has identified cellulose, glucose, and xylose fermenters, including *Bacteroidetes*, *Acidobacteria*, *Firmicutes*, *Spirochaeta*, *Actinobacteria*, and *Proteobacteria* (26–30). Metagenomic studies in a *Sphagnum* bog and in Arctic peat have indicated *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Deltaproteobacteria* as potential fermenters (14, 31, 32). A comparison of eutrophic to oligotrophic temperate peatlands found indications of functional redundancy, with similar anaerobic processes being carried out by different communities (33). This raises the question on whether and how the community composition of the anaerobic bacterial degraders relates to the eventual CH_4 production of a peatland.

We set out to compare the active anaerobic bacterial degraders in two boreal peatlands differing as CH_4 sources. These adjacent *Sphagnum* fens are rather similar in vegetation composition and pH but differ in nutrient status and, more importantly, in the pathway of organic matter degradation and the ratio of CH_4 to CO_2 produced (21). The mesotrophic fen has higher CH_4 production, higher contribution of the acetoclastic pathway of CH_4 production, and a methanogen community distinct from that of the oligotrophic fen (8, 21, 34). We targeted consumers of cellobiose-derived carbon, representing bacteria processing labile carbon from cellulose degradation, by incubating peat with [^{13}C]cellobiose and following the label in the produced CH_4 and CO_2 and in RNA by stable-isotope probing (SIP). Our aim was to (i) identify cellobiose consumers

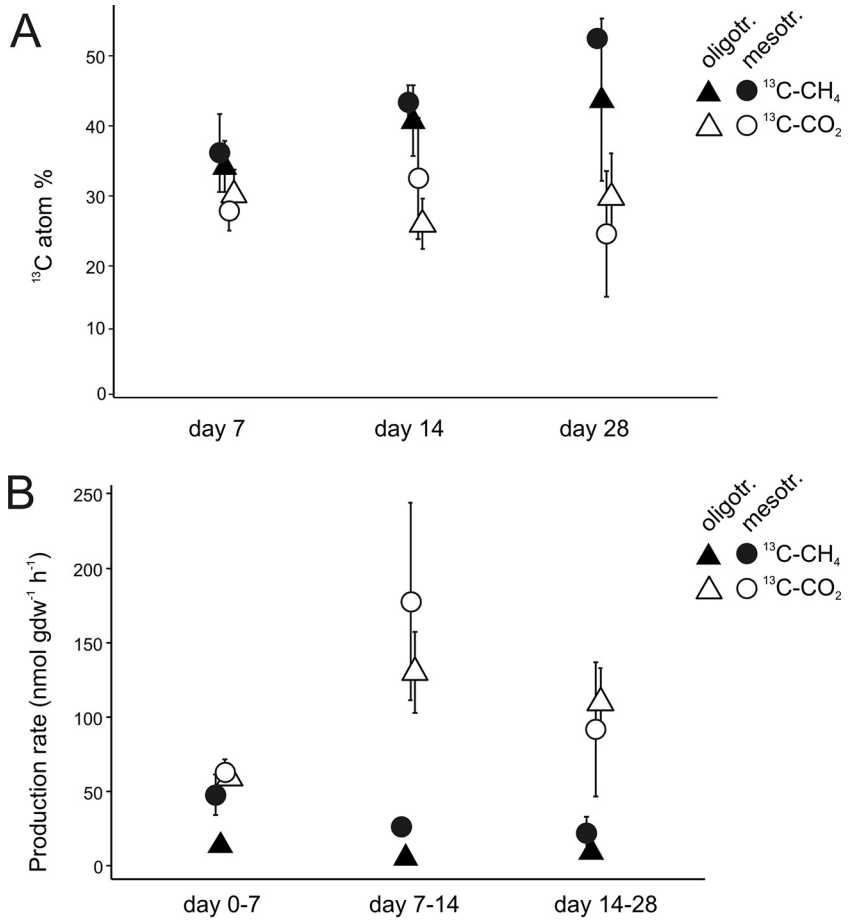

FIG 1 (A and B) ^{13}C enrichment of CH_4 and CO_2 (A) and $^{13}CH_4$ and $^{13}CO_2$ production rates (B) during labeling experiment with [^{13}C]cellobiose in oligotrophic (oligotr.) and mesotrophic (mesotr.) peat at three time points ($n = 3$, mean ± SD).

and fermenters in boreal *Sphagnum* fens and (ii) compare the active anaerobic degraders in two fens differing as CH_4 sources. By selecting outwardly similar but functionally contrasting sites, we aimed to differentiate between degraders active under high CH_4 production and under lower CH_4 production when CO_2 is the main gaseous end product.

RESULTS

Methane and anaerobic CO_2 production. Potential CH_4 production in initial measurements before cellobiose addition was higher in peat from the 20- to 30-cm layer than in the 10- to 20-cm layer (data not shown). The depth of 20 to 30 cm was therefore chosen for the labeling experiment to represent the methanogenic peat layer. Before cellobiose amendment, the mesotrophic fen showed higher CH_4 production (mean ± standard deviation [SD], 36 ± 18 nmol · g [dry weight] [gdw]$^{-1}$ · h^{-1}) than the oligotrophic fen (1.2 ± 0.8 nmol · gdw^{-1} · h^{-1}; *t* test, $P = 0.003$). The rates of anaerobic CO_2 production did not significantly differ between mesotrophic (116 ± 72 nmol · gdw^{-1} · h^{-1}) and oligotrophic peat (61 ± 10 nmol · gdw^{1} · h^{-1}, *t* test, $P = 0.32$). Rates of CO_2 production of the original unamended peat were on average 3 times higher than CH_4 production in the mesotrophic fen and 50 times higher in the oligotrophic fen.

After the addition of [^{13}C]cellobiose, ^{13}C enrichment of CH_4 demonstrated consumption of cellobiose with methanogenesis as a terminal process (Fig. 1A). The ^{13}C enrichment of CH_4 increased from days 7 to 28, whereas the enrichment of CO_2 remained fairly stable over time. The level of enrichment did not vary with peat type

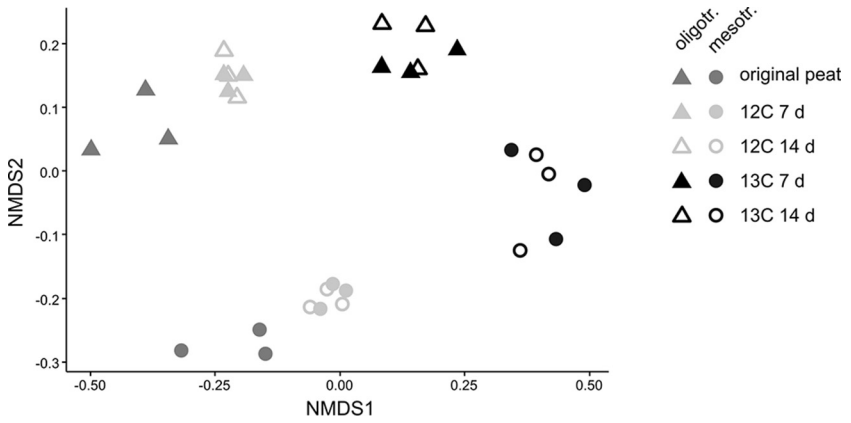

FIG 2 Nonmetric multidimensional scaling (NMDS) plot of bacterial community based on 16S rRNA gene sequencing in original peat and in the heavy (^{13}C) and light (^{12}C) stable-isotope fractions of oligotrophic and mesotrophic peat. Stress = 0.084.

(Fig. 1A). The mesotrophic peat had consistently higher $^{13}CH_4$ production, i.e., CH_4 originating from labeled cellobiose, than the oligotrophic peat ($P = 0.0001$, Fig. 1B). Methane production was highest in the first week and then decreased in both peat types. Anaerobic $^{13}CO_2$ production did not differ with peat type ($P = 0.84$, Fig. 1B). In both peat types, CO_2 production increased after the first week and was highest in the second week. Accordingly, in the first week, the ratio of $^{13}CO_2$ to $^{13}CH_4$ production was 1.4 ± 0.3 in the mesotrophic peat and 5 ± 2 in the oligotrophic peat, but in the second week, the ratio increased to 7 ± 5 in the mesotrophic peat and 32 ± 24 in the oligotrophic peat. At weeks 3 and 4, the ratio decreased to 14 ± 6 in the oligotrophic peat and 5 ± 2 in the mesotrophic peat, still being higher than in the first week.

Bacterial communities. The bacterial community of the original peat differed between the oligotrophic and mesotrophic fen (Fig. 2; permutational multivariate analysis of variance [PERMANOVA] $R^2 = 0.48$, $P = 0.001$). The most common bacterial phyla in the oligotrophic fen were *Parcubacteria* (Candidate division OD1, 15.4% ± 2.2% of reads), *Planctomycetes* (13.2% ± 3.6% of reads), and *Acidobacteria* (10.2 ± 2.0% of reads) (see Table S2 in the supplemental material). In the mesotrophic fen, the groups with the highest relative abundance were *Deltaproteobacteria* (17.6% ± 5.9% of reads), *Chloroflexi* (10.9% ± 4.6% of reads), and *Acidobacteria* (10.8% ± 4.2% of reads).

After incubation with [^{13}C]cellobiose and separation of RNA into heavy (^{13}C-enriched) and light (^{12}C) fractions, operational taxonomic units (OTUs) differed between the fractions (PERMANOVA $R^2 = 0.34$, $P = 0.001$) and between the oligotrophic and mesotrophic peat (PERMANOVA $R^2 = 0.27$, $P = 0.001$) (Fig. 2). Light fractions at day 7 differed from the original peat (PERMANOVA $R^2 = 0.17$, $P = 0.007$). No change was observed between days 7 and 14 in the overall community (PERMANOVA $R^2 = 0.01$, $P = 0.80$) or in the mesotrophic peat despite the clear decrease in CH_4 production (PERMANOVA $R^2 = 0.06$, $P = 0.35$). Unlabeled controls that received [^{12}C]cellobiose, which were used to identify the density gradient fractions containing [^{13}C]RNA and to exclude OTUs migrating to the heavy fraction without ^{13}C incorporation, grouped with the light fractions (data not shown).

Looking at the fold changes of the OTU abundances in the heavy and light fractions revealed that *Firmicutes* and *Betaproteobacteria* were strongly represented in the heavy fractions (Fig. 3 and Table S3). Several groups common in the original peat were primarily detected in the light fractions (*Planctomycetes*, *Deltaproteobacteria*, and *Chloroflexi*) or were very rare in incubated peat (*Parcubacteria*/Candidate division OD1). To identify the most likely cellobiose-C consumers, we looked in more detail for those OTUs that were consistently more abundant in the heavy fractions than the light fractions by using an approach developed for detecting differential gene expression (see Materials and Methods). In the mesotrophic peat, we identified 70 ^{13}C-enriched

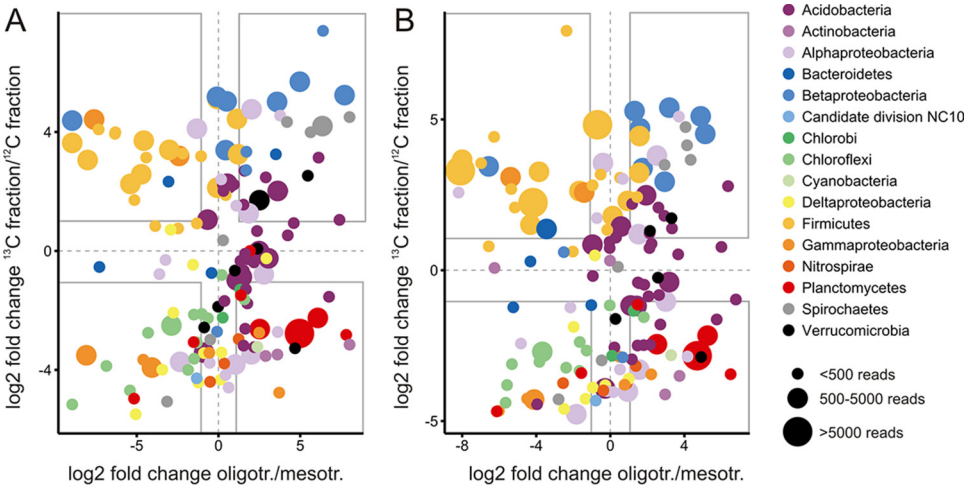

FIG 3 (A and B) Log_2 fold changes of read abundances showing the taxonomic distribution of bacterial operational taxonomic units (OTUs; 150 most abundant) in heavy (^{13}C) and light (^{12}C) fractions at day 7 (A) and day 14 (B). Reads more abundant in mesotrophic peat, *x* axis <0; in oligotrophic peat, *x* axis >0; in ^{13}C-fractions, *y* axis >0; in ^{12}C-fractions, *y* axis <0. Solid gray line separates OTUs $\geq$2-fold-more abundant than in the other peat type or fraction. Fold changes were calculated from means of three replicate incubations using data subsampled to 13,226 reads for each sample. Symbol size is based on the read number of an OTU over all the samples at each time point.

OTUs, 44 of which belonged to *Firmicutes* (Tables 1, S4, and S5). The oligotrophic peat revealed higher number and higher taxonomic variety of ^{13}C-enriched OTUs than the mesotrophic peat, with 118 enriched OTUs, and *Acidobacteria*, *Verrucomicrobia*, *Firmicutes*, *Betaproteobacteria*, and *Alphaproteobacteria* as the largest groups. Eleven OTUs in the oligotrophic peat and six OTUs in the mesotrophic peat became more enriched from days 7 to 14 (Tables 1 and S5), further supporting their ^{13}C labeling.

Firmicutes classified to the families *Clostridiaceae* and *Veillonellaceae* were identified as prominent cellobiose-C consumers in both peat types. In unincubated peat, *Firmicutes* formed only 0.1 to 1.0% of the total community, but by day 7, they had increased to 21 to 69% in the heavy fractions (Table S3). *Clostridiaceae* in the oligotrophic peat, including the two most abundant ^{13}C-enriched OTUs (Otu2 and Otu4), had high sequence similarities to several acid-tolerant and sugar-fermenting *Clostridium* species (Tables S4 and S5). In the mesotrophic peat, the two most abundant *Clostridium* OTUs (Otu1 and Otu3) showed similarities of 99 to 100% to uncultured clostridia from wetland soils (26, 28, 35, 36). Both oligotrophic and mesotrophic peat showed *Veillonellaceae* OTUs similar to genera *Pelosinus*, *Propionispira*, and *Psychrosinus* (Table S4), but several additional *Veillonellaceae* OTUs from the mesotrophic peat had no close matches to described species. Only three enriched *Firmicutes* OTUs were unique to the oligotrophic peat, and they were similar to "*Psychrosinus fermentans*" (Otu110, Otu1663, and Otu5263; Tables S4 and S5). The mesotrophic peat, on the other hand, showed 24 unique enriched *Clostridiaceae* and *Veillonellaceae* OTUs and two additional families, *Lachnospiraceae* and *Ruminococcaceae*. All four *Ruminococcaceae* OTUs (Otu40, Otu64, Otu145, and Otu316) were 94 to 96% similar to *Ethanoligenens harbinense* and 96 to 98% similar to a sequence from acidic peat (HG324862 [28]). The closest matches to *Lachnospiraceae* (Otu119) were gut microbes.

The majority of the ^{13}C-enriched *Betaproteobacteria* OTUs in both peat types were classified as *Neisseriales* and *Rhodocyclales*. Many of these OTUs (Table S4) showed high sequence similarity to two wetland isolates: fermentative *Paludibacterium yongneupense* grew at lower pH than related strains (37) and aerobic *Uliginosibacterium gangwonense* (38).

Enriched *Alphaproteobacteria* OTUs belonged to *Rhodospirillales* and *Rhizobiales*. Both the oligotrophic and the mesotrophic peat revealed several OTUs (Table S4) highly similar to *Telmatospirillum siberiense*, an acidotolerant fermentative peat isolate (39). *Rhizobiales* OTUs were mostly detected as ^{13}C-enriched in the oligotrophic peat, and

TABLE 1 Numbers of bacterial 16S rRNA gene OTUs identified as enriched in the ^{13}C-fractions of [^{13}C]cellobiose-labeled peat from oligotrophic and mesotrophic fens[a]

	No. of enriched OTUs grouped by relative abundance of reads[a]						
	Oligotrophic			Mesotrophic			
Classification of OTUs	>2.5%	>0.25%–2.5%	>0.025%–0.25%	>2.5%	>0.25%–2.5%	>0.025%–0.25%	Shared
Firmicutes							
Clostridiaceae	3	2 (1)	3 (1)	8	7 (1)	2	
Lachnospiraceae					1		
Ruminococcaceae					3	1	13
Veillonellaceae		7 (3)	2 (2)	1	9 (2)	7	
Unknown family					2	2	
Betaproteobacteria							
Burkholderiales		1					
Neisseriales	3 (1)	1	4	2		2	5
Rhodocyclales	3	2	2	2	1	3	
Myxococcales			1				
Alphaproteobacteria							
Rhizobiales	1	3 (1)	3			1	5
Rhodospirillales		4	3	1	3	3	
Deltaproteobacteria							
Anaeromyxobacter			1				0
Gammaproteobacteria							
Tolumonas		1		2			1
Acidobacteria							
Acidobacteriaceae	1	13	4				
Holophagaceae		3 (1)	3		1	1 (1)	2
Group 3		2					
Verrucomicrobia							
Subphylum 3		3	11				
Spartobacteria		1	1				0
Opitutae		2	2				
Spirochaetes							
Spirochaetales		5	5			1	0
Bacteroidetes							
Bacteroidales			2		1 (1)	1 (1)	
Sphingobacteriales						1	0
Other/unknown		2	3				
Candidate division BRC1		1					0
Actinobacteria							
Coriobacteriia		1 (1)					0
Armatimonadetes							
Group 1			2				0
Planctomycetes							
Phycisphaerae			1				0
Chlorobi							
Ignavibacteria			1				0
Melainabacteria						1	0

[a]Numbers in parentheses indicate the number of OTUs that became more ^{13}C-enriched from day 7 to day 14.

most of them were similar to one of two stalked fermentative strains able to use Fe(III) as an electron acceptor: *Rhizomicrobium electricum* (40; Otu761, Otu463, and Otu1222) and *Rhizomicrobium palustre* (41; Otu32).

Only two ^{13}C-enriched *Acidobacteria* OTUs, classified to *Holophagae*, were detected in the mesotrophic peat (Otu63 and Otu2366). In the oligotrophic peat, we identified 26 ^{13}C-enriched *Acidobacteria* OTUs, and 12 of them showed 97 to 99% sequence

similarity to cellulolytic *Telmatobacter bradus* isolated from a bog, with pH 4, and fermenting sugars, including cellobiose (42) (Table S4). The rest of the *Acidobacteria* OTUs belonging to classes *Acidobacteria* and *Holophagae* were highly similar (99 to 100%) to sequences from acidic peat (26, 28, 33, 43, 44), alpine tundra soil (45), or paddy soil (Table S5).

Verrucomicrobia were identified as consumers of cellobiose-derived carbon only in the oligotrophic fen. Fourteen out of 20 OTUs were classified to subphylum 3 (OPB35 soil group) (Table S5). These OTUs showed only ≤91% sequence similarity to the described members of subphylum 3, which all are aerobic (46–48). In addition, OTUs identified as *Spartobacteria* (Otu470 and Otu1065) and *Opitutae* (Otu224, Otu266, Otu364, and Otu613) were detected, both of which include a species fermenting sugars and polysaccharides (49, 50). All *Verrucomicrobia* OTUs were most similar to uncultured *Verrucomicrobia* from various soil, wetland, and freshwater environments.

Bacteroidetes, previously identified as a major fermentative group in peat (28), were not prominent among our ^{13}C-enriched OTUs. However, two relatively minor *Bacteroidetes* OTUs (Otu24 and Otu198) in the mesotrophic peat identified as *Porphyromonadaceae* became more ^{13}C-enriched with time (Tables 1 and S5).

DISCUSSION

We compared consumers of cellobiose-derived carbon in two peat types with similar anaerobic CO_2 production but distinct rates of CH_4 production, suggesting differences in the processes of organic matter degradation. The ratio of CO_2 to CH_4 production of close to 1 in the mesotrophic peat during the first week of the incubation implies that methanogenesis was the dominant terminal process. The considerably higher CO_2/CH_4 ratios in the oligotrophic peat throughout the incubation, ranging from 2 to 59, indicate that carbon was also directed to nonmethanogenic processes. Our CO_2/CH_4 ratios of both original and cellobiose-amended peat were higher than those of Galand et al. (21) for the same sites but support the same pattern of lower contribution of methanogenesis as a terminal process in the oligotrophic peat.

A clear separation of the bacterial community composition with peat type persisted with cellobiose amendment, and the results showed distinct cellobiose-C-consuming taxa with peat type and a higher variety of taxa in the oligotrophic peat. These included *Acidobacteria* and *Verrucomicrobia*, which were present in the mesotrophic peat at relative abundances similar to or higher than in the oligotrophic peat but were not as heavily labeled (Tables S2 and S3), suggesting they did not assimilate cellobiose-derived carbon to the same extent as in the oligotrophic peat. *Verrucomicrobia* have been associated with plant polymer degradation in soils, including anoxic peat (32, 51), and in the case of *Opitutaceae* with glucose fermentation in peat (33). Subphylum 3, the most abundant verrucomicrobial group detected here as cellobiose-C consumers, occurs in soil and peat (52–54) but contains no previously reported fermenters. According to recent metagenomic data, some members of this group possess genes for polysaccharide hydrolysis (55). Our results show that subphylum 3 contains members able to assimilate cellobiose carbon under anoxic oligotrophic conditions, extending the physiologies for subphylum 3 and revealing potential novel peatland fermenters.

Acidobacteria are common in anoxic peat and more prominent with lower pH and oligotrophy (4, 33, 34, 56–59). Accordingly, they were more prominent in processing cellobiose-C in the oligotrophic peat than in the mesotrophic peat. *Acidobacteria* closely related to *Telmatobacter bradus*, detected as ^{13}C-enriched exclusively in the oligotrophic peat, are emerging as important anaerobic and aerobic degraders of cellulose-derived carbon in peatlands with pH <5 (28, 60). Additionally, other *Acidobacteriaceae* and *Holophagaceae* appeared to participate in anaerobic carbon processing, but the roles of these taxa are more difficult to determine. Known *Holophagaceae* have diverse physiologies, but members of this group also assimilated ^{13}C from cellulose in acidic peat (28).

Firmicutes assimilating ^{13}C were particularly prominent and varied in the mesotrophic peat, and those found in the oligotrophic peat were mostly a subset of this larger

variety. Nutrient status has been previously shown to affect the distribution of *Clostridia* in a freshwater marsh between eutrophic and oligotrophic soils (35). The two *Firmicutes* families detected as ^{13}C-enriched only in the mesotrophic peat, *Ruminococcaceae* and *Lachnospiraceae*, are best known to inhabit the digestive tracts of mammals (61). Interestingly, the occurrence of *Ruminococcaceae* and *Lachnospiraceae* in sheep rumen was associated with high CH_4 emission (62), fitting their detection in the mesotrophic peat with higher CH_4 production. *Ruminococcaceae* have been indicated as fermenters in peat more acidic than our sites (28, 33), suggesting that the lack of detection in the oligotrophic peat may not be related to the slightly lower pH. *Lachnospiraceae* have been detected in the degradation of rice straw (63) but not reported as peatland fermenters previously. Both *Lachnospiraceae* and *Ruminococcaceae* have, however, been suggested to be well suited for plant material degradation (64).

Our ^{13}C-enriched *Firmicutes* OTUs closely matched sequences from other peatlands and acid-tolerant wetland isolates. These included *Clostridiaceae*, which are well-known saccharolytic and cellulolytic fermenters and have been suggested to represent important fermenters in acidic peat (26, 28, 33, 36). Another major group of *Firmicutes* was *Veillonellaceae*, which have Gram-negative cell walls and have so far been rarely detected in peat or soil habitats, although they have been actively involved in rice straw degradation (63) and detected as minor peat taxa (33, 66). *Veillonellaceae* may play a role as propionate-producing fermenters in peat: several of our OTUs were similar to propionate-producing genera *Pelosinus*, *Psychrosinus*, and *Propionispira* (Table S4), and *Propionispira*-related glucose fermenters were previously detected in acidic peat under propionate-producing conditions (26). *Veillonellaceae* also include homoacetogens producing acetate from H_2 and CO_2, but none of our OTUs were similar to known acetogenic strains.

The large amount of $^{13}CO_2$ detected throughout the incubation in the oligotrophic peat could be the result of incomplete degradation or degraders using humic substances (HS) or inorganic compounds as electron acceptors. Incomplete degradation, where electron donors and acceptors are organic substances and which results in the build-up of fermentation products instead of CH_4 production, is one of the main proposed sources for the excess CO_2 production in peat (13, 21). The various potentially fermentative groups that assimilated ^{13}C support incomplete degradation as a relevant source. The role of HS reduction (67) is more difficult to evaluate, as HS reducers are poorly known and include Fe(III)-reducing, sulfate-reducing, and fermentative bacteria (68–71). The labeled taxa in the oligotrophic peat included *Holophagaceae* and *Anaeromyxobacter* (*Deltaproteobacteria*) with members able to reduce HS analogue anthraquinone-2,6-disulfonate (AQDS) (72). None of the labeled taxa resembled known sulfate reducers but instead contained several potential Fe(III) reducers: *Rhizomicrobium*, *Clostridia* related to *Clostridium saccharobutylicum*, and *Acidobacteria* of groups 1 and 3 (73–75). Fermentative Fe(III) reducers have been shown to occur in an acidic fen (76). The putative Fe(III) reducers described above could also be speculated to reduce HS, although most have not been tested for it, and a group 3 acidobacterium, *Paludibaculum fermentans*, does not reduce AQDS (77). Whether the groups discussed above are involved in HS or Fe(III) reduction and responsible for the excess CO_2 production should be addressed in future studies. Other $^{13}CO_2$ sources that cannot be ruled out are $^{13}CO_2$ production by microbes not assimilating ^{13}C, production by organisms other than bacteria such as nonmethanogenic archaea, or anaerobic CH_4 oxidation (78). Despite attempts, anaerobic CH_4 oxidation has not been conclusively verified in this site (K. Peltoniemi, personal communication).

In the mesotrophic peat, CH_4 production decreased, and CO_2 production increased drastically after the first week, but we observed no shift in the bacterial community. The additional $^{13}CO_2$ was therefore possibly produced by *Firmicutes* and *Proteobacteria* that assimilated ^{13}C under methanogenic conditions and remained active under lower CH_4 production. The decrease in CH_4 production may be due to the inhibition of methanogenic activity by the accumulation of fermentation products, such as organic acids, as has been observed in other peat incubations (28, 79). The incubated mesotrophic

peat revealed notable amounts of deltaproteobacterial Fe(III) and sulfate reducers, such as *Geobacteraceae* (2 to 4% versus <1% in oligotrophic peat), *Syntrophaceae* (3 to 5% versus 1 to 3%), *Syntrophobacteraceae* (0.9 to 1.3% versus 0.4 to 0.7%), and *Desulfobacteraceae* (0.1 to 0.5% versus <0.1%). These taxa were, however, not enriched in the ^{13}C-fractions and thus were most likely not producing the additional $^{13}CO_2$ from [^{13}C]cellobiose, but they may be contributing to CO_2 production overall from endogenous substrates.

To avoid known sources of bias in SIP experiments, we added the ^{13}C-substrate as repeated smaller pulses instead of a large single pulse and used RNA-SIP instead of DNA-SIP to allow shorter incubation times. To exclude OTUs enriched in the heavy fraction for reasons other than ^{13}C labeling, such as high GC percentage, we sequenced the fractions corresponding to the heavy fractions in the unlabeled samples and removed such OTUs. Another source of bias, cross-feeding, should have been reduced, as the incubation flasks were flushed during the weekly sampling. Despite removal of the gases, cross-feeding could still take place via organic acids. Therefore, the detected labeled taxa may include both primary fermenters, using cellobiose and glucose, and secondary fermenters consuming the products of primary fermenters, such as organic acids. Because cellobiose-C consumers were identified based on enrichment in the heavy fractions compared to the light fractions, our analysis focuses on the most strongly ^{13}C-labeled taxa. Therefore, the analysis would miss OTUs assimilating both ^{13}C carbon and endogenous unlabeled carbon, thus appearing in the heavy fractions but in addition strongly or predominantly in the light fractions.

Several potentially fermentative groups abundant in the original peat were not labeled with ^{13}C from cellobiose. *Planctomycetes*, largely related to *Isosphaera*, became the most abundant group in oligotrophic peat with cellobiose amendment, but only one rare OTU was identified as ^{13}C-enriched. *Planctomycetes* have been described as aerobic rarely fermentative carbohydrate degraders in soil and peat (55, 80, 81) and proposed to be glucose fermenters in peat (33). Our results match those of Schmidt et al. (28), where *Planctomycetes* were not labeled from [^{13}C]cellulose. Similarly, *Chloroflexi* classified as *Anaerolineae* and *Caldilineae*, which consist of filamentous anaerobes that ferment sugars and were enriched with glucose in peat (33, 82), were mostly detected in the light fractions of the mesotrophic peat. Strong occurrence in the light fractions suggests that *Planctomycetes* and *Chloroflexi* were active but largely not assimilating cellobiose-derived carbon. *Parcubacteria* (Candidate division OD1), which have been proposed to be fermentative (83, 84), were abundant in the original oligotrophic peat but were rare and unlabeled after the incubation. Members of this group were suggested to be symbiotic (85), which could explain why they thrive in natural but not in incubated peat.

To conclude, we showed that anaerobic degradation in peat, exemplified by cellobiose-C consumers, can involve a clearly distinct set of bacteria depending on the amount of CH_4 produced. With higher CH_4 production in the mesotrophic peat, cellobiose-derived carbon was mainly processed by well-known fermenters within *Firmicutes* and by *Proteobacteria*. When CO_2 production by far exceeded CH_4 production in the oligotrophic peat, more unconventional degraders or fermenters, such as *Telmatobacter*-related *Acidobacteria* and subphylum 3 of *Verrucomicrobia*, were prevalent. The groups we detected as being ^{13}C-labeled in the oligotrophic peat, particularly those with known potential for Fe(III) or HS reduction, such as *Acidobacteria*, *Holophagaceae*, *Deltaproteobacteria*, *Rhizobiales*, and *Clostridiaceae*, should be further addressed in future studies to resolve the processes and electron acceptors behind the anaerobic CO_2 production in acidic peatlands. By differentiating anaerobic bacteria active in cellobiose processing from the rest of the community, this study contributes to assigning functions to uncharacterized bacteria in peat and to understanding the microbiological basis of differing levels of CH_4 production in peatlands.

MATERIALS AND METHODS

Study site and sampling. We sampled an oligotrophic fen and a mesotrophic fen within the Lakkasuo mire complex in southern Finland (61°47′N, 24°18′E) (86). The sampling sites are located within

500 m of each other. Both fens are minerotrophic (i.e., in contact with groundwater), with the mesotrophic fen being more nutrient-rich than the oligotrophic fen (86). Vegetation at the oligotrophic site consists mainly of sedge *Carex lasiocarpa* and mosses *Sphagnum papillosum* and *Sphagnum fallax*. The main species at the mesotrophic site are sedge *C. lasiocarpa* and mosses *Sphagnum flexuosum* and *S. fallax*. Peat at the sampled depths consists of partially degraded plant remains with no observable mineral soil. The water table depth at the time of sampling in November 2011 was 15 ± 2 cm below peat surface at the oligotrophic site and 4 ± 2 cm below surface at the mesotrophic site. Peat pH was 4.8 ± 0.1 at the oligotrophic site and 5.1 ± 0.1 at the mesotrophic site.

Three replicate peat cores were collected from each site by cutting out a 10 cm by 10 cm core with a knife to the depth of 30 cm from peat surface. Peat cores were stored overnight at 4°C and divided into two sections, 10 to 20 cm and 20 to 30 cm from peat surface. A portion of the sections was frozen for RNA analysis of the initial peat. For initial measurements of CH_4 and CO_2 production potential and for further incubation for the labeling experiment, 15 ml of peat was placed in 120-ml flasks with 30 ml of H_2O previously flushed with N_2 to remove oxygen. Each sample was bottled in duplicate. Flushing with N_2 was repeated, and the flasks were closed with rubber septa and incubated in dark at 15°C. Methane and CO_2 production were followed by gas chromatography (87) with four samplings of the headspace gas during 191 h. Production rates include gaseous and dissolved gases and are given per grams (dry weight) (gdw). The ratio of peat dry weight to peat wet weight was 0.099 ± 0.007.

Incubation and sampling for stable isotope analysis. Incubation for the labeling experiment started with addition of uniformly labeled [^{13}C]cellobiose (Omicron Biomedicals) to one of each pair of flasks in the initial measurements for the 20- to 30-cm layer, when peat had been in the flasks for 10 days. Before the addition, the flasks were flushed with N_2 to remove accumulated gases. The flasks received 0.5 ml of 18 mM cellobiose to a final concentration of 0.2 mM injected through the septum. The other flask of each pair received the same amount of [^{12}C]cellobiose as a control for ^{13}C enrichment. The flasks were shaken by hand and incubated in dark at 15°C. The cellobiose addition was repeated 12 times during 25 days (every second or third day). Altogether, each flask received 118 μmol cellobiose and 1.42 mmol ^{13}C, yielding 94 μmol ^{13}C (or ^{12}C for controls) per g (fresh weight) of peat. During the pulse labeling, CH_4 and CO_2 production was followed with three measurements per week. The flasks were sampled for analysis of $^{13}CH_4$, $^{13}CO_2$, and peat at 7, 14, and 28 days. Headspace gas was sampled through the septum and injected into N_2-flushed 12-ml vials (Labco). After gas sampling, the flasks were opened under N_2 flow, and 2× 1.5 ml of peat slurry was removed and frozen at −80°C for RNA extraction for SIP.

Isotope analysis of CO_2 and CH_4. The $^{13}C/^{12}C$ ratio of CO_2 and CH_4 was analyzed from the 12-ml vials described above. First, 1 ml was injected into a second preevacuated and N_2-flushed 12-ml vial, which was analyzed for $^{13}C/^{12}C$ ratio in CO_2. The sample was injected into a Trace gas chromatograph (GC) with a PreCon Interface connected with a continuous-flow isotope-ratio mass spectrometer (IRMS) (Finnigan DELTAplus XP; Thermo, Bremen, Germany). Separation of CO_2 from N_2O and other gases was performed with a PoraPLOT Q column (27.5 m length, 0.32 mm inside diameter [i.d.]; Varian) at 25°C using He as a carrier gas. Laboratory standards were prepared using CO_2 gas diluted in N_2 and calibrated against the International Atomic Energy Agency (IAEA; Vienna, Austria) CH-6 reference material via elemental analysis IRMS (EA-IRMS). The remaining gas in the original 12-ml vials was then analyzed for $^{13}C/^{12}C$ ratio of CH_4. The same protocol was used as described above, with the exception that an additional manual cold trap was cooled by liquid nitrogen in the PreCon unit to purge the sample gas of O_2 and N_2 while trapping CH_4 in the loop. Additionally, CH_4 was oxidized to CO_2 by reaction with nickel oxide at 1,000°C. The precision (one standard deviation) of standard gas for 10 consecutive measurements is ~0.1‰. Isotope ratios are reported in terms of ^{13}C atom (at)% values, where ^{13}C at% = $^{13}C/(^{12}C + ^{13}C) \times 100$. Natural abundance of ^{13}C was taken into account by analysis of [^{12}C]cellobiose-incubated samples as controls. The ^{13}C at% values were used to calculate the production of $^{13}CH_4$ and $^{13}CO_2$ based on the overall production of CH_4 and CO_2 (Table S1).

RNA extraction. The method of RNA extraction was modified from previously published methods (88–90). Frozen peat (0.5 g [wet weight]) was added to two 2-ml tubes containing 0.5 cm of quartz sand. Both tubes received 350 μl of lysis buffer (2% cetyl trimethylammonium bromide [CTAB], 2% polyvinyl pyrrolidone [PVP], 100 mM Tris-HCl [pH 8.0], 25 mM EDTA, 2.0 M NaCl, 0.5 g · liter^{-1} spermidine, 2% β-mercaptoethanol) (91) and 350 μl of phenol (pH 8). Cell lysis was carried out in FastPrep (Qbiogene, Illkirch, France) with setting of 5.5 m · s^{-1} for 30 s. After centrifugation at 20,800 × *g* and 4°C for 5 min, the supernatants from both tubes were combined, and 700 μl of phenol-chloroform-isoamyl alcohol (50:49:1) was added. Samples were mixed by inverting the tube repeatedly and centrifuging as described above for 3 min. Chloroform-isoamyl alcohol (700 μl, 24:1) was added to the supernatant. After mixing and centrifugation, the supernatant was passed through a polyvinylpolypyrrolidone (PVPP) column by centrifugation at 1,300 × *g* for 3 min. The flowthrough was precipitated with 1 volume of 20% polyethylene glycol in 2.5 M NaCl and 20 μg of glycogen (RNA grade; Thermo Scientific) on ice for 1.5 h. After centrifugation at 20,800 × *g* and 4°C for 30 min, the pellet was washed with 70% ethanol and dissolved in 50 μl of diethylpyrocarbonate (DEPC)-treated H_2O. Coextracted DNA was removed by treating RNA with DNase I (Thermo Scientific). The concentration of RNA was determined with the Qubit RNA high-sensitivity (HS) assay kit and a Qubit fluorometer (Invitrogen).

Density gradient centrifugation of RNA. Separation of ^{13}C-labeled RNA from [^{12}C]RNA was carried out by density gradient centrifugation in cesium trifluoroacetate (CsTFA) gradients (92) for RNA from days 7 and 14. Gradient medium was prepared by mixing 4.8 ml of CsTFA (2.0 mg · ml^{-1}; GE Healthcare), 3.4% formamide, and 938 μl of gradient buffer (0.1 M Tris-HCl, 0.1 M KCl, 1 mM EDTA) for a final volume of 6 ml. The density of CsTFA and the final gradient medium were checked before use by measuring the refractory index. Gradient medium and RNA sample (500 ng of RNA in a volume of 54.5 μl) were loaded

into 6-ml polyallomer crimp tubes (Sorvall) and centrifuged at 129,000 × *g* for 65 h in a Discovery 100 centrifuge with a TV-1665 rotor (Sorvall). Each centrifugation included two control tubes without RNA or with RNA from a cultivated strain for determining the density of fractions. After centrifugation, gradients were fractionated into 14 fractions of 400 μl by pumping water to the top of the tube using a Gilson Minipuls 3 (speed 3, ca. 0.5 ml · min^{-1}) and collecting the fractions from the bottom. Densities of the fractions from the control tubes were measured by a refractometer and by weighing the fractions.

RNA in the fractions was precipitated with isopropanol and 20 μg of glycogen at −20°C overnight, centrifuged at 20,800 × *g* and 4°C for 30 min, washed with 70% ethanol, and dissolved in 15 μl of DEPC-treated H_2O. The concentration of RNA was determined with the Qubit RNA HS assay kit and Qubit fluorometer (Invitrogen). Fractions containing ^{13}C-labeled RNA and [^{12}C]RNA were identified by comparing RNA concentrations in the density profiles of [^{13}C]cellobiose- and [^{12}C]cellobiose-incubated samples (Fig. S1). Two to three fractions were pooled to form the final heavy (^{13}C-enriched) and light (^{12}C) RNA fractions of each sample.

Reverse transcription and 16S rRNA gene amplicon sequencing. The heavy and light RNA fractions and RNA from initial peat (4-μl aliquots) were reverse transcribed with Maxima H Minus reverse transcriptase (200 U; Thermo Scientific) and random hexamers (Thermo Scientific), according to the manufacturer's instructions.

Bacterial 16S rRNA gene PCR for reverse transcription products was carried out as a two-step PCR, where the first-step primers (341f and 805r) (93) contained adapters for introducing Illumina adapters and dual barcodes in the second step. The first-step PCR primers were thus (adapter sequence, followed by primer sequence in bold) adapter-341f (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN**CCTACGGGNGGCWGCAG**-3′) and adapter-805r (5′-AGACGTGTGCTCTTCCGATCT**GACTACHVGGGTATCTAATCC**-3′). The first-step PCR was carried out in duplicate in 20-μl reaction mixtures containing 1× Q5 reaction buffer, 0.2 mM dinucleoside triphosphates (dNTPs), 0.5 μM primers, 0.4 U of Q5 high-fidelity DNA polymerase (New England BioLabs), and 1 μl of reverse transcription product (diluted if necessary) as the template. Cycling conditions were 98°C for 1 min, followed by 15 to 20 cycles of 98°C for 10 s, 62°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 2 min. The duplicate products were pooled and purified with the Agencourt AMPure XP purification system (Beckman Coulter). The second PCR step with barcoded primers (forward, AATGATACGGCGACCACCGAGATCTACAC-[index]-ACACTCTTTCCCTACACGACG; reverse, CAAGCAGAAGACGGCATACGAGAT-[index]-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT), which bind to the first-step adapters and incorporate Illumina adapters, was carried out using 1 μl of the first product (dilution 1:1 to 1:50 depending on concentration) as the template with 0.1 μM primers, annealing temperature of 66°C, and 12 to 17 cycles. Products were purified as described above and quantified with the PicoGreen kit (Life Technologies). Products were sequenced at the SciLifeLab SNP/SEQ sequencing facility at Uppsala University, Uppsala, Sweden, on an Illumina MiSeq (2 × 300 bp).

Sequence analysis. After raw amplicon sequencing data were demultiplexed into individual samples, sequence pairs were assembled using an in-house bioinformatic pipeline (94). The pipeline further removed sequences with missing primers and unassigned base pairs. The quality filtering removed 17% of reads, leaving 2.16 million reads of 400 to 430 bp and, on average, 41,379 reads per sample. The quality-filtered assembled reads were clustered into operational taxonomical units (OTUs) and filtered for chimeras using UPARSE (1.5% sequence dissimilarity cutoff) (95), which resulted in 9,243 OTUs. Singletons were removed. Taxonomy was assigned using CREST (96) and the ribosomal sequence database SilvaMod based on the release 106 of the SILVA nonredundant SSURef database (97). The sequence data were subsampled to 13,226 reads per sample, the number of reads in the sample with the fewest reads after all the filtering steps, using the command sub.sample in mothur (version 1.35.1) (98).

Statistical analyses. We used *t* tests to compare CH_4 and CO_2 production rates of the original peat of the oligotrophic and mesotrophic fens ($n = 3$). Methane production was $\log_{10}$ transformed before testing. $^{13}CH_4$ and $^{13}CO_2$ production rates between peat types during weeks 1, 2, and 3 ($n = 3$) were tested with repeated-measures analysis of variance using function lme in R package nlme (99), with week as a random factor. All analyses were carried out in R version 3.2.0 (100). *P* values were considered statistically significant at a *P* value of <0.05.

Bacterial communities in ^{13}C- and ^{12}C-fractions at days 7 and 14 and in initial peat of the fens were visualized by nonmetric multidimensional scaling (NMDS) plots based on Bray-Curtis distances of OTU data subsampled to 13,226 reads per sample ($n = 3$). Differences with peat type, fraction, and time point were tested with permutational multivariate analysis of variance (PERMANOVA; function adonis in R, Bray-Curtis distances). Analyses were carried out in R with vegan package (version 2.0) (101).

The OTUs enriched in the ^{13}C-fractions compared to the ^{12}C-fractions were identified with R package edgeR (102). Data were not subsampled for these analyses (65). Filtering was applied to keep only OTUs with more than five reads in at least four samples within each comparison of 12 samples, i.e., within ^{13}C- and ^{12}C-fractions of two time points with three replicates. The filtered data were normalized by the relative log expression (RLE) method as implemented in standard edgeR protocols. We used the general linear model (glm) approach to account for the two time points of 7 and 14 days. Trended dispersion was estimated with the power method and the tagwise dispersion with *df* of 5. In testing for enriched ("differentially expressed") OTUs, we accepted OTUs with a *P* value of <0.01, false-discovery rate (FDR) of <0.05, and $\log_2$ fold change (logFC) of >2 as enriched in the ^{13}C-fractions. To exclude OTUs migrating to the ^{13}C-fraction without actual ^{13}C incorporation, we tested for OTUs enriched in the fractions corresponding to the density of the ^{13}C-fraction in samples incubated with [^{12}C]cellobiose. Based on this test, one OTU from the mesotrophic peat was removed from the set of enriched OTUs. In further analyses, the read numbers of the enriched OTUs are from the subsampled data. Sequences similar to representative sequences of enriched OTUs were searched using Blast.

Accession number(s). Raw amplicon sequences were deposited in the NCBI Sequence Read Archive under accession number SRP075161.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02533-16.

TEXT S1, PDF file, 0.4 MB.

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