Methanol consumption drives the bacterial chloromethane sink in a forest soil

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Received: 16 February 2018 / Revised: 1 June 2018 / Accepted: 15 June 2018 / Published online: 10 July 2018 © The Author(s) 2018. This article is published with open access

Abstract
Halogenated volatile organic compounds (VOCs) emitted by terrestrial ecosystems, such as chloromethane (CH3Cl), have pronounced effects on troposphere and stratosphere chemistry and climate. The magnitude of the global CH3Cl sink is uncertain since it involves a largely uncharacterized microbial sink. CH3Cl represents a growth substrate for some specialized methylotrophs, while methanol (CH3OH), formed in much larger amounts in terrestrial environments, may be more widely used by such microorganisms. Direct measurements of CH3Cl degradation rates in two field campaigns and in microcosms allowed the identification of top soil horizons (i.e., organic plus mineral A horizon) as the major biotic sink in a deciduous forest. Metabolically active members of Alphaproteobacteria and Actinobacteria were identified by taxonomic and functional gene biomarkers following stable isotope labeling (SIP) of microcosms with CH3Cl and CH3OH, added alone or together as the [13C]-isotopologue. Well-studied reference CH3Cl degraders, such as Methylobacterium extorquens CM4, were not involved in the sink activity of the studied soil. Nonetheless, only sequences of the cmuA chloromethane dehalogenase gene highly similar to those of known strains were detected, suggesting the relevance of horizontal gene transfer for CH3Cl degradation in forest soil. Further, CH3Cl consumption rate increased in the presence of CH3OH. Members of Alphaproteobacteria and Actinobacteria were also 13C-labeled upon [13C]-CH3OH amendment. These findings suggest that key bacterial CH3Cl degraders in forest soil benefit from CH3OH as an alternative substrate. For soil CH3Cl-utilizing methylotrophs, utilization of several one-carbon compounds may represent a competitive advantage over heterotrophs that cannot utilize one-carbon compounds.

Introduction
Chloromethane (CH3Cl) is the most abundant halogenated volatile organic compound (VOC) in the atmosphere and contributes substantially to destruction of stratospheric ozone [1, 2]. Global emissions reach about 2.0 ± 0.6 Tg CH3Cl per year [3, 4]. Current knowledge also suggests that terrestrial CH3Cl emissions are mainly associated with biological activities of the aboveground part of plants and with white rot fungi in soil [5–7]. CH3Cl is produced upon burning of plant biomass, from methoxy groups of plant structural components such as lignin and pectin [8, 9]. CH3Cl is also enzymatically produced by S-adenosylmethionine-dependent methylation of chloride [10]. Current estimates for the global sink are larger than the global source [3], to which forest soil could contribute as much as 1.0 Tg per year [3, 11]. Global budgets are still uncertain, as the nature of the biotic sink activity as well as its spatial and temporal variability are not known at the regional and global scale [12].
Net emissions of CH$_3$Cl from terrestrial ecosystems are mitigated by soil and phyllosphere bacteria that utilize this VOC as a growth substrate [7, 13]. Such bacteria are aerobic methylotrophs, metabolically specialized for growth with one-carbon compounds [7]. The concentration of CH$_3$Cl in the atmosphere is low (approx. 590 ppt). This suggests similar concentrations of CH$_3$Cl in soils, although no experimental data are available to date [3, 14]. Previous studies suggest that aerobic soil methylotrophs can utilize CH$_3$Cl at environmentally relevant nanomolar to picomolar concentrations [15–20]. Such low concentrations likely do not yield sufficient energy for substantial bacterial growth with CH$_3$Cl. However, many known alphaproteobacterial CH$_3$Cl degraders also grow with methanol (CH$_3$OH) [13, 21], and this is also true in situ for soil methylotrophs of a deciduous forest [22]. Abundance of methylotrophs in O and A soil horizons is high, and ranges from 10$^6$ to 3 × 10$^8$ cells g$^{-1}$ soil, consistent with their frequent isolation from soils [23, 24].

Which organisms define the bacterial CH$_3$Cl sink in soils is largely unknown at present. The only biochemically characterized pathway for CH$_3$Cl utilization is the cmu pathway, characterized in detail for Methyllobacterium extorquens CM4 [21]. It has been found in various CH$_3$Cl-degrading bacterial strains, including several strains from forest soil [7, 18, 25–27]. The chloromethane dehalogenase gene cmuA has been used to detect CH$_3$Cl-degraders in various environments [15, 16, 18, 28–30]. Nonetheless, there is experimental and genomic evidence that the cmu pathway is not the only pathway for CH$_3$Cl utilization [13].

We hypothesized that (a) top soil (i.e., organic layer [Of, Oh] plus A horizon) is the preferable habitat for CH$_3$Cl degraders, and (b) that the as yet largely unknown CH$_3$Cl bacterial sink in soils benefits from additional energy and carbon sources, such as CH$_3$OH as a widespread methylotrophic growth substrate in soil [23, 24]. These hypotheses were tested for a European deciduous forest dominated by beech (Fagus sylvatica L.), and in a multi-treatment DNA stable isotope probing (SIP) laboratory experiment involving combinations of $^{13}$C-labeled and unlabeled CH$_3$OH and CH$_3$Cl amendment. Amplicon sequencing of 16S rRNA, cmuA, and mxaF/foxF genes as relevant gene markers was employed to identify microorganisms potentially involved in the CH$_3$Cl sink.

Materials and methods

Sampling site. Samples were collected in the natural forest reserve area Steigerwald located in South Germany (49° 37' N, 10° 17' O; sandy loam; Dystric Cambisol; pH of 4.6; mean annual temperature 7.5 °C; mean annual precipitation 725 mm). Steigerwald forest has not been managed for at least 25 years and represents a quasi-pristine deciduous forest typical of Central Europe, i.e., predominance of European beech (Fagus sylvatica L.) with minor stocks of oak (Quercus robur L.) [31].

Field closed-chamber measurements of CH$_3$Cl consumption

Eight closed top chambers were installed on-site at 12 am on 19 June 2013 and 20 August 2013. Air temperatures were 19 °C and 23 °C, respectively. The self-constructed stainless steel chambers had a total volume of 25.1 L (40 cm diameter; 20 cm height) and were equipped with an injection port. Chambers were spiked with 100 ppm CH$_3$Cl [32] and gas samples were collected over a period of 180 min with gas-tight syringes, and stored in extainers (Labco Limited, England) for subsequent analysis by gas chromatography (GC).

First order rate constants of CH$_3$Cl-degradation

Fresh beech leaves, dead leaf litter, soil horizons (Oh, Ah, B), and rotting wood were investigated. For beech phyllosphere analysis, fresh beech leaves were sampled by cutting branches from trees and a pool of cut leaves were immediately used for experiments. Samples were taken on 20 August 2013 (8 locations per compartment pooled) and on 12 July 2016 (3 locations pooled), and transferred to duplicated gas-tight 125 mL serum bottles that were subsequently flushed with synthetic air (Rießner Gase GmbH, Germany). CH$_3$Cl (Rießner Gase GmbH, Germany, purity 99.99%) was injected to final concentrations of 60 ppb or 200 ppm for samples taken on 20 August 2013 and 12 July 2016, respectively. Controls included a substrate-free control, a biological process control (amended with 20 mM KCN to inhibit biological activity), and an anoxic control (headspace was pure N$_2$). All treatments and controls were performed in triplicate. Gas samples were taken with gas-tight syringes and stored in 3 mL, pre-evacuated extainers (Labco ltm, UK) for further analysis by gas chromatography.

Soil microcosm set-up for DNA stable isotope probing (DNA SIP)

Five samples from the upper soil layer within a 20 m circle, were taken on 20 August 2013, pooled, homogenized and sieved (2 mm-mesh size). To stimulate CH$_3$Cl consumption, sieved soil was aliquoted in batches of 500 g into sealed flasks, CH$_3$Cl (Sigma Aldrich) added to the head space at a final concentration of 1%, and incubated in 1 L glass flasks at 20°C in the dark. Mixing ratios of CH$_3$Cl were followed by GC until added CH$_3$Cl was consumed. Then, for each
Table 1 Microcosm setup and carbon substrate amendment

| Carbon source added | Total carbon added (mM) | Gas phase amendment | Liquid phase amendment |
|---------------------|-------------------------|---------------------|------------------------|
| [13C]-CH3Cl         | 18                      | [13C]-CH3Cl         | H2O                    |
| [13C]-CH3Cl and CH3OH | 36                  | [13C]-CH3Cl         | CH3OH                  |
| CH3Cl and [13C]-CH3OH | 36                  | CH3Cl               | [13C]-CH3OH            |
| [13C]-CH3OH         | 18                      | –                   | [13C]-CH3OH            |
| CH3Cl               | 18                      | CH3Cl               | H2O                    |
| CH3Cl and CH3OH     | 36                      | CH3Cl               | CH3OH                  |
| CH3OH               | 18                      | –                   | CH3OH                  |
| Control             | –                       | –                   | H2O                    |

aAdded per pulse (5 pulses in total)
bA volume of 6 mL CH3Cl was added for each CH3Cl pulse. At each pulse, a total gas phase volume of 56 mL (air with or without CH3Cl) was added to 500 mL flasks in order to maintain overpressure in the flasks
cEither 1 mL milliQ water or 216 mM CH3OH stock solution was added per pulse to each microcosm

done during the DNA SIP experiment, 70 g activated soil was transferred to a 500 mL flask sealed with a Viton stopper. Eight different treatments were prepared, each performed in duplicates. Four treatments were amended with 13C-labeled substrates and four with unlabeled 12C-substrates. [13C]-CH3Cl (Sigma Aldrich), [13C]-CH3Cl together with unlabeled CH3OH (Campro Scientific), [13C]-CH3OH (Sigma Aldrich) together with unlabeled CH3Cl, and [13C]-CH3OH (Table 1). Control incubations were amended with equivalent amounts of unlabeled substrates, or left without amendment (Table 1). CH3Cl and/or CH3OH were amended as unlabeled or 13C-labeled isotopologue in 5 pulses over a period of 23 days (Fig. S1). CH3Cl and CO2 headspace mixing ratios were monitored by GC, and amended again when CH3Cl was no longer detectable. After each pulse, 5 soil aliquots (1 g each) per microcosm were retrieved and stored at −80 °C until further analysis.

pH and gravimetric water content in soil samples

pH was measured on sieved soil, before activation and after each substrate pulse using an InLab R422 pH electrode (InLab Semi-Micro; Mettler-Toledo, Gießen, Germany). Gravimetric soil moisture content was determined by weighing the soil before and after weight constancy following drying at 60 °C.

Quantification of CH3Cl, CO2, and 13CO2

On 19 June 2013 and 20 August 2013, CH3Cl and CO2 mixing ratios were determined by GC (HP 5890 Series II, Agilent) using a Porapak Q 80/100 column (Supelco, USA) and a helium-methane mixture (95:5) as the carrier gas. On 16 July 2016, CH3Cl mixing ratios were determined by ISQ™ Quadrupole mass spectrometer (MS) coupled with TRACE™ Ultra gas chromatograph (GC) (Thermo Fisher Scientific, Massachusetts, USA). CH3Cl and CO2 mixing ratios were calculated by regression analysis based on a 5-point calibration with standard mixing ratios of both gases. In the SIP experiment, GC MS analysis (Perkin–Elmer GC Clarus 600 system) was carried out as described previously [22]. Further Details of gas analysis methods are given in Supplemental Information.

Nucleic acid extraction and RNA removal

Nucleic acids from experimental replicates of all treatments and controls were extracted from each 0.5 g of soil when CH3Cl had been consumed after the third substrate pulse [33]. RNA was removed according to standard procedures including a treatment with RNAsase followed by isopropanol precipitation (Supplemental Information). Recovered DNA was resuspended in DNase-free water, quantified, and eventually stored at −80 °C until further processing through isopycnic centrifugation [22].

DNA fractionation by isopycnic centrifugation

Separation of the heavy (H) and light (L) DNA in a cesium chloride gradient was described in detail previously [22, 34]. In brief, a cesium chloride gradient solution mixed with each RNA-free DNA was loaded into an ultracentrifugation tube, placed in a Vti 65.2 vertical rotor (Beckman Coulter, Germany) and centrifuged for 40 h at 177,000×g in a LE-70 ultracentrifuge (Beckman Coulter, Germany). DNA was harvested according to established procedures in 10 gradient fractions [22, 34]. The main four fractions of H and of L DNA were pooled and DNA was quantified [22] (Fig. S2a-b, Supplemental Information). DNA concentrations ranged between 0.3 and 3.0 ng µL−1 for H fractions, and 18.5 and 50.3 ng µL−1 for L fractions.

PCR amplification, high-throughput sequencing, and data processing

PCR amplification of the 16S rRNA gene, and of CH3Cl dehalogenase CmuA and methanol dehydrogenase MxaF/XoxF encoding genes are described in detail in Table 2, and in Table S2 (Supplemental Information). New primers were designed to detect a larger spectrum of genetic diversity using NGS sequencing both for cmuA (Table S2; Fig. S7) and mxaF/ xoxF (Supplemental Information, Table S2, Fig S8). For sequencing analysis following PCR
amplification, briefly, a barcode oligonucleotide identifying sample origin was ligated to each PCR product. Equimolar pools of all libraries were assembled, and the resulting combined library was sequenced using Illumina MiSeq technology (LGC Genomics GmbH, Germany). Reads were assembled into contigs and analyzed using Mothur v.1.33.2 with default parameters (http://www.mothur.org/wiki/MiSeq_SOP) [35]. 16S rRNA reads <420 or >460 bp were discarded. Reads were pre-clustered into groups of sequences with up to 2 nucleotide differences. Chimera sequences were removed with UCHIME [36]. Remaining sequences were assigned by naïve Bayesian taxonomic classification using the SILVA reference database. Sequences that could not be assigned to Bacteria and Archaea were excluded from further analysis. OTUs were defined at 98% sequence similarity. Raw reads of cmuA and mxaF/xoxF amplicons with read lengths within 20 nucleotides of the expected amplicon length were clustered by USEARCH [37]. Sequences occurring only once in all libraries were considered artefactual and removed, but singletons within individual amplicon libraries were kept. Reads were clustered iteratively at progressively lower cut-off values, and the cut-off value at which the number of retrieved OTUs stabilized was selected [38]. These OTU sequences were compared against a gene-specific database generated from GenBank using BLAST (http://blast.ncbi.nlm.nih.gov) for identification. Taxonomic assignments of consensus sequences of each OTU were used to identify 13C-labeled gene OTUs.

**Table 2** Analyzed gene markers and amplicon characteristics

| Gene marker | Function                          | PCR Primer      | Sequence (5′-3′)*a | Amplicon size (bp) | SNP/OTUb | Total OTUs*c | Labeled OTUsd | Reference |
|-------------|-----------------------------------|-----------------|--------------------|--------------------|----------|--------------|--------------|-----------|
| 16 S rRNA gene | Ribosomal small subunit RNA       | 341for          | CTCAGGGGGGCGGCAG    | 464                | 9        | 117          | 5            | [60]      |
|             |                                   | 785/805rev      | GACTACHVGGGTATCTAATCC |                    |          |              |              | [61]      |
| cmuA        | chloromethane methyltransferase   | cmuAf422        | GARGTBBGGITAYAYGHG   | 422                | 38       | 8            | 5            | This studyf |
|             |                                   | cmuAr422        | TCRTTGCGCTCRAATGTCC  |                    |          |              |              |           |
| mxaF/ xoxF  | methanol dehydrogenase            | mdh1            | GCGGWWSCAICTGGGTYT   | 430                | 39       | 6            | 6            | This studyf |
|             |                                   | mdh2            | GCGGWWSCAICTGGGTYT   |                    |          |              |              |           |
|             |                                   | mdhR            | GAASGTYCSTYARTCCATGA |                    |          |              |              |           |

*aDegenerate base mixtures: B (C,G,T), H (A,C,T), K (G,T), N (A,C,G,T), R (A,G), S (G,C), Y (C,T), V (A, C, T), W (A,T). Inosine (I) was used instead of the N mixture [62] in some cases

*bMaximal Single Nucleotide Polymorphism (SNP) positions possible within an OTU

*cCorresponding to the sum of OTUs detected in the 8 microcosms of the SIP experiment. Sequences were affiliated to the same OTU at cutoff values of 98, 90, and 80% sequence identity at the nucleotide level for 16S rRNA gene, cmuA, and mxaF/xoxF amplicons, respectively

*dSee Material and Methods for the criteria applied to define OTUs as ‘labeled’

*ePrimer pairs allow to amplify both mxaF and xoxF types of methanol dehydrogenase (mdh) sub units Amplifications were performed with two different forward primers (mdh1, mdh2) in order to reduce primer degeneracy and improve PCR efficiency. Amplicons obtained with primers mdh1/mdhR and mdh2/mdhR were pooled before sequencing.

fSee Supplemental Information of Materials and Methods for details

**Identification of 13C-labeled OTUs**

Labeled OTUs were defined according to a previously reported protocol developed to minimize false positives in ‘H DNA’ fractions [22, 39]. The following criteria were applied to identify 13C-labeled OTUs: (1) OTU abundance in the ‘heavy’ fraction of a microcosm treated with 13C-labeled substrate higher than in the ‘heavy’ fraction of the corresponding microcosm treated with unlabeled substrate; (2) OTU abundance in the ‘heavy’ fraction higher than in the ‘light’ fraction; (3) OTU abundance in the ‘heavy’ fraction of the microcosm treated with 13C-labeled substrate ≥0.5%; (4) OTU abundance difference between ‘heavy’ and ‘light’ fractions higher by at least 0.3% (a threshold that considers the variance of each OTU abundance). Moreover, a lower limit of 5% was set for the labeling proportion (LP), (i.e., the relative frequency of a labeled OTU in a specific heavy fraction) of a given OTU to be considered as labeled [22].

**Statistical and phylogenetic analyzes**

Richness and Simpson diversity indices were determined using Mothur and PAST (http://folk.uio.no/ohammer/past). Relationships between sequence datasets in different DNA fractions and microcosms were investigated by two-dimensional NMDS (non-metric multidimensional scaling) within Mothur, and visualized with Kaleidagraph (Synergy Software, Reading, PA, USA). Details on phylogenetic tree construction are given in figure legends.
Nucleotide sequence accession numbers

Sequence datasets were deposited to the NCBI BioSample database under the study accession number SUB3319582.

Results

Localization of active CH$_3$Cl consumption in different forest compartments. Two sampling campaigns were performed, with the second campaign aiming to verify and justify that top soil samples were indeed the most active in CH$_3$Cl degradation, since only this horizon was selected to be assessed by SIP. Immediate consumption of CH$_3$Cl was observed in two field site campaigns at the temperate deciduous forest Steigerwald (Fig. 1a, b). In contrast, no net CH$_3$Cl emissions were detected from the forest floor (<1 ppb in chambers headspace). This strongly suggested that the investigated forest top soil represents a major sink for atmospheric CH$_3$Cl at the forest ecosystem level.

Results from lab-scale microcosms suggest that net CH$_3$Cl consumption was primarily due to biological activity. Indeed, CH$_3$Cl consumption was not observed in microcosms that were treated with KCN (Fig. 2a–e). O$_1$ and A$_h$ horizons were the most active layers (Fig. 2b, c). Because no CH$_3$Cl consumption was detected under anoxic conditions (data not shown), top soil aerobic microorganisms likely represent the active sink for CH$_3$Cl (Fig. 2b). Results obtained for the phyllosphere of European beech were variable, as we detected substantial consumption of CH$_3$Cl in 2013 only (Fig. 2e).

Mineralization and assimilation of CH$_3$Cl by the top soil microbial community

As expected, a net increase in CO$_2$ formation occurred in all microcosms in response to substrate amendment (Fig. S1a–c). A non-significant trend was observed towards larger CO$_2$ release upon addition of CH$_3$OH compared to CH$_3$Cl (Fig. S1c). Rates of CO$_2$ production were in the range of 0.2–0.3 mmol g$_{dry ~soil}^{-1}$ day$^{-1}$ for microcosms to which CH$_3$Cl or CH$_3$OH was added. No differences in CO$_2$ release between microcosms to which labeled or unlabeled substrates were added were observed, confirming that different carbon isotopologues did not affect carbon metabolism ($t$-test, $p > 0.10$; Fig. S1). Uncertainties were large, but about 10 mM (20%) of the added 54 mM of $^{13}$C-CH$_3$Cl were converted to $^{13}$C-CO$_2$ (Fig. S1). Hence, up to 80% of amended $^{13}$C-CH$_3$Cl was assimilated into biomass. This rather high rate may be an overestimate, given measurement uncertainties and undetected losses by precipitation of carbonate. An non-significant increase in $^{13}$C-CO$_2$ formation from labeled $^{13}$C-CH$_3$Cl was observed when unlabeled CH$_3$OH was also provided ($t$-test, $p > 0.10$; Fig. S1c). In the reverse case of microcosms amended with $^{13}$C-CH$_3$OH together with unlabeled CH$_3$Cl, the increase in $^{13}$C-CO$_2$ formation was slightly less. Moreover, CH$_3$OH was mineralized to a larger extent than CH$_3$Cl when added alone ($t$-test, $p \leq 0.05$). Taken together, these observations suggest that microbial CH$_3$Cl consumption activity is enhanced by CH$_3$OH.

Overall microbial community response to amended substrates

Based on three gene markers, amendment of substrates led to significant changes in microbial community composition, basing on statistical analyzes combining $^{13}$C-labeled and unlabeled phylotypes (Fig. S3a–c, Table S3, Tables 3 and 4). Combined with the observation that CO$_2$ formation increased in amended microcosms (Fig. S1), this suggested increased growth of specific microorganisms with amended substrates.

Diversity of $^{13}$C-labeled family-level OTUs based on the 16S rRNA gene

A total of 117 family-level OTUs (90% similarity) were detected (Table S1). Of these and across all four substrate treatments (Fig. 3a), several genera within five families
were identified that satisfied the criteria set for defining $^{13}$C-labeled OTUs (Fig. 3a): Beijerinckiaceae within Alphaproteobacteria, three families of Actinobacteria (Acidothermaceae, Pseudonocardiaceae, and Streptomyctaceae) and OTU10816S within the TM7 phylum, recently renamed as Candidatus Saccharibacteria (Fig. S4) [40]. Methylovirgula, Acidothermus, and Streptomyces represented over 95% of the Beijerinckiaceae, Acidothermaceae, and Streptomyctaceae, respectively (Fig. 3a). In the microcosm amended with $[^{13}$C]-CH$_3$Cl, three of the five family-level OTUs (Acidothermaceae, Beijerinckiaceae, and Streptomyctaceae) represented about 80% of all labeled OTUs (Fig. 3a). In the microcosm amended with $[^{13}$C]-CH$_3$OH, in contrast, mainly Beijerinckiaceae was labeled. The labeled Cand. Saccharibacteria-like OTU10816S and the Pseudonocardiaceae sp.-like OTU8516S were both found at low abundance in all $^{13}$C-amended microcosms (Fig. 3a). All labeled OTUs differed from the closest type strains as well as from previously described CH$_3$Cl-degrading isolates (Fig. S4). Another interesting observation was that several other detected Actinobacteria, i.e. Gryllotalpocila, likely represent CH$_3$OH utilizers based on labeling patterns (Fig. 3a, Fig S4).

Diversity of CH$_3$Cl utilizers based on cmuA

Chloromethane: cobalamin methyltransferase cmuA, the biomarker for CH$_3$Cl consumption by the cmu pathway, was amplified with newly designed primers (Table S2). Eight OTUs were detected among which five satisfied the criteria defined for $^{13}$C-labeled OTUs (Table S3). Consensus sequences of these 8 OTUs were compared to known
of genome-sequenced cultivated strains, and of uncultivated OTUs identified in a previous SIP study of soil amended with \(^{13} \text{C}\)-CH\(_3\)Cl \cite{15}. The eight OTUs belonged to three distinct gene clusters (Fig. 4). Labeled OTUs were closely related to sequences of known CH\(_3\)Cl-degraders including *Methylobacterium extorquens* CM4 (>99% identity) or *Hyphomicrobium* sp. MC1, as well as with *cmuA* sequences retrieved from a soil environment \cite{15}. In the CH\(_3\)Cl treatment, OTU\(_2\)\(_{cmuA}\) and OTU\(_3\)\(_{cmuA}\) were among the most dominant \(^{13} \text{C}\)-labeled OTUs. This labeling pattern was conserved in all \(^{13} \text{C}\)-labeled substrate treatments of our study (Fig. 3b). However, when methanol was the \(^{13} \text{C}\)-labeled substrate, an additional OTU\(_6\)\(_{cmuA}\) was detected (Fig. 3b). OTU\(_6\)\(_{cmuA}\) was very similar to *cmuA* genotypes of reference CH\(_3\)Cl-degrading *Hyphomicrobium* strains (Fig. 4). All other \(^{13} \text{C}\)-labeled OTUs represented minor populations with closest similarity to *cmuA* from *Methylobacterium* (Figs. 3b, 4).
Diversity of methanol utilizers based on mxAF/xoxF

Two types of methanol dehydrogenase (mdh) are predominantly found in methanol utilizers, MxaFI and XoxF, both harboring a pyrroloquinoline quinone catalytic center [41]. Previously used mxAF/xoxF primers were biased against xoxF [42]. Thus, we designed new mdh primers targeting both types of MDH (Table 2 and S2; Fig. S8). A total of 15 distinct 13C-labeled mdh OTUs were found, all but one xoxF genes (Figs. 3c and S5). These OTUs were

Fig. 4 Phylogenetic affiliations of detected cmuA OTUs. A maximum likelihood phylogenetic tree was reconstructed from a 422 nt-long sequence alignment based on the Tamura-Nei model [59]. Bold, labeled cmuA OTUs. Scale bar, 0.05 substitutions per site. Bootstrapping was performed with 1000 replicates. Reference sequences from previous studies were included: characterized CH3Cl-utilizing isolates (green diamonds); uncharacterized genome-sequenced isolates containing cmu genes (green open diamonds); sequences detected by SIP in the marine environment (blue circles [30]) or in soil (brown circles [18])

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mostly affiliated to one of the canonical XoxF clades, i.e., XoxF5 [41]. The underrepresentation of other clades might have been effected by the design strategy of the primers (Fig. S8, Supplemental Information). Only OTU15mdh was closely similar to a mxaF gene of Beijerinckiaceae (Fig. 3c and Fig. S5). Predominant $^{13}$C-labeled mxaF/xoxF OTUs associated with known xoxF genes of Bradyrhizobium and Sinorhizobium were found in all four $^{13}$C-labeled substrate treatments. On the other hand, $^{13}$C-labeled mxaF/xoxF OTUs closely related with xoxF genes from Acetobacteraceae (Acidiphilium, Gammaproteobacteria) were detected in the $^{13}$C]-CH$_3$OH-amended treatment only (OTU14$_{mdh}$) (Figs. 3c and S5). We hypothesize that OTU14$_{mdh}$ corresponds to a methanol utilizer, not a CH$_3$Cl utilizer, also because only methanol-utilizing strains are known in Acetobacteraceae [43, 44]. In both $^{13}$C]-CH$_3$Cl-amended treatments, OTU25$_{mdh}$ was dominant, whereas in both $^{13}$C]-CH$_3$OH amended treatments, OTU24$_{mdh}$ prevailed. Both OTUs correspond to Bradyrhizobium-like xoxF genes.

**Key Bacteria of the CH$_3$Cl sink in the investigated top soil**

At first glance, bacterial taxa suggested to be associated with utilization of CH$_3$Cl differ depending on which of the three investigated gene biomarkers in the $^{13}$C]-CH$_3$Cl SIP experiment is considered (Fig. 3, Tables 3 and 4). However, consideration of the labeling proportion (LP) of $^{13}$C-labeled OTUs (Fig. 3) allows to refine the analysis. A high LP suggests strong $^{13}$C-labeling, hinting at predominant transformation of the $^{13}$C labeled-substrate by Alphaproteobacteria. In support of this, the taxonomic 16S rRNA gene biomarker indeed suggests that this class, and in particular strains closely related to Methylovirgula within the Beijerinckiaceae family, includes a major part of the primary utilizers of amended $^{13}$C]-CH$_3$Cl soils (Figs. 3a, S4, S6). Beyond that, the LP analysis approach also allowed to identify Actinobacteria of the genus Kineospora as potential novel key $^{13}$C]-CH$_3$Cl degraders (Figs. 3a, S4, Table S3).

**Discussion**

We show in this study that theoxic compartment of top soil was the main sink for CH$_3$Cl in the investigated forest soil, and that this degradation process was primarily biotic, in agreement with an early exploratory study of CH$_3$Cl dissipation in various environments [27]. We performed a detailed study of potential CH$_3$Cl degraders in top soil, and focused onoxic conditions, since anoxic incubations did not show evidence for CH$_3$Cl consumption, as expected given that strictanaerobes are not frequently abundant inoxic soils [45].

### Table 3 Number of filtered sequences obtained from heavy DNA fractions

| Treatment                        | 16S rRNA gene | cmuA | mxaF/xoxF |
|----------------------------------|---------------|------|-----------|
| $[^{13}]$C]-CH$_3$Cl              | 46110         | 4282 | 786       |
| $[^{13}]$C]-CH$_3$Cl and CH$_3$OH | 178042        | 3778 | 1534      |
| CH$_3$Cl and $[^{13}]$C]-CH$_3$OH| 76690         | 614  | 918       |
| $[^{13}]$C]-CH$_3$OH              | 98332         | 1886 | 588       |
| CH$_3$Cl                          | 72522         | 370  | 2616      |
| CH$_3$Cl and CH$_3$OH             | 47236         | 922  | 862       |
| CH$_3$OH                          | 90822         | 246  | 2946      |
| Unamended control                 | 100032        | 184  | 2710      |

See Table S1 for further information

### Table 4 Diversity indices for 16S rRNA gene OTUs obtained from heavy and light fractions of SIP experiment

| Microcosm | SIP fraction | Sobs$^a$ | Shannon index$^b$ | Simpson diversity$^b$ |
|-----------|--------------|----------|-------------------|-----------------------|
| $[^{13}]$C]-CH$_3$Cl | H            | 1762     | 5.95              | 44                    |
|            | L            | 2870     | 7.82              | 223                   |
| $[^{13}]$C]-CH$_3$Cl and CH$_3$OH | H            | 1629     | 6.11              | 36                    |
|            | L            | 3150     | 7.55              | 354                   |
| CH$_3$Cl and $[^{13}]$C]-CH$_3$OH | H            | 1566     | 6.52              | 120                   |
|            | L            | 3062     | 7.86              | 283                   |
| $[^{13}]$C]-CH$_3$OH | H            | 1215     | 4.58              | 7                     |
|            | L            | 3148     | 7.40              | 322                   |
| Unamended control | H            | 1804     | 6.45              | 102                   |
|            | L            | 2904     | 7.39              | 217                   |

$^a$Calculated at the 98% sequence identity level. See Materials and Methods for definitions. Simpson diversity is considered a conservative measure of the effective number of phylotypes [63]

**Potential bacterial degraders of CH$_3$Cl in forest top soil are phylogenetically distinct from known CH$_3$Cl-utilizing isolates**

Alphaproteobacteria (i.e., Beijerinckiaceae) and Actinobacteria (i.e., Kineosporaceae) likely represent key CH$_3$Cl degraders in the investigated forest soil (Fig. 3a). The phylogenetic affiliation of these CH$_3$Cl utilizers was only distantly related to known CH$_3$Cl degraders and thus a novel finding. Moreover, metabolically active Actinobacteria show only limited sequence identity with the only known CH$_3$Cl-degrading isolate of this phylum, Nocardiodia sp. strain SAC-4 [27] (Fig. S4). In contrast, almost all soil CH$_3$Cl-degrading isolates known so far are affiliated with only a few genera of Alphaproteobacteria [15, 18, 29, 30], and are phylogenetically distinct from the key genus associated with CH$_3$Cl degradation in our study (Methylovirgula, family Beijerinckiaceae). In addition, members of the candidate division Cand. Saccharibacteria
(syn. TM7) were also \(^{13}\)C-labeled in our experiments. Physiological knowledge within this division is limited, and methylo-trophy unreported to date [40, 46]. Since cross-labeling via \(^{13}\)CO\(_2\) cannot be fully ruled out in our experimental setup due to the long incubation period, further efforts will be required to confirm that this phylum indeed includes methylo-trophs able to degrade CH\(_3\)Cl.

**Low diversity of the cmuA biomarker and potential horizontal gene transfer of cmu genes to Beijerinckiaceae**

Amplicons of cmuA retrieved from \(^{13}\)C-labeled DNA were most closely similar to the cmuA gene of Methylobacterium extorquens CM4 in both \(^{13}\)C-CH\(_3\)Cl and \(^{13}\)C-CH\(_3\)OH treatments, despite the fact that newly designed cmuA primers cover a broader diversity of known cmuA sequences than previous ones (Fig. S7). However, abundance of Methylobacteriaceae in 16S rRNA gene datasets was <1% (data not shown), and no OTU affiliated to this family was defined as \(^{13}\)C-labeled with the applied criteria. Notably, Beijerinckiaceae was the key family associated with CH\(_3\)Cl degradation based on the 16S rRNA gene biomarker. This may suggest that CH\(_3\)Cl-degraders from Beijerinckiaceae use another CH\(_3\)Cl degradation pathway than the cmu pathway, or that they possess a cmuA gene that escapes detection with the used primers. Alternatively, such degraders may have acquired a known cmuA gene by horizontal gene transfer. Indeed, the cmu pathway is plasmid-borne in *M. extorquens* CM4 [47], and further experimental [48] as well as sequence-based [13] evidence for horizontal transfer of the capacity for chloromethane degradation is also available.

**Evidence for novel CH\(_3\)Cl degraders based on mxaF/xoxF**

Whereas mxaF genes had been detected in a previous study of the same soil with canonical primers [22] (Fig. S5), mainly xoxF sequences were retrieved here with newly designed primers that detect both mxaF and xoxF (Fig. S5, Supplemental Information). Of the detected 25 mxaF/xoxF OTUs, only three OTUs, most similar to xoxF genes found in Bradyrhizobium and Sinorhizobium genomes, dominated microcosms irrespectively of the performed treatment (Fig. 3c). Worthy of note, growth with CH\(_3\)Cl has not been reported to date for representatives of these genera [23, 49, 50]. In addition, xoxF genes similar to those of *Methylobacterium* were also \(^{13}\)C-labeled upon \(^{13}\)C-CH\(_3\)Cl amendment. Although some cross-feeding of \(^{13}\)CO\(_2\) cannot be entirely excluded since Bradyrhizobium, Sinorhizobium, and Methylobacterium can assimilate CO\(_2\) [49, 51], this possibility was minimized by regular exchange of the gas phase to remove formed CO\(_2\). 16S rRNA gene OTU analysis indicated members of Beijerinckiaceae as main alphaproteobacterial CH\(_3\)Cl degraders (Fig. 3a). Basing on phylogenetic analysis (Fig. S5), the major \(^{13}\)C-labeled xoxF OTUs appear quite distinct from known xoxF genes of Beijerinckiaceae Sinorhizobium and Bradyrhizobium. Hence, we hypothesize that OTU24\(_{mxa}\) and OTU25\(_{mxa}\) represent hitherto unknown xoxF genes in Beijerinckiaceae, or that used primers discriminated against the genotype of Beijerinckiaceae. A XoxF-type MDH might be advantageous as it directly leads to formate, avoiding the more toxic formaldehyde produced by MxaFI-type MDH [52].

**Consumption of CH\(_3\)OH by CH\(_3\)Cl degraders and implications for the CH\(_3\)Cl sink in soil**

Beijerinckiaceae were newly identified here as novel potential CH\(_3\)Cl and also CH\(_3\)OH utilizers. The latter finding agrees well with a previous study on the microbial methanol sink in the same soil [22]. None of the methylo-trophic Beijerinckiaceae characterized so far utilize methyl halides [53]. Beijerinckiaceae also comprise typical methanotrophs [54], and one such isolate was shown to transform CH\(_3\)Cl under laboratory conditions, but not to grow with this compound [55, 56]. Basing on the functional gene marker pmoA (encoding the beta-subunit of particulate methane monoxygenase), representatives of the uncultivated upland soil cluster α taxon represented the most abundant group of methanotrophs in Steigerwald forest soil. This pmoA type, however, is phylogenetically distinct from pmoA of Beijerinckiaceae [32]. However, a recent study suggest that USC\(_\alpha\) is indeed a member of Beijerinckiaceae [57]. Thus, we presently cannot rule out that methanotrophs were not involved in the observed CH\(_3\)Cl sink activity in Steigerwald forest soil.

The presence of CH\(_3\)OH as an alternative methylo-trophic growth substrate supports the notion of enhanced assimilation of carbon from CH\(_3\)Cl by Beijerinckiaceae during CH\(_3\)OH-driven growth. This idea is supported by the observed high labeling percentages in combined substrate amendments with CH\(_3\)OH and \(^{13}\)C-CH\(_3\)Cl (Fig. 3). Similarly, the reverse combined amendment of unlabeled CH\(_3\)OH with \(^{13}\)C-CH\(_3\)Cl led to increased mineralization of \(^{13}\)C-CH\(_3\)Cl (Fig. S1). Taken together, these findings suggest that activity and growth of soil microorganisms that define the bacterial CH\(_3\)Cl sink in the investigated soil strongly depend on availability of CH\(_3\)OH.

Many methylo-trophs can simultaneously utilize several one-carbon compounds [58]. This is likely a selective advantage in natural environments when availability of potential substrates is variable and often limiting. As shown in a previous study on the same soil, Beijerinckiaceae can be either methylo-trophic or non-methylo-trophic [22].
the basis of the data reported here, we suggest that key CH$_3$Cl degraders in soil may be capable of assimilating several one-carbon substrates to optimize their growth. Such a metabolic lifestyle is likely to be of advantage in order to compete with other aerobes in the complex top soil environment, and suggests that the microbial CH$_3$Cl sink is linked to the availability of other key carbon sources in soil such as CH$_3$OH.

Conclusions

Our study provides a first deep coverage exploration of bacterial diversity functionally linked with the CH$_3$Cl sink in soil. It has revealed that CH$_3$Cl consumption in forest soil may be driven by alternative carbon sources such as CH$_3$OH. It also uncovered new taxa associated with CH$_3$Cl degradation, including genera of Alphaproteobacteria and Actinobacteria that had not yet been identified in the context of previous SIP experiments with CH$_3$Cl, and for which no isolates are yet available. The used CH$_3$Cl concentrations, which are much higher than those encountered in the troposphere, might have harmed some bacteria that cannot grow with 1% CH$_3$Cl. However, we focused in the study on those soil bacteria that can deal with these concentrations and degraded it. We are aware that the used SIP approach might have been biased by label transfer. However, the low limit of detection of DNA SIP combined with the very conservative approach chosen to identify potential chloromethane degraders, only strongly-labeled microorganisms were identified, which maximizes the likelihood that they were directly labeled from amended labeled chloromethane and not through crossfeeding. Taxa corresponding to cultivated model CH$_3$Cl degraders, such as Methylobacterium extorquens CM4, were not relevant for CH$_3$Cl degradation in the investigated forest top soil. Thus, cultivation of new isolates requires future efforts to improve coverage of existing diversity chloromethane degraders by isolates. Detection of cmuA genes closely similar to those of such strains (>99%) suggests that horizontal transfer of the ability to degrade CH$_3$Cl is an important aspect of the CH$_3$Cl sink in soil. Our study also suggests that methylotrophs in top soil may have a competitive advantage over non-methylotrophs by their ability to utilize diverse one-carbon substrates simultaneously. Testing this hypothesis, and addressing alternative metabolic strategies of CH$_3$Cl degradation in soil, will be the topic of future investigations.

Acknowledgements PC was funded through a PhD grant from Région Alsace (France) and the Deutsche Forschungsgemeinschaft (Germany). LB was supported by the French Agence Nationale de la Recherche ANR (ANR-14-CE35-0005-01). MM was financed through the Deutsche Forschungsgemeinschaft DFG (Ko2912/5-1 and Ko2912/10-1). The Deutsche Akademischer Austauschdienst (DAAD) program PROCOPE funded travel expenses. We thank Y. Louhichi-Jelail for technical assistance in performing PCR amplifications, and H.L. Drake and M.A. Hor for hosting the project at the Department of Ecological Microbiology of the University of Bayreuth (Germany).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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