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A Putatively New Family of Alphaproteobacterial Chloromethane Degraders from a Deciduous Forest Soil Revealed by Stable Isotope Probing and Metagenomics

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Abstract

Background:

Chloromethane (CH₃Cl) is the most abundant halogenated organic compound in the atmosphere and substantially responsible for the destruction of the stratospheric ozone layer. Since anthropogenic CH₃Cl sources have become negligible with the application of the Montreal Protocol (1987), natural sources, such as vegetation and soils, have increased proportionally in the global budget. CH₃Cl-degrading methylotrophs occurring in soils might be an important and overlooked sink.

Results&Conclusions:

The objective of our study was to link the biotic CH₃Cl sink with the identity of active microorganisms and their biochemical pathways for CH₃Cl degradation in a deciduous forest soil. When tested in laboratory microcosms, biological CH₃Cl consumption occurred in leaf litter, senescent leaves, and organic and mineral soil horizons. Highest consumption rates, around 2 mmol CH₃Cl g⁻¹ dry weight h⁻¹, were measured in organic soil and senescent leaves, suggesting that top soil layers are active (micro-)biological CH₃Cl degradation compartments of forest ecosystems. The DNA of these [¹³C]-CH₃Cl-degrading microbial communities was labelled using stable isotope probing (SIP), and the corresponding taxa and their metabolic pathways studied using high-throughput metagenomics sequencing analysis. [¹³C]-labelled Metagenome-Assembled Genome closely related to the family *Beijerinckiaceae* may represent a new methylotroph family of *Alphaproteobacteria*, which is found in metagenome databases of forest soils samples worldwide. Gene markers of the only known pathway for aerobic CH₃Cl degradation, via the methyltransferase system encoded by the CH₃Cl utilisation genes (*cmu*), were undetected in the DNA-SIP metagenome data, suggesting that biological CH₃Cl sink in this deciduous forest soil operates by a *cmu*-independent metabolism.

Keywords: Stable Isotope Probing, Chloromethane, VOCs, methylotrophy

Background

Chloromethane (CH₃Cl) is a well-documented halocarbon and one of the most abundant volatile organic compounds in the atmosphere. The global atmospheric concentration of CH₃Cl is currently 600 ppt and is responsible for 17% of halogen-dependent ozone depletion in the stratosphere [1, 2]. Abiotic and biotic processes in plants, grassland, soil, salt marshes, and oceans are the key drivers of global chloromethane formation [2-6]. The major CH₃Cl sinks in the troposphere are photochemical processes involving OH radicals [1, 7], i.e. currently estimated as 2.8 Tg yr⁻¹ [2]. Furthermore, microbial degradation in top soils is a major global sink of the atmospheric trace compound CH₃Cl [8-10]. However, discrepancies in the CH₃Cl flux estimates (range between 1.5 and 2.5 million t yr⁻¹) have been observed between known sources and calculated sinks [1]. Thus, the imbalance of the global budget of CH₃Cl is due to underestimated sinks, notably forest soils and their microbial degradation activity.

Biological sink activity within the forest soils may represent a significant CH₃Cl contribution of as much as 1.0 Tg yr⁻¹ [2, 7]. Strong evidence of the CH₃Cl metabolism by soil bacteria, by both oxidation and hydrolysis in earlier investigations suggests that they are the potential sinks of CH₃Cl [11-13]. Keppler et al. [14] suggested that microbial soil sink could be 1600 Gg yr⁻¹, which might still be considerably underestimated due to the ubiquitous presence of yet unquantified impact of CH₃Cl degraders in soils [13].

The microorganisms active in terrestrial sink of CH₃Cl are poorly characterized compared to other atmospheric trace gases such as methane or nitrous oxide. Among methylotrophs, a few are capable of utilizing CH₃Cl as their primary growth substrate [15-17]. CH₃Cl-degrading bacteria were isolated from diverse natural environments [11, 13, 18], including representatives from *Alpha*-, *Beta*-, and *Gammaproteobacteria*, *Actinobacteria*, and *Firmicutes* [15, 19]. Taxonomically diverse CH₃Cl-degrading bacteria have been isolated from the soil such as alphaproteobacterial genera *Aminobacter*, *Hyphomicrobium*, *Methylobacterium*, *Methylocella*, *Acidithiobacillus* and *Bradyrhizobium* [13, 20, 21]. The most comprehensively characterized metabolic pathway of aerobic CH₃Cl degradation is the *cmu* pathway, studied in detail in a model methylotroph, *Methylobacterium extorquens* (previously *Methylobacterium extorquens*) strain CM4 [22]. Genes *cmuA* and *cmuB* encoding methyltransferases essential for CH₃Cl dehalogenation have been detected in various environments, including forest soils [16, 20, 23-25]. However, the absence of a *cmu* genes in active CH₃Cl degraders in metagenomics and labelling studies suggests this pathway is not the only CH₃Cl degradation pathway [15, 17, 19] and limits CH₃Cl-degrading bacteria detection by overlooking *cmu*-independent microbial sink in soils.

Stable Isotope Probing (SIP) is a powerful method used in microbial ecology in order to identify specific functional groups of microorganisms that incorporate distinct (labeled) substrates without knowing how to cultivate them in standard laboratory conditions. Such a SIP approach enabled the identification of key bacterial degraders of naturally occurring CH₃Cl in a temperate German European beech forest soil [20] using 16S rRNA gene-targeted metagenomics. The major consumers of CH₃Cl-derived carbon were identified by 16S rRNA gene-targeted belonged to the family *Beijerinckiaceae* of the class of *Alphaproteobacteria* [23]. However, in these methylotrophic aerobes, the metabolic CH₃Cl-utilization pathway has not been identified. Also, the precise phylogenetic affiliation of these *Alphaproteobacteria* CH₃Cl degraders remained elusive. Using the same forest soil location where these consumers of CH₃Cl-derived carbon have been identified, this study aims to identify genomic and metabolic traits of active aerobic microbial CH₃Cl degraders [20]. A combination of DNA-SIP and high-throughput metagenomic sequencing was used to unravel the active CH₃Cl-degrading bacteria and to study their methylotrophic metabolism.

Methods

Environmental sampling and microcosm incubations

Soil samples and degraded leaf samples were taken from the Steigerwald, Germany, in June 2017. Three organic soil samples, three mineral soil samples and three degraded leaf samples were extracted, transported to the laboratory on the same day, and stored overnight at 4°C before processing in the following morning. DNA stable isotope labelling (SIP) experiments were set up with three biological replicates to determine the most active layers for chloromethane degradation. Furthermore, no substrate-added control incubations and control incubations with potassium cyanide (20 mM) were set up to determine intrinsic chloromethane formation and non-microbial chloromethane dissipation, respectively.

Two grams of organic or mineral soil or degraded leaves were incubated in a 125-mL serum vial and flushed with synthetic air (Linde AG, Germany). Microcosms were set up in three biological replicates by adding ^{13}C -CH₃Cl (Campro Scientific, Germany) or ^{12}C -CH₃Cl (Linde AG, Germany), to a final concentration of 250 mM g⁻¹ DW (dry weight). In more detail, serum vials were sealed with butyl rubber stoppers, evacuated to a level of 0.01 bar and flushed three times with synthetic air containing 20% oxygen and 80% dinitrogen gas (Linde AG, Germany) to a pressure of 1.2 bar. Finally, CH₃Cl (Linde, Germany) was added using a 100 µL gas-tight syringe (Hamilton, Romania). Gas samples were taken with gas-tight syringes and stored in 3-mL, pre-evacuated Exetainers (Labco Limited, England) for further analysis by gas chromatography (GC). After complete degradation of CH₃Cl, vials were ventilated, evacuated and flushed with synthetic air as before, and again CH₃Cl was added. Microcosms were monitored for CH₃Cl depletion at regular intervals until an incorporation of 100 µmol carbon per gram sample (soil or degraded leaves) was achieved. Three biological replicated microcosms were then destructively sampled and frozen at - 20 °C for subsequent DNA isolation.

Gas chromatography

CH₃Cl was measured in headspace gas volume by injecting 100 µL of a headspace gas sample into a ISQTM Quadrupole GC-MS System using a TRACETM Ultra gas chromatograph (Thermo Fisher Scientific, USA) fitted with a 60 m, 0.32 mm GS-GasPro capillary column (Agilent Technologies, California, USA) with helium as the carrier gas (constant column flow rate, 1.5 mL min⁻¹). Headspace gas samples (100 µL) were injected into the column with a temperature ramp from 40 °C to 200 °C (15 °C increase min⁻¹). High-throughput measurements were carried out by the MultiPurpose autosampler MPS (Gerstel Inc., USA). Chromatograms were analyzed with the software Openchrome® (Lablicate GmbH, Germany). CH₃Cl concentrations were calculated by regression analysis based on a six-point calibration with CH₃Cl standard ranging from 1 to 75 ppm.

DNA isolation, ultracentrifugation, and high-throughput sequencing

DNA extractions from unincubated samples (T_0) and samples after incubation with CH_3Cl were carried out using the FastDNA Spin Kit for Soil (MP Bio Science, Derby, UK). ^{13}C -labelled heavy DNA was subsequently separated from unlabeled light ^{12}C -DNA using a caesium chloride density gradient ultracentrifugation, as described previously [26]. Density formation across 12 fractions (200 μL each) was confirmed by measuring refractive indices using a digital refractometer (Reichert AR2000). DNA was subsequently extracted from caesium chloride using PEG6000 and glycogen, as described previously [26].

To determine the microbial populations in T_0 and 'heavy' and 'light' fractions of the [^{13}C]- CH_3Cl incubations and a "Kitome" sample, Terminal Restriction Fragment Length Polymorphism (T-RFLP) was carried out as described previously [27]. Subsequent 16S rRNA amplicon sequencing was performed on an Illumina Miseq platform at LGC Genomics GmbH, Berlin, Germany. Amplicon reads were analysed using the QIIME pipeline, and singletons and chimaeras were removed using USEARCH v7 [28] and UCHIME [29]. OTU binning was carried out against the GreenGenes database [30].

Metagenomics and bioinformatics

Metagenome sequencing was carried out using DNA from [^{13}C]- CH_3Cl -amended microcosms in three biological replicates, together with three replicates of [^{12}C]- CH_3Cl -amended microcosms. Library preparation and sequencing were performed at LGC Genomics GmbH, Berlin, Germany.

Reads of Raw Fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 [31]. The reads were further trimmed by Sickle version 1.200 [32] applying a minimum window quality score of 20. Quality trimmed metagenome reads were then assembled using metaSPAdes v3.11.1 [33] and binned with MyCC version MyCC_2017 [34] using default settings. Estimation of genome completeness and contamination was carried out using the CheckM program [35]. Taxonomic assignment of each bin was carried out by submitting bins to the Rapid Annotation using Subsystem Technology (RAST) annotation pipeline ('Classic RAST' pipeline) [36]. Average Nucleotide/Amino Acid Identity (ANI/AAI) [37] between different genomes was estimated using one-way ANI (best hit) and two-way ANI (reciprocal best hit) based on Goris et al. [38]. In order to infer phylogenetic relatedness whole genome phylogenetic trees were constructed using Composition Vector Tree (CVTree) [39]. Furthermore, Bins were screened for 16S RNA-encoding gene sequences using rnammer version 1.2 [40] with option -S bac and -m ssu. To search for presence of functional genes involved in CH_3Cl degradation within the bins, bins were annotated using Prokka (v1.12) [41] and BlastP [42] searches (cutoff $1\text{e}-30$, > 70% identity, manual check of chromosomal region) were carried out against annotated bins (MAGs) using characterized proteins of *cmuA*. Furthermore, ShortBRED (version 0.9.3) [43] and GraftM (version 0.13.0) [44] were used to identify *cmuA* in unassembled metagenomic reads, as described previously [17]. To estimate the distribution of MAGs in public available datasets, metagenomics reads from various regions (Supplementary Table S2) were downloaded from sequence read archive (SRA) using sra-tools 2.8.0. Metagenomic short reads were mapped to Mineral Soil Bin2 using BBmap (v38.79) [45] with an identity threshold of 97% (--idfilter=0.97). Bedtools intersect (v2.29.2)

[46] was used to exclude regions that also displayed high identity alignments of length > 50 bp to the RefSeq database. These ambiguous regions were previously identified by aligning the bin against ncbi's RefSeq database (downloaded 26.08.2021) using blast+ [47] and filtering for pident >=97 (detailed code: <https://github.com/SonWende/MAG2metagenome>).

Accession numbers for datasets

SIP 16S rRNA amplicon sequencing and SIP metagenome read data of the Steigerwald soils and degraded leaves have been submitted to the National Center for Biotechnology Information (NCBI) under the BioProject number PRJNA742226.

Results and Discussion

We measured the CH₃Cl degradation potential and identified the bacterial players involved in CH₃Cl degradation in deciduous forest soil samples by stable isotope labelling and probing experiments using leaf litter, degraded (senescent, partially decomposed) leaves, organic and mineral soil. These samples were incubated in triplicates with either [¹³C]-CH₃Cl or [¹²C]-CH₃Cl and CH₃Cl concentrations were monitored over 650 hours.

CH₃Cl degradation potential is highest in top layers of the forest floor

Rapid and steady consumption of CH₃Cl was observed in all the tested samples of leaf litter, degraded leaves, organic and mineral soils. Degraded leaves and organic soil displayed the highest CH₃Cl dissipation rates around 1.47 and 1.40 mM CH₃Cl g⁻¹ DW h⁻¹ in [¹³C]-CH₃Cl supplemented condition, respectively (Supplementary Figure S1A). CH₃Cl dissipation rates in [¹²C]-CH₃Cl incubated samples reached 2.05 and 2.02 mM CH₃Cl g⁻¹ DW h⁻¹ in degraded leaves and leaf litter, respectively (Supplementary Figure S1B). On the other hand, the lowest CH₃Cl dissipation rates were observed with the mineral soil (Supplementary Figure S1A,B). Furthermore, control incubations with potassium cyanide did not lead to any CH₃Cl degradation, confirming that the process is biotic. Thus, the top layers of the forest ground (leaf litter, degraded leaves, and organic horizon) are the most active layers for CH₃Cl degradation in the tested conditions, as previously found in a study of the same deciduous forest sampling site by Chaignaud et al. 2018 [23].

Identification of 2 bacterial genome bins exclusively found in [¹³C]-CH₃Cl treated microcosms

In order to gain insights into the active CH₃Cl-degrading community in the forest soil samples, DNA-SIP microcosms using [¹³C]-CH₃Cl or [¹²C]-CH₃Cl were combined with high-throughput sequencing of the 16S RNA 'heavy' and 'light' fractions and of the metagenomic DNA of [¹³C]-CH₃Cl and [¹²C]-CH₃Cl fractions. 16S rRNA amplicon sequencing showed e.g., an enrichment of *Actinobacteria* in the [¹³C]-CH₃Cl 'heavy' fractions compared to [¹³C]-CH₃Cl 'light' fraction (Supplementary Figure S3). Metagenome reads were assembled and assigned into individual bins (Figure 1). This resulted in the assignment of a total of 105 bins, comprising of 14-24 bins from each 'heavy' fraction ([¹³C]-CH₃Cl and [¹²C]-CH₃Cl 'heavy' fractions for degraded leaf samples and organic and mineral soil samples). Two taxa of ¹³C-labelled bacterial genome bins did not occur in the ¹²C control treatments and occurred exclusively in the [¹³C]-CH₃Cl treatment: i.e. 'Mineral Soil Bin 2' and 'Degraded leaves bin 11' (Figure 1). We reconstructed genome fragments (so called metagenome assembled genomes, MAGs) of the [¹³C]-CH₃Cl 'heavy' samples, and chose 7 MAGs with the highest quality (>70% completeness and <10% contamination) for further analyses (Supplementary Table S1). Among those, 'Mineral Soil Bin 2' and 'Degraded leaves bin 11' harboured no 16S RNA-encoding genes. Taxa assignment was proposed according to the closest representative found in the RAST database (Supplementary Table S1). The first represented a member of the *Alphaproteobacteria* (retrieved from mineral soil) and the second represented a member of *Acidobacteria* (retrieved from the organic layer and degraded leaves). We reconstructed - based on annotations - relevant metabolic pathways of these two MAGs and refined their phylogenetic affiliation.

Figure 1. Visual comparison of binned ‘heavy’ control [^{12}C]- CH_3Cl and labelled [^{13}C]- CH_3Cl incubated met-
agenomes from degraded leaves, organic soil and mineral soil. Microbial names that are written in grey
in the ^{13}C - CH_3Cl ‘heavy’ incubation sample are present in ^{12}C - and ^{13}C -‘heavy’ fractions and are therefore
not involved in CH_3Cl degradation. Microbial names that are written in black are enriched in the ^{13}C -‘heavy’
fraction compared to the ^{12}C -‘heavy’ fraction. Names that are written in bold are microorganisms that are
present exclusively in ^{13}C -‘heavy’ fractions and therefore most likely involved in the degradation of CH_3Cl
and were used for further analysis.

Recovery of a population genome of putatively novel family of Alphaproteobacteria retrieved from metagenomes of ¹³C-labelled DNA

The MAG exclusively found in the [¹³C]-CH₃Cl-labelled mineral soil metagenome was related to genomes of *Methylocella* (Mineral Soil Bin2) (Figure 1). This MAG had a completeness of about 75% and a contamination of about 4% (Supplementary Table 1). A phylogenetic tree based on whole genome sequences suggested that this MAG most likely represents a new family closely related to the families *Beijerinckiaceae* and *Methylobacteriaceae* within the class of *Alphaproteobacteria* (Figure 2). We performed comparative genome analyses of average nucleotide identity (ANI) and average amino acid identity (AAI) against closely related genomes from the families *Beijerinckiaceae* and *Methylobacteriaceae*, and the data placed this MAG in a novel clade (Figure 2).

The MAG showed 54.4% AAI to the closely related bacterium *Methylocella silvestris* and between 52–54% AAI to genomes of the family *Methylobacteriaceae* (Figure 2). Therefore, phylogenetic analyses of the whole genome and ANI analysis both strongly suggest that this unclassified group of CH₃Cl-enriched bacteria forms a novel family within the class of *Alphaproteobacteria*. Similarly, in the previous study by Chaignaud et al. [23] on the same deciduous forest soil, microorganisms related to *Beijerinckiaceae* were also enriched in CH₃Cl-amended stable isotope experiments and only identified on the basis of a partial 16S rRNA sequence so that its phylogenetic positioning remained approximate. To assess the breath of distribution of ‘Mineral Soil Bin 2’ in the environment, genome mapping was performed by recruiting metagenomic reads from environmental metagenomes using its ~75% complete genome. Total number of reads that are mapped to ‘Mineral Soil Bin 2’ can be highly variable (Supplementary Table S2) but detected at high abundance in forest soil samples across the globe (Figure 4). Moreover, reads mapped to the ‘Mineral Soil Bin 2’ were also detected in soil with different land uses (Figure 4). Highly related OTUs to the closely related *Beijerinckia* detected by Chaignaud et al. are present in the same forest soil samples across the globe (Supplementary Table S2).

Figure 2. Phylogenetic analysis of whole genome sequences, average nucleotide identity (ANI) and average amino acid identity (AAI) analyses [34] suggest that the MAG “Mineral soil bin 2” is a novel *Alphaproteobacterium*.

Reconstruction of the metabolic pathways revealed that the ‘Mineral soil bin 2’ MAG contained two known pathways to oxidize aerobically methyl-groups to carbon dioxide (Figure 3) – the tetrahydrofolate (H₄F) and the tetrahydromethanopterin (H₄MPT)-dependent formaldehyde oxidation pathways. However, we could not identify any genes that may code for a dehalogenating enzyme, such as genes *cmuAB* (assessed by BLAST searches and via ShortBRED and GraftM – data not shown). Also, since Chaignaud et al. could not detect *cmuA* from *Beijerinckiaceae* via PCR amplification, the authors suggested that it uses another CH₃Cl degradation pathway [23]. Despite not having characterized a dehalogenating enzyme, the same predominantly ¹³C-labelled taxon may have been labelled in this study and in the previous SIP study by Chaignaud et al. as both studies were conducted with the same forest soil sampling site [23]. Thus, we conclude that this ‘Mineral soil bin 2’ MAG is most likely responsible for the degradation of the methyl-group CH₃Cl in these deciduous forest mineral soil samples. Nonetheless, the CH₃Cl dehalogenating enzyme has yet to be characterized in this taxon.

The ~75% complete genome sequences retrieved from the [¹³C]-CH₃Cl DNA SIP-derived MAG provided the opportunity to explore the metabolic potential in this novel bacterium. Figure 3 summarizes its reconstructed metabolic pathways. Complete gene sets for gluconeogenesis, glycolysis and the Entner-Doudoroff pathway were found. Acetyl-phosphate, which is channelled to the central carbon metabolism through acetyl-CoA is converted to generate ATP, and the gene encoding an acetate kinase is also present in the genome. A gene coding for a succinate dehydrogenase is also found. This MAG contains the genes for two known pathways for oxidation of methyl-groups – the H₄F and the H₄MPT-dependent pathways, which result in the formation of formate and subsequent carbon dioxide. Genes for the serine cycle or a methane monooxygenase (sMMO/pMMO) were not detected. On the other hand, several alcohol dehydrogenases (EC 1.1.1.1) were found in the MAG. *In situ* and microcosm studies on forest soils pointed towards soil methylotrophs utilizing CH₃Cl at low concentration in ppt range that may lead to a limitation of energy conservation needed for bacterial growth [22]. In soils, CH₃Cl amounts for methylotrophic consumption are much lower than methanol, and key CH₃Cl degraders use alternative carbon sources such as methanol to subsidize the energy requirements [23]. Many *Methylobacteriaceae* are motile and possess a single polar, subpolar or lateral flagellum [48]. The ‘Mineral soil bin 2’ MAG does not possess genes encoding for flagellar proteins and is therefore distinct from many members of the closely-related *Methylobacteriaceae* (Figure 2 and 3). In addition, *Methylobacteriaceae* normally possess the serine cycle for formaldehyde assimilation [49], which is also absent in ‘Mineral soil bin 2’ MAG (Figure 3) and hence distinct from these members. Some members of the family *Beijerinckiaceae*, such as *Beijerinckia* and *Methylocella* are diazotrophs [50] with the ability to reduce nitrogen (N₂) to ammonia (NH₃) or ammonium (NH₄⁺). The closely related ‘Mineral soil bin 2’ MAG does not possess genes encoding for nitrogen fixation and is therefore distinct from members of *Beijerinckia* and *Methylocella* (Figure 2 and 3). These observations strengthen our conclusion that this on CH₃Cl enriched MAG represents a novel family within the class of *Alphaproteobacteria*.

A second ¹³C-labelled MAG suggested involvement of *Acidobacteria* in C fluxes from CH₃Cl 350

The second MAG ‘Senescent Leaves bin 11’ was highly similar to genomes of the *Acidobacterium* 351
Granulicella mallensi, of which members are Gram-negative aerobe rods that have been isolated from 352
soils [51]. The high relatedness of ANI >87% suggests that it represents a strain of this species (Sup- 353
plementary Figure S4). Here, a H₄F-dependent pathway for formaldehyde oxidation was present (Sup- 354
plementary Figure S5). This pathway is well known to function for formaldehyde detoxification. Whether 355
the MAG ‘Senescent Leaves bin 11’ was labelled by ¹³C-carbon dioxide or even formaldehyde cross- 356
feeding or directly through utilization of CH₃Cl cannot be excluded. Nonetheless, MAG ‘Senescent 357
Leaves bin 11’ was ¹³C-labelled. 358

Figure 4. Global distribution of ‘Mineral soil bin 2’ MAG in forest, grassland and agricultural soil. The map 360
was created with Tableau 2021.3 361

Conclusions 362

Our results suggest that a new key family of aerobic *Alphaproteobacteria*, ‘Mineral soil bin 2’ MAG, may 363
be responsible for the degradation of the methyl group of CH₃Cl. However, this organism has not been 364
cultivated and the mechanisms of CH₃Cl dehalogenation have not been identified. We did not detect 365
cmu genes in the MGA corresponding to this taxon. This may be explained by the *cmu* gene plasmid 366
location, as found in *M. extorquens* CM4 [52], which might have thereby escaped metagenome assem- 367
bly and binning within this MAG. Nonetheless, no *cmuA* genes were detected in the unassembled met- 368
agenomic reads, which strongly suggests an alternative *cmu*-independent mechanism or co-metabolic 369
conversion through other soil organisms. 370
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Combining DNA-SIP with metagenomics and assembly allowed the retrieval of a genomes of a puta- 373
tively novel family of *Alphaproteobacteria* involved in CH₃Cl degradation in a deciduous forest soil. The 374
results presented in this work demonstrated the power of multi-methodological approaches combining 375
stable isotope labelling and omics to uncover metabolic functions in as-yet uncultivated novel environ- 376
mental microbes. 377

Declarations	413
<i>Ethics approval and consent to participate</i>	414
Not applicable	415
	416
<i>Consent for publication</i>	417
Not applicable	418
	419
<i>Availability of data and material</i>	420
Read data of the Steigerwald soil and degraded leave SIP metagenomes have been submitted to the	421
National Center for Biotechnology Information (NCBI) under the BioProject number PRJNA742226.	422
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<i>Competing interests</i>	424
The authors declare that they have no competing interests.	425
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<i>Authors' contributions</i>	430
Conceptualization, S.Ko. and E.K.; methodology, E.K.; software, E.K. and S.W.; data analysis and	431
pathway reconstruction, E.K.; writing - original draft preparation, E.K. and S.Ka.; writing - review and	432
editing, S.Ko. F.B.; visualization, E.K.; supervision, S.Ko.; project administration, S.Ko.; funding	433
acquisition, S.Ko. All authors read and approved the final manuscript.	434
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Supplementary Information	443
	444
Supplementary Figure S1: CH ₃ Cl degradation in deciduous forest soil samples during SIP incubations.	445
	446
Supplementary Figure S2: Relative distribution of terminal restriction fragments detected by bacterial	447
T-RFLP of fractionated DNA from [¹³ C]-CH ₃ Cl and [¹² C]-CH ₃ Cl SIP incubations of mineral and or-	448
ganic soil samples and degraded leave samples from Steigerwald after incorporation of 100 µmol	449
C g ⁻¹ sample,	450
	451
Supplementary Figure S3: Relative distribution and phylogenetic affiliation of OTUs detected by bac-	452
terial 16S rRNA high-throughput amplicon sequencing of samples before SIP experiments (T0)	453
and fractionated DNA from [¹³ C]-CH ₃ Cl and [¹² C]-CH ₃ Cl SIP incubations of mineral and organic	454
soil samples and degraded leave samples from Steigerwald after incorporation of 100 µmol C g ⁻¹	455
sample.	456
	457
Supplementary Figure S4: Average nucleotide identity (ANI) analysis of “Degraded leaves bin 11” and	458
the closely related <i>Granulicella mallensis</i> and AAI analysis between those two species.	459
	460
Supplementary Figure S5: Metabolic reconstruction of [¹³ C]-CH ₃ Cl labelled metagenome assembled	461
genome “Degraded leaves bin 11” (closely related to <i>Granulicella mallensis</i>).	462
	463
Supplementary Table S1: Summary of metagenome-assembled genomes (MAGs) from the CH ₃ Cl-	464
stable isotope probing enrichment.	465
	466
Supplementary Table S2: Metagenomes used for read-recruitment against ‘Mineral soil bin 2’ MAG	467
and the closely related OTU from Chaignaud et al. 2018.	468
	469
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