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► **To cite this version:**

Nicole Jaeger, Ludovic Besaury, Amelie Ninja Röhlings, Fabien Koch, Anne-Marie Delort, et al.. Chloromethane formation and degradation in the fern phyllosphere. *Science of the Total Environment*, 2018, 634, pp.1278-1287. 10.1016/j.scitotenv.2018.03.316 . hal-01801618

**HAL Id: hal-01801618**

**<https://hal.science/hal-01801618v1>**

Submitted on 2 Jul 2018

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## Chloromethane formation and degradation in the fern phyllosphere



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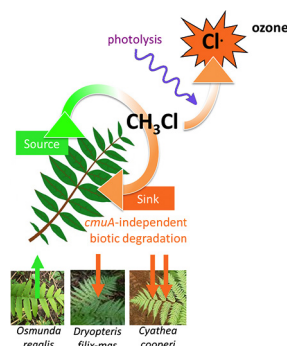
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### HIGHLIGHTS

- Ferns both produce and degrade atmospheric CH<sub>3</sub>Cl with large individual variations.
- Ferns degrade CH<sub>3</sub>Cl at rates ranging from 0.3 to 17 μg·g<sub>(dry weight)</sub><sup>-1</sup> day<sup>-1</sup>.
- CH<sub>3</sub>Cl degradation was correlated to a large ε<sub>C</sub> and almost no ε<sub>H</sub> isotope effect.
- Involvement of the bacterial *cmu* pathway in CH<sub>3</sub>Cl degradation was not detected.
- Still unknown CH<sub>3</sub>Cl biodegradation processes in plants contribute to the CH<sub>3</sub>Cl cycle.

### GRAPHICAL ABSTRACT



### ARTICLE INFO

#### Article history:

Received 27 September 2017

Received in revised form 25 March 2018

Accepted 25 March 2018

Available online 18 April 2018

Editor: P. Kassomenos

#### Keywords:

Biodegradation  
Chloromethane  
Isotope fractionation  
Ferns  
Phyllosphere  
Methylotrophic bacteria

### ABSTRACT

Chloromethane (CH<sub>3</sub>Cl) is the most abundant halogenated trace gas in the atmosphere. It plays an important role in natural stratospheric ozone destruction. Current estimates of the global CH<sub>3</sub>Cl budget are approximate. The strength of the CH<sub>3</sub>Cl global sink by microbial degradation in soils and plants is under discussion. Some plants, particularly ferns, have been identified as substantial emitters of CH<sub>3</sub>Cl. Their ability to degrade CH<sub>3</sub>Cl remains uncertain. In this study, we investigated the potential of leaves from 3 abundant ferns (*Osmunda regalis*, *Cyathea cooperi*, *Dryopteris filix-mas*) to produce and degrade CH<sub>3</sub>Cl by measuring their production and consumption rates and their stable carbon and hydrogen isotope signatures. Investigated ferns are able to degrade CH<sub>3</sub>Cl at rates from 2.1 to 17 and 0.3 to 0.9 μg g<sub>dw</sub><sup>-1</sup> day<sup>-1</sup> for *C. cooperi* and *D. filix-mas* respectively, depending on CH<sub>3</sub>Cl supplementation and temperature. The stable carbon isotope enrichment factor of remaining CH<sub>3</sub>Cl was  $-39 \pm 13\%$ , whereas negligible isotope fractionation was observed for hydrogen ( $-8 \pm 19\%$ ). In contrast, *O. regalis* did not consume CH<sub>3</sub>Cl, but produced it at rates ranging from 0.6 to 128 μg g<sub>dw</sub><sup>-1</sup> day<sup>-1</sup>, with stable isotope values of  $-97 \pm 8\%$  for carbon and  $-202 \pm 10\%$  for hydrogen, respectively. Even though the 3 ferns showed clearly different formation and consumption patterns, their leaf-associated bacterial diversity was not notably different. Moreover, we did not detect genes associated with the only known chloromethane utilization

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pathway “*cmu*” in the microbial phyllosphere of the investigated ferns. Our study suggests that still unknown CH<sub>3</sub>Cl biodegradation processes on plants play an important role in global cycling of atmospheric CH<sub>3</sub>Cl.

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## 1. Introduction

Since the Montreal Protocol came into force in 1987, anthropogenic emissions of ozone-depleting substances have been strongly reduced, and the relative proportion of halocarbon emissions from natural sources has increased (Carpenter and Reimann, 2014). Chloromethane (CH<sub>3</sub>Cl, also named methyl chloride) is the most abundant chlorinated organic compound in the atmosphere, with an estimated global CH<sub>3</sub>Cl emission rate of 4 to 5 Tg (1 Tg = 10<sup>12</sup> g) per year (Xiao et al., 2010; Keppler et al., 2005). This estimate is based on its mean mixing ratio of 540 ± 5 parts per trillion by volume (pptv) and atmospheric lifetime of 1.0–1.2 years.

CH<sub>3</sub>Cl is predominantly emitted from natural sources, including tropical and senescent plants (Yokouchi et al., 2002; Hamilton et al., 2003; Yokouchi et al., 2015; Blei et al., 2010), grasslands (Rhew and Abel, 2007; Teh et al., 2008; Rhew, 2011), peatlands (Dimmer et al., 2001), salt marshes (Rhew et al., 2000; Valtanen et al., 2009), aerated and flooded soil (Keppler et al., 2000; Redeker et al., 2000), wood-rotting fungi (Harper, 1985) and oceans (Moore et al., 1996), but also from anthropogenic sources, like biomass burning or industrial emissions (Carpenter and Reimann, 2014). However industrial sources have been considered minor (Keene et al. 1999), although very recently it has been reported that CH<sub>3</sub>Cl emission from industrial sources particularly from China might be much higher than previously assumed (Li et al., 2016). Within natural sources in particular, ferns are strong CH<sub>3</sub>Cl emitters under diverse climate conditions, including tropical, subtropical, temperate and cool-temperate zones (Yokouchi et al., 2002, 2015), with CH<sub>3</sub>Cl emission rates up to a few μg g<sub>dw</sub><sup>-1</sup> h<sup>-1</sup>. The best characterized biogenic CH<sub>3</sub>Cl production is an enzymatic mechanism. A protein encoded by the gene *HOL* (harmless to the ozone layer) catalyzes the *S* adenosyl L methionine (SAM)-dependent methylation of chloride (Cl<sup>-</sup>) (Wuosmaa and Hager, 1990; Ni and Hager, 1999; Rhew et al., 2003). A high *HOL*-type enzyme activity was detected in the highly CH<sub>3</sub>Cl-forming fern *C. lepifera* (Yokouchi et al., 2002; Itoh et al., 2009). Nonetheless, the factors controlling this rate are poorly understood. Furthermore, abiotic production of CH<sub>3</sub>Cl in plant material has also been reported, with carbon originating from pectin and lignin methoxyl groups (Hamilton et al., 2003; Wishkerman et al., 2008; Sallaukhanuly et al., 2014). This ubiquitous process is active in terrestrial ecosystems at ambient temperatures, but is much more efficient at higher temperatures, such as during biomass burning (Hamilton et al., 2003; McRoberts et al., 2015).

The dominant sink for atmospheric CH<sub>3</sub>Cl results from the reaction with photochemically-produced OH radicals, currently estimated at about 2.8 Tg year<sup>-1</sup> (Carpenter and Reimann, 2014). Microbial CH<sub>3</sub>Cl degradation in soils may be a relevant additional global sink (McAnulla et al., 2001b; Harper and Hamilton, 2003; Miller et al., 2004; Jaeger et al., 2018), but its impact on the global CH<sub>3</sub>Cl budget is uncertain. The microbial CH<sub>3</sub>Cl soil sink strength has been estimated to range from 0.1 to 1.6 Tg year<sup>-1</sup> (Carpenter and Reimann, 2014; Harper and Hamilton, 2003; Keppler et al., 2005). Different types of methylotrophic bacteria capable of utilizing CH<sub>3</sub>Cl as the only carbon and energy source have been isolated from various environments, including soils (Doronina et al., 1996; McAnulla et al., 2001b; Miller et al., 2004), sludge (Hartmans et al., 1986; Trauneker et al., 1991; Freedman et al., 2004.), seawater (Schäfer et al., 2005), and plant leaves (Nadalig et al., 2011). Consumption potentials of CH<sub>3</sub>Cl in soils (Miller et al., 2004; Jaeger et al., 2018) and the plant environment of the phyllosphere (above-ground portions of plants) (Saito et al., 2013) have been investigated. When both foliar production and consumption

of CH<sub>3</sub>Cl were evaluated, large net emissions of CH<sub>3</sub>Cl were found (Saito et al., 2013). Indeed, plants colonized with methylotrophic microorganisms able to consume volatile one-carbon compounds exhibit lower emission rates (Ababda-Nkpwatt et al., 2006). Bacteria able to degrade CH<sub>3</sub>Cl have been isolated from plant leaves and may utilize the *cmu* chloromethane utilization pathway (Nadalig et al., 2014). The only CH<sub>3</sub>Cl degradation pathway characterized in detail, was mainly studied in *Methylobacterium extorquens* CM4, an aerobic methylotrophic strain able to use CH<sub>3</sub>Cl as the sole source of carbon and energy (Chaignaud et al., 2017; Roselli et al., 2013; Vanelli et al., 1999). In this pathway, the CH<sub>3</sub>Cl dehalogenase includes a corrinoid methyltransferase (*CmuA*) and a tetrahydrofolate-dependent methyltransferase (*CmuB*) (Studer et al., 2001, 1999). Bacterial isolates known to degrade CH<sub>3</sub>Cl by the *cmu* pathway are taxonomically diverse and include organisms affiliated with 7 genera so far (*Acetobacterium*, *Aminobacter*, *Hyphomicrobium*, *Leisingera*, *Methylobacterium*, *Pseudomonas* and *Roseovarius*) (Nadalig et al., 2011). Nevertheless, other as yet uncharacterized pathways for CH<sub>3</sub>Cl degradation exist, such as in *Leisingera methylohalidivorans* MB2, as demonstrated using stable isotope fractionation of carbon and hydrogen (Nadalig et al., 2014).

Two-dimensional stable isotope approach of hydrogen and carbon can be used to better understand the processes of CH<sub>3</sub>Cl biodegradation and formation. Combined with CH<sub>3</sub>Cl flux measurements, it can help constrain the strengths of CH<sub>3</sub>Cl sinks and sources within the global CH<sub>3</sub>Cl budget (Keppler et al., 2005; Saito and Yokouchi, 2008). Isotope fractionation occurs due to enzymatic discrimination of heavier isotopes against their lighter counterparts, because of the lower energy required for reaction by the latter. In the case of chloromethane, carbon directly present in the key C—Cl bond defines the so-called primary isotope effect, whereas hydrogen is affected by a secondary isotope effect (Elsner et al., 2005). A few studies have measured the stable isotope fractionation of carbon and hydrogen during CH<sub>3</sub>Cl biodegradation by bacterial isolates (Miller et al., 2001; Nadalig et al., 2013, 2014) or in soils under laboratory conditions (Miller et al., 2004; Jaeger et al., 2018). For plants, several studies have investigated the stable isotope signature of CH<sub>3</sub>Cl produced via biotic (Saito and Yokouchi, 2008; Harper et al., 2003) and abiotic processes (Keppler et al., 2004; Greule et al., 2012).

The main aim of this study was to investigate the CH<sub>3</sub>Cl degradation potential by ferns known to produce CH<sub>3</sub>Cl (Yokouchi et al., 2002, 2015). We determined both the production and consumption rates of CH<sub>3</sub>Cl of several abundant ferns. Using a stable isotope tracer technique, we differentiated between formation and degradation processes within the same fern, and assessed the correlation between ability for CH<sub>3</sub>Cl biodegradation and phyllospheric occurrence of bacterial taxa previously described to be associated with CH<sub>3</sub>Cl degradation.

## 2. Material and methods

### 2.1. Plant material and sampling

Plant samples were collected in the Botanical Garden of Heidelberg University. Leaf samples of *Cyathea cooperi* (accession number: 108562), *Osmunda regalis* (accession number: 004923) and *Dryopteris filix-mas* (accession number: 002834) were collected in May, July and September 2016, respectively. In the laboratory, fern leaves were separated in detached pinnules according to Yokouchi et al. (2015) (for *D. filix-mas* the primary and for *C. cooperi* and *O. regalis* the secondary division of a pinnate leaf of a fern). The incubation of the collected leaves started within 2 h after sampling.

## 2.2. Stable isotope tracer-based measurements for chloromethane consumption and production rates

Fresh fern leaf samples (5 g) were incubated in 170 mL gastight incubation flasks at 20 °C and spiked with 5 parts per million by volume (ppmv) of the stable isotope  $^{13}\text{CH}_3\text{Cl}$  (99.7 at.% labeled). The incubations were stopped after a maximum of 6 h. The simultaneous measurement of  $^{12}\text{CH}_3\text{Cl}$  and  $^{13}\text{CH}_3\text{Cl}$  concentrations at different time points was used to determine gross production and uptake rates. The headspace was investigated for at least 4 time points. Therefore, 200  $\mu\text{L}$  sample were injected in a flow of 1 mL/min of helium (split ratio 5:1) on a GasPro column (60 m  $\times$  0.32 mm i.d., Agilent Technologies) and isothermally (150 °C) quantified by a Hewlett Packard HP 6890 gas chromatograph coupled to a MSD 5973 mass spectrometer (GC–MS, Agilent Technologies, Palo Alto, CA). By single ion monitoring mode the four stable isotopologues of  $\text{CH}_3\text{Cl}$  were detected ( $m/z = 50$  for  $^{12}\text{CH}_3^{35}\text{Cl}$ ,  $m/z = 52$  for  $^{12}\text{CH}_3^{37}\text{Cl}$ ,  $m/z = 51$  for  $^{13}\text{CH}_3^{35}\text{Cl}$ ,  $m/z = 53$  for  $^{13}\text{CH}_3^{37}\text{Cl}$ ). However, during the detection via the quadrupole mass spectrometer ion fragmentation occurs and need to be corrected in such isotope tracer studies (Rhew and Abel, 2007), since e.g.  $^{13}\text{CH}_2\text{Cl}^+$  yields the same  $m/z$  as  $^{12}\text{CH}_3\text{Cl}$ . Therefore  $^{12}\text{CH}_3\text{Cl}/^{13}\text{CH}_3\text{Cl}$  fragmentation ratios were determined experimentally and detected areas corrected for it. Gross production ( $^{12}\text{CH}_3\text{Cl}$ ) and uptake ( $^{13}\text{CH}_3\text{Cl}$ ) rates were calculated by the slope of a linear fit applied to the dry weight based concentration of the respective isotopologues ( $\mu\text{g g}_{\text{dw}}^{-1}$ ) versus time (day).

Additionally, to check for biotic degradation and to exclude abiotic production with increasing temperature, the ferns that showed net  $\text{CH}_3\text{Cl}$  degradation (*C. cooperi*, *D. filix-mas*) were spiked with 10 ppmv  $^{13}\text{CH}_3\text{Cl}$  and incubated at increasing temperature in 10 °C steps until net  $\text{CH}_3\text{Cl}$  uptake rates decreased.

Furthermore *C. cooperi* and *D. filix-mas* samples were incubated to assess their production potential. These incubations did not include the  $^{13}\text{CH}_3\text{Cl}$  stable isotope tracer described in the section before, thereby avoiding the possibility of reversing an enzyme mediated production reaction (i.e., making a source into a sink).

## 2.3. Determination of chloromethane consumption rates

*C. cooperi* samples (20 g) were incubated in triplicate in gastight incubation flasks (800 mL) at different temperatures (5, 20, 30 °C). Samples were first equilibrated to the respective incubation temperature, before headspace was spiked with 10 and 100 ppmv  $\text{CH}_3\text{Cl}$  (99.8%). The headspace was sampled at least at 4 time points until the remaining fraction was <50%. Chloromethane was directly quantified by GC–MS and net uptake rates were calculated by a linear fit applied to the dry weight based concentration ( $\mu\text{g g}_{\text{dw}}^{-1}$ ) versus time (day). The resulting slope represents  $-k_{\text{net}}$ . For IRMS measurements, at each time point, 25 mL of the headspace was stored in 12 mL Exetainer® (Labco Limited, Lampeter, UK) until analyses. For the bacterial investigations, plant leaf samples were collected and stored at  $-18$  °C, both at the beginning and at the end of the incubation experiments (maximal 6 h).

## 2.4. Determination of chloromethane production rates

*O. regalis* samples (5 g) were incubated in triplicate in gastight incubation flasks (170 mL) at different temperatures (5, 20, 30, 40 °C). The headspace was sampled at least at 4 time points and  $\text{CH}_3\text{Cl}$  was directly quantified by GC–MS. Net production rates were calculated by the slope of a linear fit applied to the dry weight based concentration ( $\mu\text{g g}_{\text{dw}}^{-1}$ ) versus time (day). Additionally, at the end of the incubation we sampled 25 mL of the headspace for IRMS measurements, stored in 12 mL Exetainer® until analyses. At both the beginning and the end of the incubation experiments (maximal 6 h), we collected *O. regalis* samples for the bacterial investigations and stored them at  $-18$  °C.

## 2.5. Stable isotope analysis of chloromethane

Stable hydrogen and carbon isotope ratios of  $\text{CH}_3\text{Cl}$  were measured by an in-house built cryogenic pre-concentration unit coupled to a Hewlett Packard HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) and an isotope ratio mass spectrometer (IRMS) (Isoprime, Manchester, UK) according to Nadalig et al. (2013) and Jaeger et al. (2018). The conventional delta notation, expressing the isotopic composition of the sample relative to that of V-SMOW standard (Vienna Standard Mean Ocean Water) for hydrogen ( $\delta^2\text{H}_{\text{V-SMOW}}$ ) and V-PDB standard (Vienna Pee Dee Belemnite) for carbon ( $\delta^{13}\text{C}_{\text{V-PDB}}$ ) on per mil basis is used. The isotope fractionation factor  $\alpha$  and the isotope enrichment factor  $\epsilon$  are derived from the slope of the Rayleigh plot according to Elsner et al. (2005) and Elsner (2010). Negative  $\epsilon$  indicates that the remaining  $\text{CH}_3\text{Cl}$  is enriched in the heavier isotope and corresponds to an  $\alpha < 1$ , meaning that the heavier [ $^{13}\text{C}$ ]- $\text{CH}_3\text{Cl}$  reacted more slowly by this factor than the lighter [ $^{12}\text{C}$ ]- $\text{CH}_3\text{Cl}$  during incubation.

## 2.6. Determination of stable isotope values of plant methoxyl groups

Stable hydrogen and carbon isotope values of plant methoxyl groups ( $\text{OCH}_3$ ) can be measured without isotope fractionation as iodomethane ( $\text{CH}_3\text{I}$ ) upon treatment of plant matter with hydriodic acid. Stable isotope values of methoxyl groups of the fern samples were measured according to the procedure described by Greule et al. (2008, 2009). Briefly, 250  $\mu\text{L}$  hydriodic acid (55–58%; Fluka, Buchs, Switzerland) were added to 40 mg of dried and milled *O. regalis* samples and heated at 130 °C for 30 min. After equilibration to room temperature, the stable isotope values of produced  $\text{CH}_3\text{I}$  were measured by Hewlett Packard HP 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to Delta<sup>PLUS</sup> XL isotope ratio mass spectrometer (ThermoQuest Finnigan, Bremen, Germany).

## 2.7. DNA extraction from fern phyllosphere

To harvest the microbial cells associated with the plant, leaf samples (5 to 10 g) were immersed in sterile 50 mL of potassium phosphate buffer (0.1 M, pH 8.0) in a 100 mL sterile Schott-bottle plunged into an ultrasonication bath for 5 min. The bottles were then shaken manually and vortexed twice. The microbial cells of the supernatant were collected by filtration on a GNWP 0.22  $\mu\text{m}$  filter (Millipore, France) and stored at  $-20$  °C. Microbial DNA was extracted directly from the filters using MoBio's Power Water DNA Isolation kit according to the manufacturer's protocol. DNA was quantified using the ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). DNA quality was controlled after electrophoresis on a 0.8% (w/v) agarose gel made with Tris acetate EDTA buffer (TAE 1  $\times$  buffer) and stored at  $-20$  °C.

## 2.8. PCR amplification, and sample pooling

DNA was used as template for 16S rRNA gene amplification in PCR reactions with universal primers 799F and 1193r targeting the V5, V6 and V7 regions of the gene and barcoded for multiplexed sequencing (Table SM1) as described (Bulgarelli et al., 2015). Each 50  $\mu\text{L}$  PCR reaction contained 1 ng of DNA, 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM  $\text{MgCl}_2$ , 200 mM dNTP, 200 nM of each reverse and forward primer and 0.8 units of Taq DNA Polymerase (MP Biomedicals). The targeted amplicons sizing 394 bp were isolated on 2% agarose gel using QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions, and quantified using Qubit (ThermoFisher, France) and pooled at equimolar concentrations. Sequencing was done at a final library concentration of 5 pM and incorporation of the control library PhiX at 15% (V/V). This control shotgun "library" was built from the genome of the PhiX174 phage. The resulting pool was sequenced on Illumina Miseq 2  $\times$  250 bp (GenoScreen, France).

DNA was also used to amplify an inter-domain region of the protein-encoding gene *cmuA* of 422 bp. The 50  $\mu$ L PCR reaction volume contained 1  $\times$  buffer including  $MgCl_2$ , 0.8 mM dNTPs (0.2 mM each dNTP), 1121 nM of primer *cmuA*f422 and 258 nM of primer *cmuA*r422 (Table SM 1, 2 U Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, Carlsbad, USA), template DNA (1–10 ng). PCR program had an initial step at 95 °C for 10 min, followed by 30 cycles of 15 s at 94 °C (denaturation) and 56 °C for 60 s (hybridization/elongation), and a final extension step at 72 °C for 5 min.

### 2.9. Bioinformatics analysis

Illumina reads (2,805,832 sequences with an average contig length of 411 bp) were analyzed using Mothur software package (v 1.39.1; Schloss et al., 2009) with the Miseq standard operating procedure (<http://www.mothur.org/wiki/MiSeqSOP>). Briefly, mate pairs were assembled, subjected to quality filtering (phred quality score  $Q \geq 28$ ) and aligned and classified against SILVA SSURef database v128 (Quast et al., 2012). Unique sequences were screened and further de-noised based on pre-clustered command for up to 2 differences between sequences. Putative chimera sequences were removed using UCHIME (Edgar et al., 2011). Sequences assigned to plastid-chloroplast-mitochondria taxa were removed (16S rRNA amplification using primer 799F only minimized amplification of the plant host chloroplast DNA and the mtDNA sequences; Bringel and Couée, 2015). Remaining sequences were clustered into operational taxonomic units (OTUs) at an identity level of 97%.

### 2.10. Ecological and statistical analysis

Total richness and alpha diversity indices were calculated on datasets rarefied to similar sequence number (613 sequences) using Mothur software (Schloss et al., 2009). Rarefaction curves were plotted and diversity indices calculated using the Mothur “summary single” command. For each tested  $CH_3Cl$ -incubation condition, OTUs enrichment factors were calculated as their mean relative abundance of triplicates in sequence datasets at the end-point of the incubation experiments compared to corresponding “time zero”. Multivariate and univariate statistical analyses and graphical representations were performed using SigmaPlot 13, PAST v3 (Hammer et al., 2001), and R version 3.3.1.

### 2.11. Capture sequencing of *cmuA* gene

A set of nineteen 80-mer degenerate non-overlapping probes (Table SM3) covering the complete *cmuA* gene was designed using KASPOD software (Parisot et al., 2012). Adaptor sequences were added to the ends of the probes to enable their amplification by PCR, resulting in “ATCGCACCAGCGTGT-N<sub>80</sub>-CACTGCGGCTCTCA” sequences, with N<sub>80</sub> representing the *cmuA* gene-specific capture probe. Biotinylated RNA probes were then synthesized as described by Ribière et al. (2016). In brief, adaptors containing the T7 promoter were added to the capture probes through ligation-mediated PCR, and the final biotinylated RNA probes were obtained after in vitro transcription and purification. A NGS library was constructed from an equimolar pool of DNA extracted from the microbial communities of leaves of all fern *C. cooperi* samples using Nextera kit (Illumina), according to the manufacturer's instructions. Hybridization capture was then conducted as described in Ribière et al. (2016). Briefly, 500 ng of biotinylated RNA probes and 500 ng of denatured Illumina library were incubated at 65 °C for 24 h. The probe/target heteroduplexes were captured using streptavidin-coated paramagnetic beads, and the captured fragments were eluted with NaOH. To increase the enrichment efficiency, a second round of hybridization capture was performed using the first-round capture products. Enriched DNA was then sequenced using 1/8 of an Illumina MiSeq 2  $\times$  250 bp run. Sequence data files (510,036 paired-end reads

total) were filtered for quality using FastQC (Andrews, 2010), trimmed using Prinseq-Lite (Schmieder and Edwards, 2011), de novo assembled using SPAdes (Bankevich et al., 2012), and mapped using Bowtie2 (Langmead and Salzberg, 2012) to a pre-built *cmuA* database containing 122 *cmuA* nucleotide sequences of lab-isolated strain to non-cultivated environmental taxa. The sorted reads were then blasted on a local server on that same *cmuA* database in order to reveal the diversity of the plant sample retrieved *cmuA* sequences.

### 2.12. Nucleotide sequence accession numbers

Nucleotide sequences generated during this study were deposited in GenBank under Bioproject accession no. PRJNA393101.

## 3. Results and discussion

### 3.1. Measurements of chloromethane production and degradation

We first monitored potential  $CH_3Cl$  emissions from 3 representative fern species *Cyathea cooperi*, *Osmunda regalis* and *Dryopteris filix-mas* collected from the Botanical Garden of Heidelberg University incubated at 20 °C in the laboratory. While *O. regalis* showed clear  $CH_3Cl$  production, the other two ferns did not show measurable  $CH_3Cl$  production (Table 1). Based on the detection limit of the applied analytical system,  $CH_3Cl$  production rates for *C. cooperi* and *D. filix-mas* were below 0.05  $\mu g\ g_{dw}^{-1}\ day^{-1}$ . The lack of production in *C. cooperi* observed in our study differs from previously studied *Cyathea* species (Table 1). Production rates of *O. regalis* were in the range of 0.6 to 128  $\mu g\ g_{dw}^{-1}\ day^{-1}$ , with a high variability of rates from different *O. regalis* plant individuals and sampling times, which may due to different plant vitality, as also noted previously (Yokouchi et al., 2007). Measured emission rates were in agreement with previous results on other *Osmunda* species, with observed rates varying from 2 to 144  $\mu g\ g_{dw}^{-1}\ day^{-1}$  (Yokouchi et al., 2007, 2015; Table 1).

Production and consumption processes might be simultaneously active in plants. A stable carbon isotope tracer technique was used to differentiate a possible overlay of uptake and production processes (Rhew et al., 2003; Saito et al., 2013). We found contrasting behavior between ferns with different roles as  $CH_3Cl$  source and sink. *O. regalis* showed a strong natural  $^{12}CH_3Cl$  production, while the supplemented  $^{13}CH_3Cl$  remained constant during the incubation (when 5 ppmv  $^{13}CH_3Cl$  was supplemented), suggesting that no measurable degradation of  $CH_3Cl$  occurred during incubation (Fig. 1B). Here it should be noted, that due to the strong production of *O. regalis*, uptake is possibly underestimated (Rhew, 2011).

In contrast, *C. cooperi* and *D. filix-mas* investigated in our study consumed the supplemented  $^{13}CH_3Cl$  (5 ppmv), while no  $^{12}CH_3Cl$  production was detectable (Fig. 1A, C). At 20 °C *C. cooperi* showed degradation rates from 2.1 to 17  $\mu g\ g_{dw}^{-1}\ day^{-1}$ , which were higher

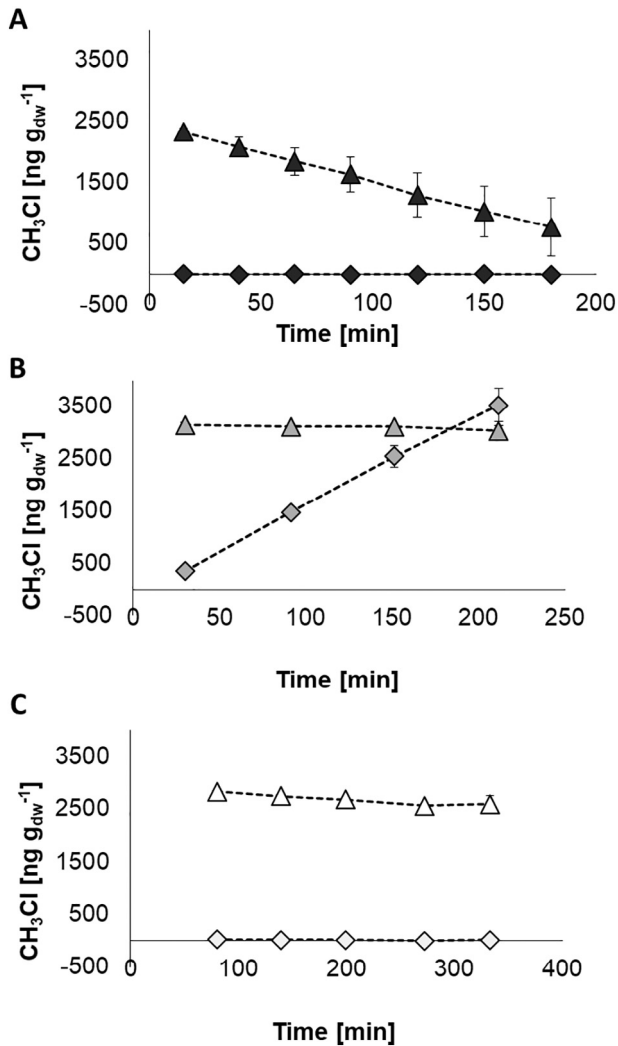
**Table 1**

$CH_3Cl$  sinks and sources associated with fern leaves. Comparison of rates determined by stable isotope tracer technique in this work with the literature.

Fern plants	$CH_3Cl$ flux rate ( $\mu g\ g_{dw}^{-1}\ day^{-1}$ )		Reference
	Degradation	Production	
<i>C. cooperi</i>	2.1–17 <sup>a</sup>	<0.05	This work
<i>C. lepifera</i>	n.d.	1.7–88.3	Yokouchi et al., 2002, 2007 Saito and Yokouchi, 2006, 2008
<i>C. podophylla</i>	n.d.	57.6 $\pm$ 14.4	Yokouchi et al., 2007 Saito and Yokouchi, 2006, 2008
<i>D. filix-mas</i>	0.3–0.9 <sup>a</sup>	<0.05	This work
<i>O. banksiifolia</i>	n.d.	26	Yokouchi et al., 2007
<i>O. japonica</i>	n.d.	2–144	Yokouchi et al., 2015
<i>O. regalis</i>	<0.2	0.6–128	This work

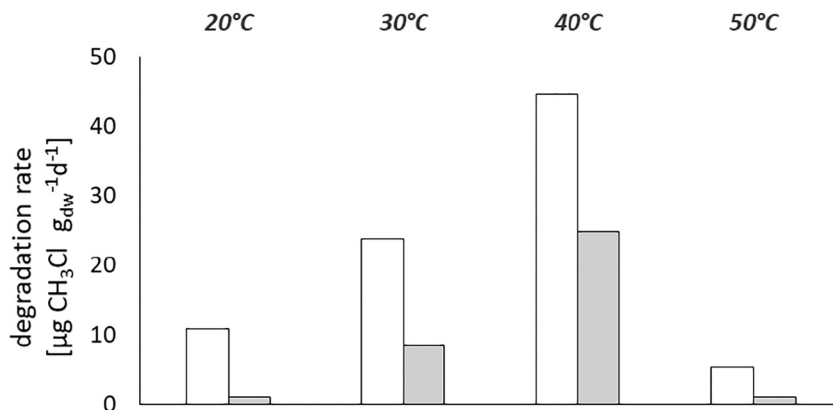
n.d. not determined.

<sup>a</sup> Spiked with 5 ppmv  $CH_3Cl$  at 20 °C.



**Fig. 1.** Stable isotope tracer experiments showing  $^{13}\text{CH}_3\text{Cl}$  (triangles) and  $^{12}\text{CH}_3\text{Cl}$  (rhombus) concentrations during incubation of *C. cooperi* (black,  $n = 3$ ) (A), *O. regalis* (grey,  $n = 3$ ) (B) and *D. filix-mas* (white,  $n = 2$ ) (C) plant samples, spiked with 5 ppmv of  $^{13}\text{CH}_3\text{Cl}$  at 20 °C. Error bars indicate standard deviation of the mean of three independent experiments. Some error bars lie within the symbol.

compared to rates measured for *D. filix-mas* of about  $0.3$  to  $0.9 \mu\text{g g}_{\text{dw}}^{-1} \text{day}^{-1}$ . However, measured  $\text{CH}_3\text{Cl}$  consumption rates were much higher than that of tropical trees ( $0.1 \pm 0.07 \mu\text{g g}_{\text{dw}}^{-1} \text{day}^{-1}$ ; Saito et al., 2013). Furthermore, both degrading ferns showed increasing uptake rates with increasing



**Fig. 2.** Temperature dependence of gross chloromethane degradation rates in ferns. Values were determined for *C. cooperi* (white bars) and *D. filix-mas* (grey bars) spiked with 10 ppmv  $^{13}\text{CH}_3\text{Cl}$  at 20 °C, 30 °C, 40 °C and 50 °C; ( $n = 1$ ;  $R^2 = 0.98$  for *C. cooperi* (20–40 °C);  $R^2 = 0.96$  for *D. filix-mas* (20–40 °C)).

temperature up to 40 °C, which then strongly decreased at 50 °C (Fig. 2). As noted previously, this indicates that  $\text{CH}_3\text{Cl}$  degradation originates mainly from biotic processes such as bacterial degradation (Farhan ul Haque et al., 2017). However, plant related enzymatic processes might be also involved in  $\text{CH}_3\text{Cl}$  consumption.

### 3.2. Biogenic chloromethane production and its stable isotopic signature

As expected,  $\text{CH}_3\text{Cl}$  production rates by *O. regalis* were dependent on temperature: lowest at 5 °C, highest at 20 or 30 °C (Fig. 3) and substantially decreased at 40 °C, which altogether indicates a predominately biotic process of  $\text{CH}_3\text{Cl}$  production by *O. regalis*. Wuosmaa and Hager (1990) discovered a methyl chloride transferase catalyzing the formation of  $\text{CH}_3\text{Cl}$  via reaction of S adenosyl L methionine (SAM) with chloride ion for the white rot fungus *Phellinus pomaceus*. A similar process of enzymatic  $\text{CH}_3\text{Cl}$  production was reported in *Arabidopsis thaliana* (Rhew et al., 2003), and suggested to be widespread among vascular plants.

Stable carbon isotope signature ( $\delta^{13}\text{C}$  value) of  $\text{CH}_3\text{Cl}$  produced by *O. regalis* at 20 °C was  $-97 \pm 8\%$ , a value more negative than the reported mean  $\delta^{13}\text{C}$  values of ferns including *Angiopteris lygodiiifolia*, *C. lepifera*, *C. podophylla* ( $-61.9 \pm 9.7\%$ ; Saito and Yokouchi, 2008), *Angiopteris evecta* and *C. smithii* (around  $-71\%$ ; Harper et al., 2003). In these studies, although biodegradation processes were not assessed, they may impact overall  $\delta^{13}\text{C}$  values of  $\text{CH}_3\text{Cl}$  (see discussion below for the other two ferns). The stable hydrogen isotope signature ( $\delta^2\text{H}$  value) of emitted  $\text{CH}_3\text{Cl}$  by *O. regalis* at 20 °C was  $-202 \pm 10\%$ . As far as we know, this is the first  $\delta^2\text{H}$  value of  $\text{CH}_3\text{Cl}$  reported for biogenic emissions from a living fern, which is in the range of what has been reported for abiotic  $\text{CH}_3\text{Cl}$  release from other dried plants ( $-155$  to  $-290\%$ ) when heated at temperatures between 30 and 300 °C (Greule et al., 2012). In their study,  $\delta^2\text{H}$  values of thermally emitted  $\text{CH}_3\text{Cl}$  were not affected by varying temperatures but correlated with  $\delta^2\text{H}$  values of the plant source water (meteoric water) and plant methoxyl groups ( $\text{OCH}_3$ ) (Greule et al., 2012). Under elevated temperatures such as during biomass burning, the plant structural components lignin and pectin (both containing methoxyl groups) can be chlorinated under different environmental conditions, leading to the release of substantial amounts of  $\text{CH}_3\text{Cl}$  (Hamilton et al., 2003; Keppler et al., 2004; McRoberts et al., 2015). However, at low ambient temperatures of 20 °C the abiotic  $\text{CH}_3\text{Cl}$  release from air-dried pinnules of *O. regalis* was found to be below the detection limit of the analytical system. Moreover, the measured  $\delta^2\text{H}$  value of methoxyl groups from *O. regalis* of  $-259 \pm 0.2\%$  was in the range published by Greule et al. (2012) for other plants ( $-200$  to  $-296\%$ ). Carboxyl groups in pectin and lignin are esterified by the enzyme pectin O methyltransferase (PMT) using S adenosylmethionine (SAM) as methyl donor (Weilacher et al., 1996). We thus considered that the methyl groups provided for chloride methylation during biogenic and abiotic formation both originate

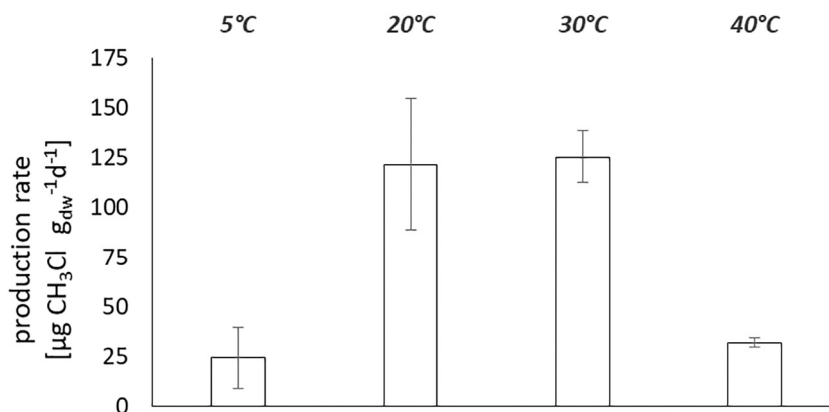


Fig. 3. Temperature dependence of chloromethane emission rates in *O. regalis*. Error bars indicate standard deviation of the mean of three independent experiments.

from the SAM methyl pool with similar  $\delta^2\text{H}$  value which is reflected by our measurement of the methoxyl groups ( $-259 \pm 0.2\%$ ). Thus, assuming that  $\text{CH}_3\text{Cl}$  ( $\delta^2\text{H}$  value of  $-202 \pm 10\%$ ) released by *O. regalis* is catalyzed by the reaction with SAM then the chloride ion methyltransferase must exhibit a rather small kinetic isotope effect. Moreover, the stable hydrogen fractionation ( $\Delta = \delta^2\text{H}_{\text{CH}_3\text{Cl}} - \delta^2\text{H}_{\text{HOCH}_3}$ ) during biotic  $\text{CH}_3\text{Cl}$  production from *O. regalis* of around 50‰ is similar to the stable hydrogen fractionation of abiotic  $\text{CH}_3\text{Cl}$  production from methoxyl groups (1 to 50‰) (Greule et al., 2012).

### 3.3. Degradation of chloromethane and its stable isotopic signature

Chloromethane degradation rates of *C. cooperi* were lowest at 5 °C with a mean value of  $0.30 \pm 0.03 \mu\text{g g}_{\text{dw}}^{-1} \text{day}^{-1}$  (Fig. 4A) and increased drastically up to  $5.8 \pm 1.3 \mu\text{g g}_{\text{dw}}^{-1} \text{day}^{-1}$  with increasing temperature. A 10-fold increase of spiked  $\text{CH}_3\text{Cl}$  mixing ratio resulted only in 3-fold higher degradation rates (Fig. 4A), indicating biotic processes that follow typical Michaelis-Menten-kinetics of enzymatic reactions. During degradation experiments at 20 °C and 30 °C as well as increasing supplementation of  $\text{CH}_3\text{Cl}$  almost no isotope effect was observed for hydrogen as indicated by the stable hydrogen enrichment factor  $\epsilon_{\text{H}}$  with a mean value of  $-8 \pm 19\%$  (Fig. 4B). The stable hydrogen isotope fractionation is slightly smaller as observed for  $\text{CH}_3\text{Cl}$  biodegradation in soils ( $\epsilon_{\text{H}} -50 \pm 19\%$ , Jaeger et al., 2018), but is in a similar range as it was reported by Nadalig et al. (2014) for  $\text{CH}_3\text{Cl}$  degradation by *Leisingera methylohalidivorans* MB2, a strain able to degrade  $\text{CH}_3\text{Cl}$  by an unknown dehalogenase. In contrast during incubations at 5 °C, the remaining  $\text{CH}_3\text{Cl}$  was considerably more enriched in  $^2\text{H}$  ( $\epsilon_{\text{H}}: -142 \pm 19\%$ ), potentially indicating a different process of  $\text{CH}_3\text{Cl}$  dissipation at much lower ambient temperatures. However, due to the low degradation rates (one to two orders of magnitude lower compared to higher ambient temperatures), we do not consider the results at 5 °C to be of environmental relevance. Furthermore, considerable enrichment was measured for stable carbon isotopes, with a mean  $\epsilon_{\text{C}}$  of  $-43 \pm 12\%$ . This is in the reported  $\epsilon_{\text{C}}$  range for  $\text{CH}_3\text{Cl}$  degrading bacterial strains (Nadalig et al., 2013) and at the lower end of the  $\epsilon_{\text{C}}$  range for  $\text{CH}_3\text{Cl}$  removal in soils (Jaeger et al., 2018) ( $-38$  to  $-11\%$ ). Both hydrogen and stable carbon isotope fractionation were unaffected by temperature at 20 °C and 30 °C and the amount of added  $\text{CH}_3\text{Cl}$  (Fig. 4C). Increased carbon isotope fractionation compared to hydrogen isotope fractionation seems to be the result of the primary isotope effect in cleavage of the carbon-halogen bond during  $\text{CH}_3\text{Cl}$  dehalogenation, as suggested by Elnner et al. (2005).

### 3.4. Composition and diversity of phyllosphere bacterial communities

The 3 ferns showed clearly different  $\text{CH}_3\text{Cl}$  formation and consumption patterns. Since the phyllosphere microbiota interacts with volatile

organic compounds and atmospheric trace gases (Bringel and Couée, 2015; Farhan ul Haque et al., 2017), we investigated fern leaf-associated bacterial diversity further. A total of 30,746 distinct prokaryotic OTUs were detected in the samples investigated. Sequences were assigned to 29 bacterial distinct phyla, 143 families and 314 genera.

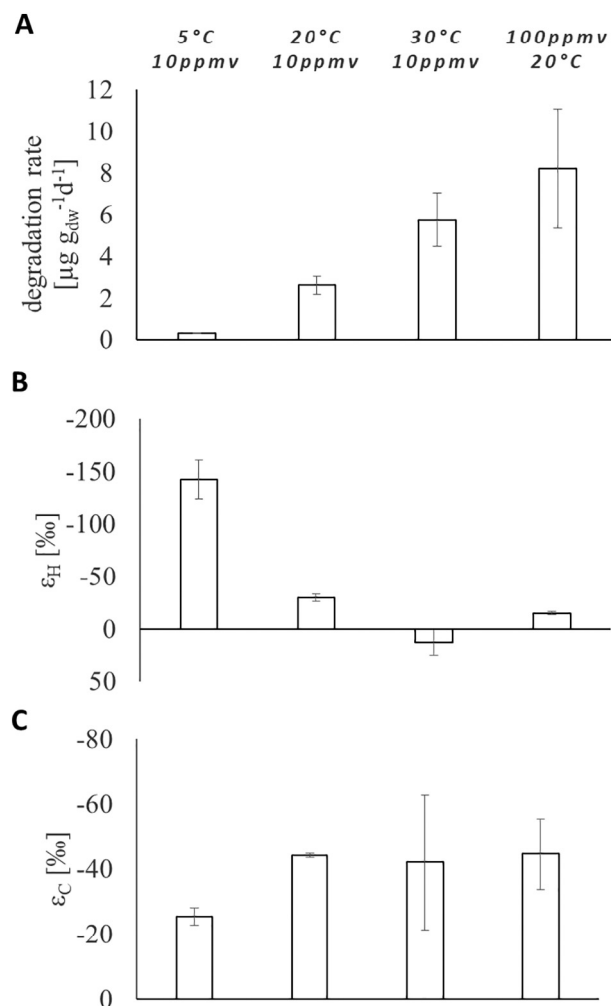


Fig. 4. Net  $\text{CH}_3\text{Cl}$  degradation rates (A) and stable hydrogen (B) and carbon (C) isotope enrichment factors ( $\epsilon_{\text{H}}$ ,  $\epsilon_{\text{C}}$ ) for *C. cooperi*. Values were determined at 5 °C, 20 °C and 30 °C after spiking with 10 ppmv  $\text{CH}_3\text{Cl}$  and at 20 °C spiked with 100 ppmv  $\text{CH}_3\text{Cl}$ . Error bars indicate standard deviation of the mean of three independent experiments. Please note that leaf material of *Cyathea cooperi* collected for incubation experiments was different to experiments presented in Fig. 2.

Gammaproteobacteria was by far the predominant phylum in the 3 fern phyllospheres (85.4% of total reads) (Fig. SM1). Although uncommon (Bringel and Couée, 2015), similar cases of dominance of Gammaproteobacteria in plant phyllosphere communities have been reported (Ren et al., 2014). Notably, at least 53% of OTUs belonged to the *Pseudomonadaceae* family (56.5% of total reads) (Fig. SM2). The genus *Pseudomonas* alone represented 53.9% of total reads and at least half of OTUs at the genus level in the investigated ferns (Fig. SM3). Other abundant OTUs were affiliated to Actinobacteria, Alphaproteobacteria and Betaproteobacteria, representing 6, 3.3 and 2.9% of the total reads, respectively (Fig. SM1). No significant difference was observed in the proportion of these taxa among phyllosphere types (ANOVA;  $p$ -value > 0.05) (data not shown). Within the phylum Proteobacteria, four dominant *Enterobacteriaceae* (*Enterobacteriaceae*\_unclassified, *Buttiauxella*, *Erwinia* and *Pantoea*) were found, with higher abundance in the *C. cooperi* (55.4%), *D. filix-mas* (14.9%) than in *O. regalis* (11.3%) (Fig. SM3).

Community richness indices were calculated on datasets rarefied to similar sequence number averaging 225, 215 and 239 OTUs respectively for the *C. cooperi*, *D. filix-mas* and *O. regalis* phyllospheres, but no significant difference between them was found (ANOVA;  $p$ -value > 0.05) (Table SM2).

### 3.5. Occurrence of previously characterized chloromethane-degrading taxa and *cmuA* in fern leaf bacterial communities

We investigated whether the microbiota associated with the three fern samples contributes to  $\text{CH}_3\text{Cl}$  degradation. To do so, we searched for the presence of taxa previously assigned to bacterial  $\text{CH}_3\text{Cl}$  degraders, and for the presence of the signature gene *cmuA*. This gene found in contrasting environments is essential for the only known pathway for  $\text{CH}_3\text{Cl}$  degradation (McAnulla et al., 2001a; Nadalig et al., 2011; Schäfer et al., 2005). Of the 7 genera that have been previously described to have chloromethane-degrading members (Nadalig et al., 2013), within the bacterial taxa identified in the fern samples, only three were detected in this study: i) *Pseudomonas* was the most abundant genus in the three fern leaves; ii) *Methylobacterium* was detected in 30 out of 33 samples and averaged 0.17%, 0.06% and 0.89% respectively for the *C. cooperi*, *D. filix-mas* and *O. regalis* phyllosphere; and iii) *Hyphomicrobium* was representing around 0.001% of the total relative abundance in a single *Dryopteris* sample. In these genera, *cmu* genes are rare and have only been found in a few  $\text{CH}_3\text{Cl}$ -degrading isolates (Freedman et al., 2004; Doronina et al., 1996; McDonald et al., 2001; Nadalig et al., 2011). When we investigated the occurrence of *cmuA* in the studied fern leaf bacterial communities, we were not able to detect *cmuA* using either a direct endpoint PCR amplification (detection limit of 200 *cmuA* copies per ng DNA; data not shown) nor a highly sensitive hybridization capture method (Gasc et al., 2016; Denonfoux et al., 2013) for the detection and recovery of rare sequences, with a detection limit of 0.00006% (sequencing depth of 9.3 million reads) (Gasc and Peyret, 2018). The hybridization-capture-gene sequencing approach was tested for *cmuA* with DNA extracted from the microbial communities of leaves of the fern *C. cooperi* displaying the highest  $\text{CH}_3\text{Cl}$  degrading abilities, but despite the sensitivity of the method no *cmuA* sequences were retrieved (data not shown). Altogether, the lack of *cmuA* detection suggests that the *cmu* pathway is not driving  $\text{CH}_3\text{Cl}$  bacterial consumption in the phyllosphere of *C. cooperi*. Consistently, no *cmu* pathway gene or protein sequence has been previously detected by metagenomics and metaproteomics investigations of environmental foliar samples (Delmotte et al., 2009; Knief et al., 2012). Also, <0.1% of bacteria on leaf surfaces carried the *cmuA* gene (Nadalig et al., 2011). Besides the very low abundance of *cmuA* in the tested fern plants, and possibly in other environments, unknown *cmuA*-independent  $\text{CH}_3\text{Cl}$  degrading pathways may be involved in microbial  $\text{CH}_3\text{Cl}$  consumption as previously suggested (Nadalig et al., 2014).

### 3.6. Influence of temperature, $\text{CH}_3\text{Cl}$ production and degradation parameters on bacterial fern leaf communities

A PCA analysis was used to correlate bacterial community composition with the variations in  $\text{CH}_3\text{Cl}$  production and consumption in the different fern samples. Analyzed parameters were  $\text{CH}_3\text{Cl}$  added, temperature, measured  $\text{CH}_3\text{Cl}$  production rate, measured  $\text{CH}_3\text{Cl}$  degradation rate and isotopic enrichment factors ( $\epsilon_{\text{H}}$  [‰],  $\epsilon_{\text{C}}$  [‰]) and the biological community descriptors (diversity and richness indices). There was no discrimination of the samples following these variables (Fig. 5A), indicating no relationship between microbial community composition and the incubation conditions, chloromethane biodegradation and isotope fractionation (Fig. 5B).

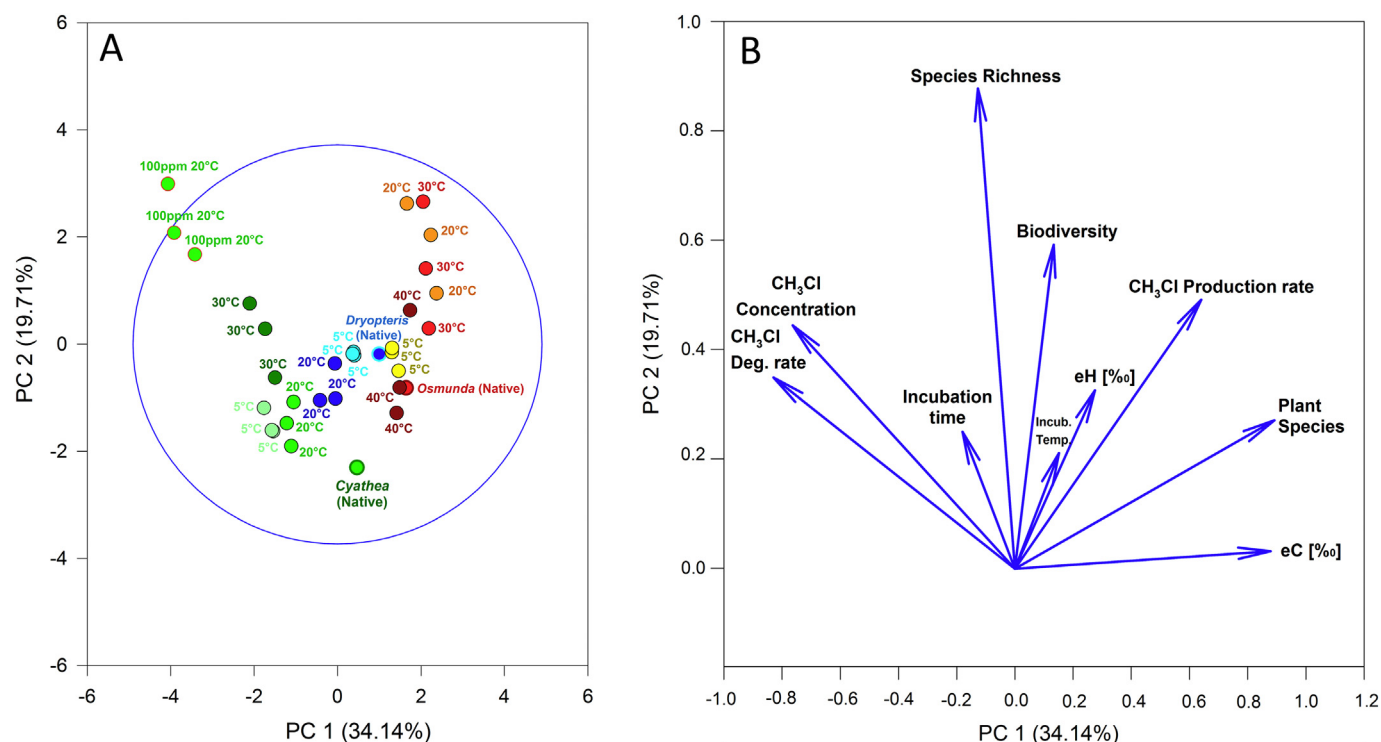
## 4. Conclusion

Ferns investigated in this study displayed either  $\text{CH}_3\text{Cl}$  strong degradation (*Cyathea cooperi* and *Dryopteris filix-mas*) or production (*Osmunda regalis*). Table 1 summarizes  $\text{CH}_3\text{Cl}$  flux variations observed between plant families (*Osmunda* versus *Dryopteris*), plants of the same species (*Cyathea*), and individual plants (Yokouchi et al., 2002; Saito and Yokouchi, 2006; Yokouchi et al., 2007, this work). These variations may be the result of complex interactions between genetic, physiological and environmental factors. Factors that may impact directly or indirectly  $\text{CH}_3\text{Cl}$  biogenic emission rates include: i) the plant-halogenating enzyme such as halide/thiocyanate methyl transferase (HTMT) for which a single residue substitution impairs its reactivity with  $\text{Cl}^-$  (Schmidberger et al., 2010); ii) the plant-halogenating enzyme activity (Yokouchi et al., 2002; Itoh et al., 2009) and its speculated reverse dehalogenating reaction (Jeffers et al., 1998); iii) the HTMT expression that can be modulated by glucosinolate metabolism (Nagatoshi and Nakamura 2009), plant development and exposure to pesticides (Bringel and Couée, 2018); iv) the plant metabolic state when stress modifies the substrate SAM methyl donor content (discussed in Saito and Yokouchi, 2006); v) the plant ion content of the substrate methyl acceptor halide ( $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ ) and pseudohalide ( $\text{NCS}^-$ ) ions (Itoh et al., 2009; Toda and Itoh, 2011; Attieh et al., 2000; Rhew et al., 2003; Blei et al., 2010); vi) the leaf structure and stomatal openness (Niinemets et al., 2004); and finally, vii) the most recently discovered factor, the phyllosphere microbiota (Farhan ul Haque et al., 2017). In this study, no differences in phyllospheric bacterial communities were detected that could account for differences in  $\text{CH}_3\text{Cl}$  consumption. Nonetheless, the stable carbon isotope enrichment factor of remaining  $\text{CH}_3\text{Cl}$  was  $-39 \pm 13\text{‰}$  and corresponds to previous results obtained with *Methylobacterium extorquens* CM4 and *Hyphomicrobium* sp. MC1 (Nadalig et al., 2013) featuring the *cmu* pathway for  $\text{CH}_3\text{Cl}$  degradation. Indeed, DNA evidence using very sensitive methods strongly suggests that *cmuA* is most likely not involved in the observed degradation of  $\text{CH}_3\text{Cl}$ . On the other hand, the stable isotope enrichment observed for hydrogen ( $-8 \pm 19\text{‰}$ ) is different than that previously published for *cmu* pathway  $\text{CH}_3\text{Cl}$  degrading strains (Nadalig et al., 2013). This suggests that biodegradation pathways other than *cmu* might be involved in the investigated ferns. Uncharacterized  $\text{CH}_3\text{Cl}$  degradation pathways could be assayed by molecular techniques such as stable isotope probing coupled with metagenomics and metatranscriptomics (Jameson et al., 2016). Despite the technical difficulties involved, we recommend that future studies investigate  $\text{CH}_3\text{Cl}$  biodegradation at atmospheric levels ( $\sim 600$  pptv), both in the laboratory and in the field, to quantify the microbial  $\text{CH}_3\text{Cl}$  sink in plants under environmentally relevant conditions.

## Acknowledgements

The CHLOROFILTER project (DFG KE 884/10-1, DFG KO 2912/10-1, ANR-14-CE35-0005-01) was funded by the German Research Foundation (DFG) and The French National Research Agency (ANR).





**Fig. 5.** PCA analysis of phyllospheric fern communities. Samples (A) and variables (B) are shown for the 2 main components of PCA analysis. Unless indicated, experiments were conducted in the presence of 10 ppm CH<sub>3</sub>Cl. Parameters included in the analysis were physico-chemical variables (amount of CH<sub>3</sub>Cl added in incubation experiments, measured CH<sub>3</sub>Cl production and degradation rates, temperature of incubation, and isotopic enrichments  $\epsilon\text{H}$  [‰],  $\epsilon\text{C}$  [‰]), fern type, and the biological community descriptors (diversity and richness indices).

Furthermore, we thank the Botanical Garden of Heidelberg University for providing fern samples.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.03.316>.

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