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Chloromethane formation and degradation in the fern phyllosphere

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HIGHLIGHTS
• Ferns both produce and degrade atmospheric CH3Cl with large individual variations.
• Ferns degrade CH3Cl at rates ranging from 0.3 to 17 μg (g dry weight)−1 day−1.
• CH3Cl degradation was correlated to a large εC and almost no εH isotope effect.
• Involvement of the bacterial cmu pathway in CH3Cl degradation was not detected.
• Still unknown CH3Cl biodegradation processes in plants contribute to the CH3Cl cycle.

GRAPHICAL ABSTRACT

ABSTRACT
Chloromethane (CH3Cl) 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 CH3Cl budget are approximate. The strength of the CH3Cl global sink by microbial degradation in soils and plants is under discussion. Some plants, particularly ferns, have been identified as substantial emitters of CH3Cl. Their ability to degrade CH3Cl 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 CH3Cl by measuring their production and consumption rates and their stable carbon and hydrogen isotope signatures. Investigated ferns are able to degrade CH3Cl at rates from 2.1 to 17 and 0.3 to 0.9 μg (g dry weight)−1 day−1 for C. cooperi and D. filix-mas respectively, depending on CH3Cl supplementation and temperature. The stable carbon isotope enrichment factor of remaining CH3Cl was −39 ± 13‰, whereas negligible isotope fractionation was observed for hydrogen (−8 ± 19‰). In contrast, O. regalis did not consume CH3Cl, but produced it at rates ranging from 0.6 to 128 μg (g dry weight)−1 day−1, with stable isotope values of −97 ± 8‰ for carbon and −202 ± 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 pathways (cmu).

Keywords: Biodegradation, Chloromethane, Isotope fractionation, Ferns, Phyllosphere, Methylotrophic bacteria.

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pathway “cmu” in the microbial phyllosphere of the investigated ferns. Our study suggests that still unknown CH3Cl biodegradation processes on plants play an important role in global cycling of atmospheric CH3Cl.
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 \[^{13}\text{C}\text{H}_3\text{Cl}\] (99.7 at.% labeled). The incubations were stopped after a maximum of 6 h. The simultaneous measurement of \[^{13}\text{C}\text{H}_3\text{Cl}\] and \[^{12}\text{C}\text{H}_3\text{Cl}\] 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 μL sample was injected in a flow of 1 mL/min of helium (split ratio 5:1) on a GasPro column (60 m × 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 \[^{12}\text{C}\text{H}_3\text{Cl}\] were detected (m/z = 50 for \[^{12}\text{C}\text{H}_3\text{Cl}\], m/z = 52 for \[^{13}\text{C}\text{H}_3\text{Cl}\], m/z = 51 for \[^{12}\text{C}\text{H}_3\text{Cl}\] and m/z = 53 for \[^{13}\text{C}\text{H}_3\text{Cl}\]). However, during the detection via the quadrupole mass spectrometer ion fragmentation occurs and need to be corrected in such isotope tracer studies (Rhwew and Abel, 2007), as e.g. \[^{13}\text{C}\text{H}_3\text{Cl}\] yields the same m/z as \[^{12}\text{C}\text{H}_3\text{Cl}\]. Therefore \[^{13}\text{C}\text{H}_3\text{Cl}\]/\[^{12}\text{C}\text{H}_3\text{Cl}\] fragmentation ratios were determined experimentally and detected areas corrected for it. Gross production (\[^{13}\text{C}\text{H}_3\text{Cl}\]) and uptake (\[^{12}\text{C}\text{H}_3\text{Cl}\]) rates were calculated by the slope of a linear fit applied to the dry weight based concentration of the respective isotopologues (\(μ\ g\ mu\ −1\)) versus time (day).

Additionally, to check for biotic degradation and to exclude abiotic production with increasing temperature, the ferns that showed net \[^{12}\text{C}\text{H}_3\text{Cl}\] degradation (\(C.\ cooperi, D.\ flilx-mus\)) were spiked with 10 ppmv \[^{13}\text{C}\text{H}_3\text{Cl}\] and incubated at increasing temperature in 10 °C steps until net \[^{12}\text{C}\text{H}_3\text{Cl}\] uptake rates decreased.

Furthermore \(C.\ cooperi\) and \(D.\ flilx-mas\) samples were incubated to assess their production potential. These incubations did not include the \[^{13}\text{C}\text{H}_3\text{Cl}\] 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 \[^{12}\text{C}\text{H}_3\text{Cl}\] (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 (\(μ\ g\ mu\ −1\)) versus time (day). The resulting slope represents \(−k_{net}\). 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 \[^{12}\text{C}\text{H}_3\text{Cl}\] 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 (\(μ\ g\ mu\ −1\)) 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 \(^2\text{H}_3\text{Cl}\) 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 (\(δ^2\text{H}_3\text{Cl}_{\text{V-SMOW}}\)) and V-PDB standard (Vienna Pee Dee Belemnite) for carbon (\(δ^{13}\text{C}_{\text{PDB}}\)) on per mil basis is used. The isotope fractionation factor \(α\) and the isotope enrichment factor \(ε\) are derived from the slope of the Rayleigh plot according to Elnser et al. (2005) and Elnser (2010). Negative \(ε\) indicates that the remaining \[^{12}\text{C}\text{H}_3\text{Cl}\] is enriched in the heavier isotope and corresponds to an \(α < 1\), meaning that the heavier \[^{13}\text{C}\]–\[^{12}\text{C}\]–\[^{12}\text{C}\]–\[^{12}\text{C}\text{H}_3\text{Cl}\] reacted more slowly by this factor than the lighter \[^{13}\text{C}\]–\[^{13}\text{C}\]–\[^{13}\text{C}\]–\[^{12}\text{C}\text{H}_3\text{Cl}\] during incubation.

2.6. Determination of stable isotope values of plant methoxyl groups

Stable hydrogen and carbon isotope values of plant methoxyl groups (\[^{13}\text{C}\text{H}_3\text{O}\]) can be measured without isotope fractionation as iodomethane (\(^2\text{H}_3\text{Cl}\)) 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 μL 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 \(^2\text{H}_3\text{Cl}\) were measured by Hewlett Packard HP 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to DeltaPlus 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 μm 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 aceta EDTA buffer (TAE 1× 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 μL PCR reaction contained 1 ng of DNA, 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 200 mM dNTP, 200 mM 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 * 250 bp (GenoScreen, France).
DNA was also used to amply an inter-domain region of the protein-encoding gene cmuA of 422 bp. The 50 μL PCR reaction volume contained 1 × buffer including MgCl2, 0.8 mM dNTPs (0.2 mM each dNTP), 1121 nM of primer cmuA4f22 and 258 nM of primer cmuA4r22 (Table SM 1; 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 ≥ 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 CH3Cl-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 “ATCCACCGGTGT-N80P-CACGCCGCTCTCA” sequences, with N80 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 × 250 bp run. Sequence data files ($10,036 paired-end reads)

### Table 1

<table>
<thead>
<tr>
<th>Fern plants</th>
<th>CH3Cl flux rate (μg g\text{dw}^{-1} day^{-1})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. cooperi</td>
<td>2.1–17a</td>
<td>−0.05</td>
</tr>
<tr>
<td>C. leptifera</td>
<td>n.d.</td>
<td>1.7–88.3</td>
</tr>
<tr>
<td>C. podophylla</td>
<td>n.d.</td>
<td>57.6 ± 14.4</td>
</tr>
<tr>
<td>D. flīx-mas</td>
<td>0.3–0.9a</td>
<td>−0.05</td>
</tr>
<tr>
<td>G. banksii-folia</td>
<td>n.d.</td>
<td>26</td>
</tr>
<tr>
<td>G. japonica</td>
<td>n.d.</td>
<td>2–144</td>
</tr>
<tr>
<td>G. regalis</td>
<td>−0.2</td>
<td>0.6–128</td>
</tr>
</tbody>
</table>

n.d. not determined.

* a Spiked with 5 ppmv CH3Cl at 20 °C.
compared to rates measured for D. filix-mas of about 0.3 to 0.9 μg g⁻¹ dw⁻¹ day⁻¹. However, measured CH₃Cl consumption rates were much higher than that of tropical trees (0.1 ± 0.07 μg g⁻¹ dw⁻¹ day⁻¹: Saito et al., 2013). Furthermore, both degrading ferns showed increasing uptake rates with increasing temperature up to 40 °C, which then strongly decreased at 50 °C (Fig. 2). As noted previously, this indicates that CH₃Cl 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 CH₃Cl consumption.

3.2. Biogenic chloromethane production and its stable isotopic signature

As expected, CH₃Cl 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 CH₃Cl production by O. regalis. Wuosmaa and Hager (1990) discovered a methyl chloride transferase catalyzing the formation of CH₃Cl via reaction of S adenosyl L methionine (SAM) with chloride ion for the white rot fungus Phellinus pomaceus. A similar process of enzymatic CH₃Cl production was reported in Arabidopsis thaliana (Rhew et al., 2003), and suggested to be widespread among vascular plants.

Stable carbon isotope signature (δ¹³C value) of CH₃Cl produced by O. regalis at 20 °C was −97 ± 8‰, a value more negative than the reported mean δ¹³C values of ferns including Angiopteris lygodifolia, C. lepifera, C. podophylla (−61.9 ± 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 δ¹³C values of CH₃Cl (see discussion below for the other two ferns). The stable hydrogen isotope signature (δ²H value) of emitted CH₃Cl by O. regalis at 20 °C was −202 ± 10‰. As far as we know, this is the first δ²H value of CH₃Cl reported for biogenic emissions from a living fern, which is in the range of what has been reported for abiotic CH₃Cl release from other dried plants (−155 to −290‰) when heated at temperatures between 30 and 300 °C (Greule et al., 2012). In their study, δ²H values of thermally emitted CH₃Cl were not affected by varying temperatures but correlated with δ²H values of the plant source water (meteoric water) and plant methoxyl groups (OCH₃) (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 CH₃Cl (Hamilton et al., 2003; Keppler et al., 2004; McRoberts et al., 2015). However, at low ambient temperatures of 20 °C the abiotic CH₃Cl release from air-dried pinnules of O. regalis was found to be below the detection limit of the analytical system. Moreover, the measured δ²H value of methoxyl groups from O. regalis of −259 ± 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 from SAM.

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**Fig. 1.** Stable isotope tracer experiments showing ¹³CH₃Cl (triangles) and ¹²CH₃Cl (rhombs) 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 ¹³CH₃Cl at 20 °C. Error bars indicate standard deviation of the mean of three independent experiments. Some error bars lie within the symbol.

**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 ¹³CH₃Cl at 20 °C, 30 °C, 40 °C and 50 °C; (n = 1; R² = 0.98 for C. cooperi (20–40 °C); R² = 0.96 for D. filix-mas (20–40 °C)).
from the SAM methyl pool with similar δ²H value which is reflected by our measurement of the methoxyl groups (−259 ± 0.2‰). Thus, assuming that CH₃Cl (δ²H value of −202 ± 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 (Δ = δ²H(CH₃Cl) − δ²H(HOCH₃)) during biotic CH₃Cl production from O. regalis of around 50‰ is similar to the stable hydrogen fractionation of abiotic CH₃Cl 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 ± 0.03 μg g⁻¹ dw day⁻¹ (Fig. 4A) and increased drastically up to 5.8 ± 1.3 μg g⁻¹ dw day⁻¹ with increasing temperature. A 10-fold increase of spiked CH₃Cl 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 CH₃Cl almost no isotope effect was observed for hydrogen as indicated by the stable hydrogen enrichment factor εH with a mean value of −8 ± 19‰ (Fig. 4B). The stable hydrogen isotope fractionation is slightly smaller as observed for CH₃Cl biodegradation in soils (εH = −50 ± 19‰, Jaeger et al., 2018), but is in a similar range as it was reported by Nadalig et al. (2014) for CH₃Cl degradation by Leisingera methylhalidivorans MB2, a strain able to degrade CH₃Cl by an unknown dehalogenase. In contrast during incubations at 5 °C, the remaining CH₃Cl was considerably more enriched in δ²H (εH: −142 ± 19‰), potentially indicating a different process of CH₃Cl 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 εC of −43 ± 12‰. This is in the reported εC range for CH₃Cl degrading bacterial strains (Nadalig et al., 2013) and at the lower end of the εC range for CH₃Cl 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 CH₃Cl (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 CH₃Cl dehalogenation, as suggested by Elsner et al. (2005).

3.4. Composition and diversity of phyllosphere bacterial communities

The 3 ferns showed clearly different CH₃Cl 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.
Fern samples contribute to CH$_3$Cl degradation. To do so, we searched for the presence of taxa previously assigned to bacterial CH$_3$Cl degraders, and for the presence of the signature gene cmuA. This gene is found in contrasting environments is essential for the only known pathway for CH$_3$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) *Methyllobacterium* was detected in 30 out of 33 samples and averaged 0.17%, 0.06% and 0.89% respectively for the *C. cooperi*, *D. flexis-mas* and *O. regalis* phyllospheres; and iii) *Hyphomicrobiurn* 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 CH$_3$Cl-degrading isolates (Freedman et al., 2004; Dornina 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 CH$_3$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 CH$_3$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 (Delmote et al., 2009; Knief et al., 2012). Also, $<$0.1% of bacteria on leaf surfaces carried the cmuA gene (Nadalig et al., 2011). Besides the very low abundance of cmuA in the tested fern plants, and possibly in other environments, unknown cmuA-independent CH$_3$Cl degrading pathways may be involved in microbial CH$_3$Cl consumption as previously suggested (Nadalig et al., 2014).

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

A PCA analysis was used to correlate bacterial community composition with the variations in CH$_3$Cl production and consumption in the different fern samples. Analyzed parameters were CH$_3$Cl added, temperature, measured CH$_3$Cl production rate, measured CH$_3$Cl degradation rate and isotopic enrichment factors ($\delta$C$_{\text{CH}_3}\text{Cl}$) 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 CH$_3$Cl strong degradation (*Cyathoa cooperi* and *Dryopteris flexis-mas*) or production (*Osmunda regalis*). Table 1 summarizes CH$_3$Cl flux variations observed between plant families (*Osmunda versus Dryopteris*), plants of the same species (*Cyathoa*), 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 CH$_3$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 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 (Cl$^-$, Br$^-$, I$^-$) and pseudohalide (NCS$^-$) ions (Itoh et al., 2009; Toda and Itoh, 2011; Attieh et al., 2000; Rhew et al., 2003; Blei et al., 2010); vii) the leaf structure and stomatal openness (Niinemets et al., 2004); and finally, viii) 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 CH$_3$Cl consumption. Nonetheless, the stable carbon isotope enrichment factor of remaining CH$_3$Cl was $-39$ ± 13‰, and corresponded to previous results obtained with *Methyllobacterium extorquens* CM4 and *Hyphomicrobiurn* sp. MCI (Nadalig et al., 2013) featuring the cmu pathway for CH$_3$Cl degradation. Indeed, DNA evidence using very sensitive methods strongly suggests that cmuA is most likely not involved in the observed degradation of CH$_3$Cl. On the other hand, the stable isotopic enrichment observed for hydrogen ($-8$ ± 19‰) is different than that previously published for cmu pathway CH$_3$Cl degrading strains (Nadalig et al., 2013). This suggests that biodegradation pathways other than cmu might be involved in the investigated ferns. Uncharacterized CH$_3$Cl degradation pathways could be assayed by molecular techniques such as stable isotopic probing coupled with metagenomics and metatranscriptomics (Jameson et al., 2016). Despite the technical difficulties involved, we recommend that future studies investigate CH$_3$Cl biodegradation at atmospheric levels ($-600$ pptv), both in the laboratory and in the field, to quantify the microbial CH$_3$Cl sink in plants under environmentally relevant conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.scitotenv.2018.03.316](https://doi.org/10.1016/j.scitotenv.2018.03.316).

**References**


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 CH₃Cl. Parameters included in the analysis were physico-chemical variables (amount of CH₃Cl added in incubation experiments, measured CH₃Cl production and degradation rates, temperature of incubation, and isotopic enrichments δH [%], δC [%]), fern type, and the biological community descriptors (diversity and richness indices).
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