Chlorine Isotope Fractionation of the Major Chloromethane Degradation Processes in the Environment

Frank Keppler,[*,†] *Jaime D. Barnes,*[#] *Axel Horst,*[§] *Enno Bahlmann,*[‡] *Jing Luo,*[∥] *Thierry Nadalig,*[∥] *Markus Greule,*[†] *S. Christoph Hartmann,*[†,⊥] *and Stéphane Vuilleumier*[∥]

[†]Institute of Earth Sciences, Heidelberg University, Im Neuenheimer Feld 236, Heidelberg, Germany

[#]Department of Geological Sciences, University of Texas, Austin, TX 78712, United States

[§]Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research – UFZ, Permoserstr.15, 04318 Leipzig, Germany

[‡]Leibniz Institute for Baltic Sea Research Warnemünde, Seestrasse 15, 18119 Rostock, Germany

[∥]UMR 7156 CNRS Génétique Moléculaire, Génomique, Microbiologie, Université de Strasbourg, 4 allée Konrad Roentgen, 67000 Strasbourg, France

[⊥]Max Planck Institute for Chemistry, Hahn-Meitner-Weg 1, 55128 Mainz, Germany

ABSTRACT

Chloromethane (CH_3Cl) is an important source of chlorine in the stratosphere, but detailed knowledge of the magnitude of its sources and sinks is missing. Here we measured the stable chlorine isotope fractionation (ε_{Cl}) associated with the major abiotic and biotic CH_3Cl sinks in the environment, namely CH_3Cl degradation by hydroxyl (·OH) and chlorine (·Cl) radicals in the troposphere, and by reference bacteria *Methylorubrum extorquens* CM4 and *Leisingera methylohalidivorans* MB2 from terrestrial and marine environments, respectively. No chlorine isotope fractionation was detected for reaction of CH_3Cl with ·OH and ·Cl radicals, whereas large chlorine isotope fractionation (ε_{Cl}) of -10.9 ± 0.7‰ (n=3) and -9.4 ± 0.9 (n=3) was found for CH_3Cl degradation by *M. extorquens* CM4 and *L. methylohalidivorans* MB2, respectively. The large difference in chlorine isotope fractionation observed between tropospheric and bacterial degradation of CH_3Cl provides an effective isotopic tool to characterize and distinguish between major abiotic and biotic processes contributing to the CH_3Cl sink in the environment. Finally, our findings demonstrate the potential of emerging triple-element isotopic approaches including chlorine to carbon and hydrogen analysis for the assessment of global cycling of organochlorines.

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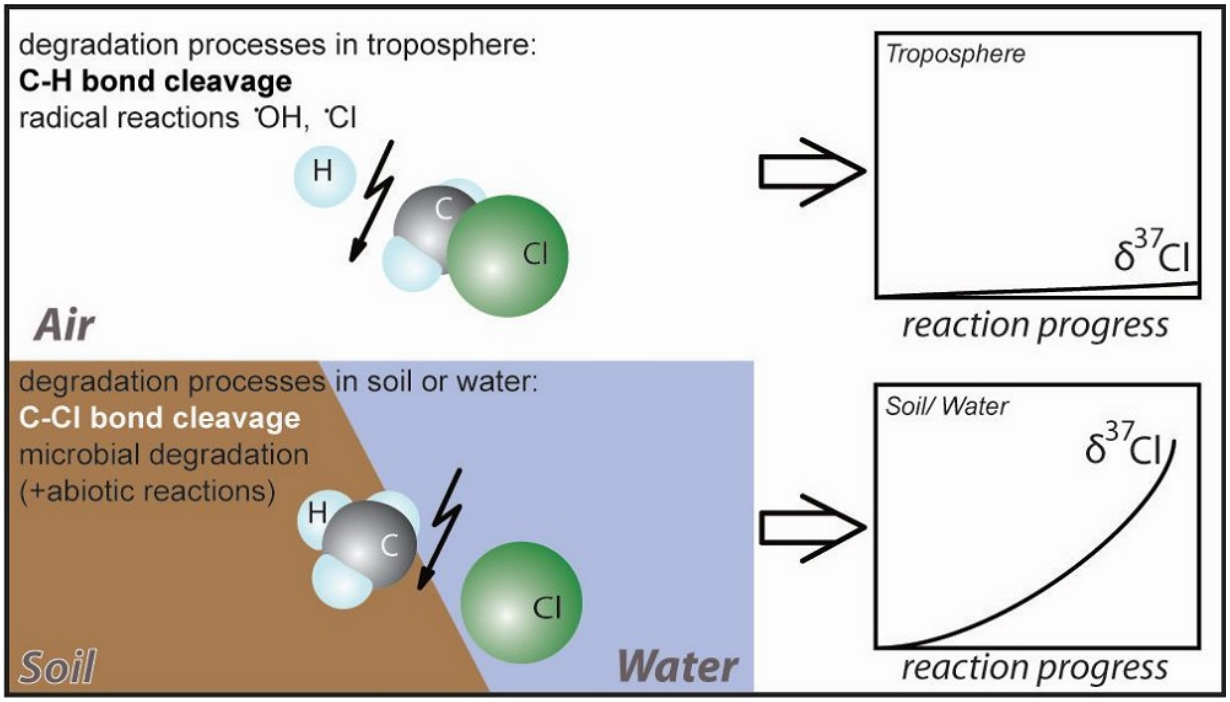
34 

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INTRODUCTION

Chloromethane is the simplest chlorinated organic molecule and the most abundant chlorine containing trace gas in the Earth atmosphere, currently with a global mean mixing ratio of ~553 ± 5 parts per trillion by volume (pptv), and an atmospheric lifetime of 0.9 years[1]. Global emissions of CH_3Cl have been estimated at 4 to 5 Tg yr^{-1} (1 Tg = 10^{12} g), and are known to originate predominantly from natural sources[2, 3]. However, current estimates of the CH_3Cl global budget, and apportionment between sources and sinks, are still highly uncertain[4]. Due to phasing out of anthropogenic emissions of chlorofluorocarbons, CH_3Cl will largely control future levels of stratospheric chlorine. In the last three decades, many natural sources of CH_3Cl have been discovered, including emissions from tropical plants[5-7], mangroves[8, 9], wood decay driven by fungi[10], algae and bacteria in oceans[11, 12], plants and salt marshes[13, 14], aerated, flooded soil and saline soils from semi-arid areas[15-18], senescent leaves[19], and from thermal destruction of plant matter such as by wild fires[20, 21]. Anthropogenic CH_3Cl is released to the atmosphere mainly by combustion of fossil fuels and biomass, with minor emissions from cattle[22], food production[23], and humans[24]. Worthy of note, CH_3Cl emissions from industrial sources, particularly in East Asia, may be much higher than previously assumed[25]. Recent studies have addressed in more detail the contribution of each CH_3Cl source[3, 25, 26].

Removal processes for CH_3Cl (often termed ‘loss’ or ‘sinks’) are driven by both abiotic and biotic reactions (Table 1). The dominant process for atmospheric CH_3Cl removal results from reaction with photochemically-produced hydroxyl radicals (·OH), currently estimated at about
2.8 Tg yr^{-1} [3]. The reaction of CH_3Cl with chlorine radicals (·Cl) in the marine boundary layer represents another sink, estimated at up to 0.4 Tg yr^{-1} [2, 27]. CH_3Cl is degraded abiotically by

nucleophilic substitution (S_N2 mechanism) of chlorine with water (hydrolysis), yielding methanol[28, 29]. Biotic CH_3Cl degradation mainly originates from methyl transfer reactions by methylotrophic bacteria[30-33]. The global significance of abiotic reactions compared to biotic processes is not yet clear. Yvon-Lewis and Butler[34] estimated that abiotic degradation in oceans is responsible for about 2 % of overall degradation. Another study suggested that microbial degradation may be more important in these compartments, particularly in higher latitude cold waters[26]. Overall, the magnitude of both types of reactions on a global scale is highly uncertain, estimated at between 0.1 and 1.6 Tg yr^{-1} in soils, [3, 4, 31, 35] and 370 Gg yr^{-1} in oceans[3]. A minor proportion of tropospheric CH_3Cl is lost to the stratosphere (~150 Gg yr^{-1}). Thus, current estimates of the magnitude of individual sources and sinks that define the CH_3Cl global budget are still highly uncertain overall[1, 3]. In this context, the use of stable isotope ratios represents a potentially powerful tool in investigations of the atmospheric CH_3Cl budget[4, 35-38]. The general underlying concept is that the atmospheric isotope ratio of a compound such as CH_3Cl may be considered to equal the sum of isotopic fluxes from all sources, corrected for the weighted average kinetic isotope effect (KIE) of all degradation processes, thereby allowing to attempt to deconvolute distinct sources and sinks of known isotopic signatures, as outlined in equation 1.

$$\delta^h E^{atm} = \sum_{i=l}^{n} \Phi_i^{source} \times \delta^h E_i^{source} + \sum_{j=l}^{n} \Phi_j^{loss} \times \varepsilon_j^{loss} \qquad (1)$$

where δ^hE^{atm} and δ^hE^{source} (^hE indicates ^2H, ^{13}C, ^{37}Cl) are the stable isotope values of CH_3Cl in the atmosphere and of the different sources i in per mil. Φ_i and Φ_j are the CH_3Cl flux fraction in per mil for each source and loss process. ε_j is the isotope fractionation of each loss j, respectively. Thus, the isotopic composition of atmospheric CH_3Cl is controlled by the KIEs for processes of physical, chemical and biological loss.

Carbon isotope fractionation associated with ·OH and ·Cl radical-driven degradation of CH_3Cl in the troposphere was recently reanalysed[4], and much smaller values than previously reported were obtained [39]. These new data applied to a global model suggest a large missing CH_3Cl source of 1530 ± 200 Gg yr^{-1} in the environment.

So far, most isotopic investigations of CH_3Cl have focused on the stable carbon and hydrogen isotope compositions of sources and sinks of CH_3Cl[33, 38, 40-43]. A recent study provided chlorine isotope fractionation data, in addition to those for carbon and hydrogen, for abiotic hydrolysis of CH_3Cl[44] (Table 1). This study demonstrated that chlorine isotope analyses might deliver additional process-level information. Chlorine, in contrast to carbon, is only involved in one potential degradation reaction (C-Cl bond cleavage but not C-H bond cleavage) and may thus help to identify and quantify underlying processes. All available information (from the literature and from this study) on stable hydrogen, carbon and chlorine isotope fractionation associated with known CH_3Cl sinks is listed in Table 1. To further improve knowledge about the global CH_3Cl budget, triple-element isotope analyses (hydrogen, carbon, and chlorine) may thus contribute important new information. Stable chlorine isotope analysis has been used to investigate the fate of several chlorinated organic compounds (e.g. [45-49]) including chlorofluorocarbons[50]. For CH_3Cl, apart from the aforementioned study[44], no data about stable chlorine isotope fractionation (ε_{Cl}) of degradation processes in the environment has yet become available.

Here, we present results from kinetic studies of chlorine isotope fractionation of CH_3Cl by bacterial degradation and atmospheric ·OH and ·Cl driven destruction processes measured by IRMS, GC-MS and GC-MC-ICPMS. In addition, we provide new data for triple element isotope analysis of bacterial consumption of CH_3Cl, also including carbon and hydrogen analysis from the same samples. Two bacterial strains growing with CH_3Cl as carbon energy source were chosen as representative of microbial CH_3Cl degradation in terrestrial and

marine environments, respectively. *Methylorubrum* (formerly *Methylobacterium*[51]) *extorquens* CM4, isolated from industrial soil contaminated by halogenated chemicals, is the model strain for CH_3Cl degradation by the *cmu* (chloromethane utilization) pathway, which is the only characterised CH_3Cl degradation pathway to date[52]. The halophilic marine strain *Leisingera methylohalidivorans* MB2, isolated from a coastal tide pool, is also capable of using CH_3Cl as carbon and energy source for growth, and belongs to the *Roseobacter* clade widely distributed in marine environments. Its genome[53] lacks *cmu* genes[41], and its pathway for CH_3Cl degradation remains unknown. Isotopic fractionation observed in bacterial degradation experiments are compared to chlorine isotope fractionation caused by radical driven processes (·OH and ·Cl radicals). Finally, we compare chlorine isotope fractionation data from the current study with previously published hydrogen and carbon isotope fractionation data to discuss the usefulness of triple element isotopic analyses toward an improved understanding of the global CH_3Cl budget.

MATERIALS AND METHODS

Bacterial growth

Methylorubrum (formerly *Methylobacterium*[51]) *extorquens* and *Leisingera methylohalidivorans* MB2 (DSM 14336) were laboratory stocks and cultivated in M3 medium and MAMS medium, respectively, as described previously[41]. Strains were cultivated at 30℃ in 300 mL custom-made Erlenmeyer vessels fitted with gas-tight mininert® screwcaps (Sigma), in 50 mL liquid medium with 250 mL headspace, under shaking at 120 rpm (Multitron, Infors HT). A total of 12 mL CH_3Cl gas (approx. 0.5 mmol; Sigma-Aldrich, France, >99.5% purity) was initially added to the flasks as the sole carbon and energy source. Chloromethane will partition between the liquid phase and the gas phase basing on a dimensionless Henry's law constant of 0.424[54]. Under the used growth conditions, chloride is released in the liquid phase up to a final concentration of approx. 10 mM upon complete consumption of CH_3Cl by the bacterial culture. Otherwise identical control experiments without bacteria were performed in parallel. Three replicate cultures were analysed for each strain. Bacterial growth was monitored by optical density at 600 nm after gas phase sampling (see below), from 1 mL liquid culture aliquots removed from the culture using a 1 mL plastic disposable syringe.

Analysis of bacterial chloromethane consumption

Chloromethane consumption was followed by analysing 100 µL headspace samples from the cultures ,retrieved with gastight syringes (Hamilton Bonaduz AG, Switzerland) at regular intervals (every 2-4 hours depending on the growth stage), by gas chromatography coupled with a flame ionization detector (GC-FID; Agilent Technologies France SAS, Courtabeuf, France), as described previously[42].

In addition, duplicate headspace samples, containing approximately 50 µg chlorine based on the estimated remaining CH_3Cl at each sampling timepoint, were also retrieved and transferred to 12 mL Exetainer® tubes (Labco Limited, Lampeter, UK) filled with N_2 gas for subsequent determination of chlorine, carbon and hydrogen isotope ratios. Following sampling, equivalent volumes of N_2 gas were injected to the culture vessels to maintain pressure balance. Initial concentrations of CH_3Cl were measured after two hours of incubation to allow for gas-liquid equilibration. For calculation of isotope fractionations (see below), we also considered the bias of mass removal by repetitive sampling as recently suggested[55]. Corrections were calculated (applying method IV of Buchner et al.[55]), but no significant differences for isotope fractionation of hydrogen, carbon and chlorine were detected.

CH_3Cl concentration was determined in gas samples using GC-MS as described previously[33]. Obtained values allowed for calculation of the remaining CH_3Cl fraction, which was used for determination of kinetic isotope effects of chlorine, hydrogen and carbon.

Degradation of chloromethane by ·OH and ·Cl radicals in smog chamber experiments

Experiments were performed in a 3,500 L Teflon smog-chamber with initial mixing ratio of 10 parts per million by volume (ppmv). Details of smog chamber design and performed CH_3Cl degradation experiments are given in Keppler et al.[38], Bahlmann et al.[4], and in the supporting information. In brief, elemental chlorine was generated via photolysis of molecular chlorine (Cl_2). Hydroxyl radicals were generated via photolysis of ozone (O_3) at 253.7 nm in the presence of water vapor (relative humidity = 70%). Perfluorohexane (PFH) was used as an internal standard to correct for dilution. The temperature was set to 20±1°C and monitored along with relative humidity. Mixing ratios of CH_3Cl and PFH were quantified by GC (Hewlett Packard HP 6890) coupled to a MSD 5973 mass spectrometer (GC-MS,

Agilent Technologies, Palo Alto, CA). The abundance of CH_3Cl relative to PFH was used to calculate the remaining fraction of CH_3Cl (equation 3, see below). The relative standard deviation (SD) of this procedure was determined prior to each experiment, and also in control experiments, and ranged between 1.3% and 1.9%.

Under these experimental conditions, typically over 70% of CH_3Cl was degraded within 6 to 10 h (Table S1). From each experiment (CH_3Cl + ·OH, and CH_3Cl + ·Cl), 10 to 15 canister samples (2 L stainless steel, evacuated $<10^{-4}$ mbar) were collected at regular time intervals for subsequent measurements of stable chlorine isotope values of CH_3Cl ($\delta^{37}Cl(CH_3Cl)$) using GC-MC-ICPMS. In addition, $\delta^{37}Cl(CH_3Cl)$ values were also derived from measurements of mixing ratios using GC-MS (see supporting information; Method S1 and Figures S1-S4).

Stable chlorine isotope analysis

Stable chlorine isotope analyses using CF-IRMS

Chlorine isotope ratios in bacterial CH_3Cl degradation experiments were measured using continuous flow isotope ratio mass spectrometry (CF-IRMS) from CH_3Cl gas samples obtained as described above. Samples were purged from vials into an ultra high purity (99.999 %; 5.0) helium stream. The helium stream was routed through the vial by puncturing the septum with two needles: one needle (the helium inlet) penetrated all the way to the bottom of the vial, whereas the other needle (the vent) penetrated only a few mm below the septum. The sample was carried through the vent needle and cryofocused in a glass U-trap frozen in liquid nitrogen. After 3 minutes of purging at 30 mL/min, sample transfer to the U-trap was complete. The U-trap was subsequently warmed in room-temperature water,

transferring the CH_3Cl sample through a custom-built open split, which was interfaced with a ThermoElectron MAT 253 for isotope analyses. Details of the online system are given in Barnes and Sharp (2006)[56] and in the supporting information (Method S2 and Fig. S5). $\delta^{37}Cl(CH_3Cl)$ values are reported in standard per mil notation vs. SMOC (Standard Mean Ocean Chloride; $\delta^{37}Cl_{SMOC} = 0‰$). Uncertainty (± 1σ) in chlorine isotope analyses is ± 0.2‰ based on the long-term precision of seawater standards. Error on seawater standards analyzed during this study was also ± 0.2‰. Seawater standards were prepared following the methods outlined in Eggenkamp (1994), in which $Cl^-(aq)$ is precipitated as AgCl via reaction with $AgNO_3$, and AgCl subsequently reacted with excess CH_3I to produce CH_3Cl. The uncertainty from repeated analyses of the internal UT CH_3Cl reference gas transferred to Exetainer® vials flushed with helium and analyzed in the same manner as samples was ± 0.1‰.

Stable chlorine isotope analyses using GC-MC-ICPMS

Stable chlorine isotope analysis of CH_3Cl samples (2 L stainless steel canisters) from ·OH and ·Cl radical reaction experiments were carried out by using recently reported analytical protocols[57, 58]. Briefly, gas chromatography (GC) is coupled via a heated transfer line to a multiple-collector inductively coupled plasma mass spectrometer (Neptune, ThermoFisher Scientific, Germany). Gas samples of 2 to 20 mL were taken directly from the stainless steel canisters using a gas-tight syringe equipped with a push-button valve (VICI precision sampling). These gas samples were injected into the GC operated in split-less mode. Analytes were trapped on a custom-built cryotrap cooled with liquid nitrogen, in order to allow for injection of volumes larger than 1 mL, while maintaining satisfactory chromatographic peak shape. This cryotrap consists of a 1/16” inch (1.59 mm) stainless steel tube (U-shape, 100 mm long), connected in line with the chromatographic column (ZB1 Phenomenex, 60m, 0.32 ID, operated at 2mL/min constant flow). Once trapped, analytes were released by immersing

the cryotrap into a warm water bath (~40°C). The GC temperature was kept at 30°C. Two analyses of a reference CH_3Cl sample were carried out in addition to each experimental sample to determine raw-$\delta^{37}Cl$ values, as relative differences of the sample $^{37}Cl/^{35}Cl$-ratio from the reference $^{37}Cl/^{35}Cl$ ratio. These raw $\delta^{37}Cl(CH_3Cl)$ values were then normalized to the SMOC scale by applying a two-point calibration approach using three organic in-house reference compounds (TCE-2, TCE-6, and CH_3Cl). Overall uncertainty for analysis by this method was usually better than 0.2‰ at the 1σ level.

Comparison of $\delta^{37}Cl(CH_3Cl)$ values measured by CF-IRMS and GC-MC-ICPMS

Subsamples of chloromethane (CH_3Cl 2.8, 99.8%, Air Liquide, Düsseldorf, Germany) were transferred to 12 mL septum-capped Exetainer® and 2 L stainless steel canisters and measured at University of Texas and the Helmholtz Centre for Environmental Research in Germany using CF-IRMS and GC-MC-ICPMS, respectively. The commercial CH_3Cl source material (Sigma-Aldrich) was analyzed along with the experimental samples using the methods outlined above. $\delta^{37}Cl(CH_3Cl)$ values for commercial CH_3Cl sources were in good agreement, yielding values of of 6.4 ± 0.3‰ (n=9) and 5.99 ± 0.15‰ (n=18), respectively.

Kinetic isotope effect, fractionation constant *α*, isotope fractionation *ε*, and lambda values

The KIE is typically expressed as isotope fractionation factor α or isotope fractionation ε (also termed isotope enrichment factor/constant). In this manuscript, we use ε for isotope effects in CH_3Cl degradation experiments.

Stable chlorine isotope fractionation (ε_{Cl}) was derived from the slope of the Rayleigh plot according to Clark and Fritz[59], Elsner et al.[60] and equation 2:

$$\ln\frac{R_t}{R_0} = \left(\frac{\delta^{37}Cl_t+1}{\delta^{37}Cl_0+1}\right) = ln\frac{(\delta^{37}Cl_0+\Delta\delta^{37}Cl+1)}{(\delta^{37}Cl_0+1)} \cong (\alpha-1)\cdot lnf = \varepsilon_{Cl}\cdot lnf \quad (2)$$

Where R_t and R_0 are the $^{37}Cl/^{35}Cl$ ratios in CH_3Cl at the different time points and time zero, respectively, and f is the remaining CH_3Cl fraction at different time points. Negative values of ε_{Cl} indicate that the remaining CH_3Cl is enriched in the heavier isotopologue ($CH_3{}^{37}Cl$).

To account for analyte dilution (airflow through smog chamber) during CH_3Cl degradation by ·OH and ·Cl radicals, the remaining fraction f was calculated as follows

$$f = c_{xT}*c_{i0}/(c_{x0}*c_{iT}) \quad (3)$$

where c_{x0} and c_{xT} are the mixing ratios of CH_3Cl at time zero and time t and c_{iO} and c_{iT} are the respective concentrations of the internal tracer perfluorohexane. The uncertainty for the remaining fraction was better than 2 %.

For comparison of multi-element compound-specific isotope fractionation during experiments of CH_3Cl degradation by growing cultures of bacterial strains *M. extorquens* CM4 and *L. methylohalidivorans* MB2, we determined lambda (Λ) values[61], expressing the slope of changing carbon and chlorine stable isotope ratios as CH_3Cl degradation progressed, for each strain growing with CH_3Cl. The following relationships were used for determination of $\Lambda^{C/Cl}$ and $\Lambda^{H/C}$, respectively:

$$\Lambda^{C/Cl} = \frac{\Delta\delta^{13}C}{\Delta\delta^{37}Cl} \quad (4)$$

$$\Lambda^{H/C} = \frac{\Delta\delta^{2}H}{\Delta\delta^{13}C} \quad (5)$$

The reader is referred to the supporting information (Method S3) for a detailed description of methods for measurement of stable carbon and hydrogen isotopes of CH_3Cl from bacterial degradation experiments.

Alternatively, Λ-values may also be estimated according to the following relationship:[44]

$$\Lambda^{C/Cl} \approx \frac{\varepsilon_C}{\varepsilon_{Cl}} \quad (6)$$

where ε_C and ε_{Cl} are the fractionations of carbon and chlorine for the same degradation mechanism in CH_3Cl. This relationship was applied to determine $\Lambda^{C/Cl}$ and $\Lambda^{H/C}$ values of CH_3Cl degradation associated with ·OH and ·Cl reactions.

Statistics

Linear regressions of the Rayleigh plots as shown in Figures 1, 2 and 3 were calculated using MATLAB® Version R2018a, in which errors of each data point were considered. Error bars of single points were calculated by error propagation including uncertainties in $\delta^{37}Cl(CH_3Cl)$ values and the remaining fraction *f*. Dashed lines shown in the Figures represent 95% confidence intervals of linear regressions (bold lines).

RESULTS AND DISCUSSION

Chlorine isotope fractionation associated with bacterial degradation of CH_3Cl

Biotic CH_3Cl degradation experiments were performed with bacterial strains *Methylorubrum extorquens* CM4 and *Leisingera methylohalidivorans* MB2 growing with CH_3Cl. The two strains were isolated from terrestrial and marine environments, respectively, and grow with CH_3Cl under aerobic conditions. Both strains were grown in biological triplicates with CH_3Cl as the sole carbon and energy source. Under the chosen experimental conditions, between 51 and 86 % of the initial CH_3Cl was consumed within 29 h upon bacterial growth. Results of CH_3Cl isotopic analysis are shown in Figures 1 and 2 for strain CM4 and strain MB2, respectively. Samples retrieved throughout the course of bacterial growth were analyzed for 10 time points for one biological replicate (Figs. 1 and 2), and for 4 time points for the two other biological replicates (Figs. S6 and S7). Calculated ε_{Cl} values from the three independent biological replicate experiments of *M. extorquens* CM4 were consistent and in good agreement (-10.3 ± 1.2‰, -10.7 ± 0.6 ‰, and -11.8 ± 0.4 ‰, respectively, Fig. 1b and Fig. S6), with a correlation coefficient R^2 of the slope of the regression line ranging from 0.87 to of 0.97 in the three independent bacterial growth experiments. For *L. methylohalidivorans* MB2, calculated ε_{Cl} values from the corresponding three independent biological replicate experiments were also in good agreement (-8.3 ± 0.2 ‰, -9.9 ± 0.7 ‰, and -9.9 ± 0.7 ‰ respectively, with R^2 of the slope of the regression line between 0.93 and 0.99 (Fig. 2b and Fig. S7). Overall, this yielded mean ε_{Cl} values for *M. extorquens* CM4 and *L. methylohalidivorans* MB2 of -10.9 ± 0.7 ‰ and -9.4 ± 0.6 ‰, respectively. No significant CH_3Cl degradation, and no change in $\delta^{37}Cl(CH_3Cl)$ values, (-7.20 ± 0.32 ‰ and -7.25 ± 0.25 ‰ for *M. extorquens* CM4 and *L. methylohalidivorans* MB2, respectively) were detected in

otherwise identical experiments without bacterial inoculation performed as controls in parallel.

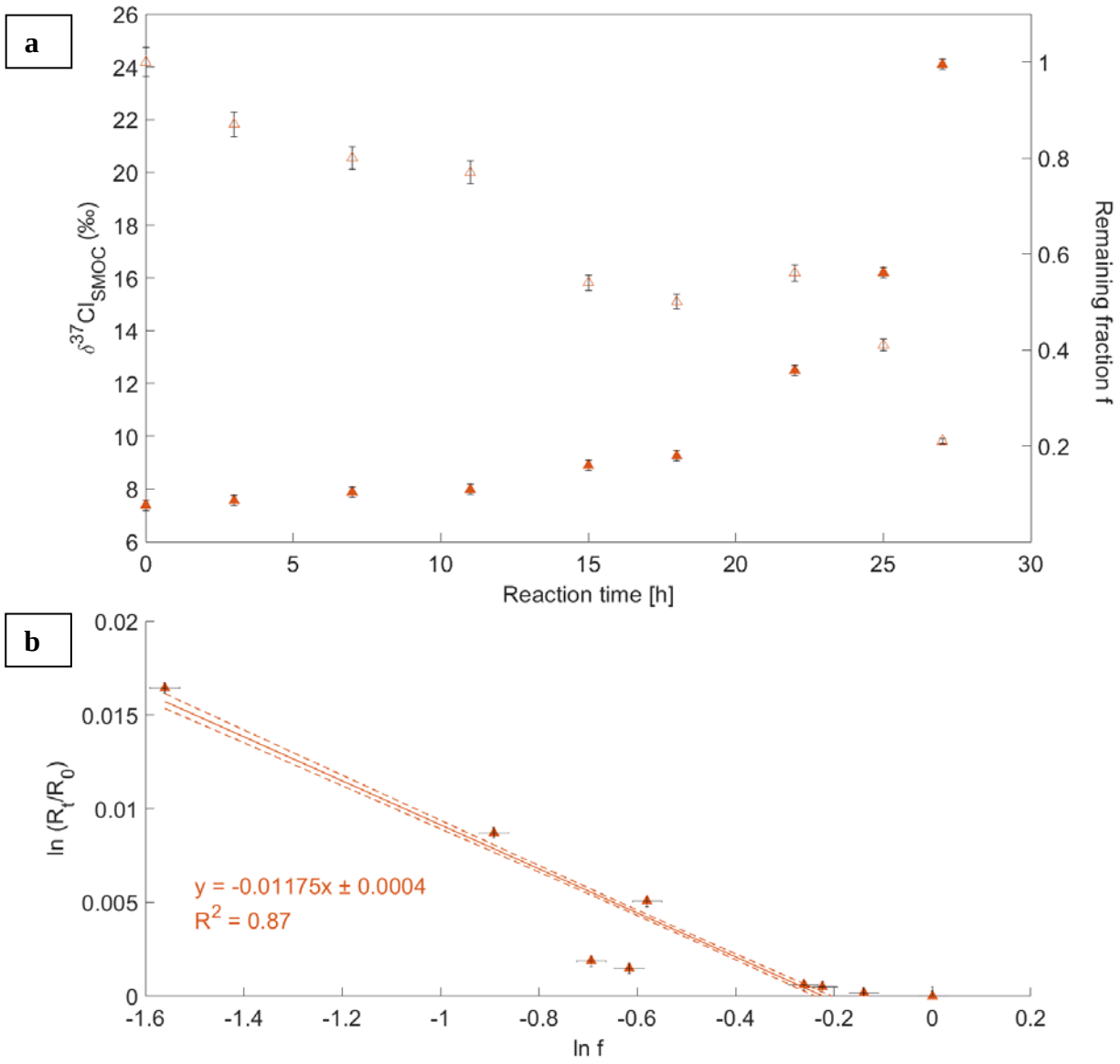


Figure 1: Chlorine isotope fractionation for degradation of CH_3Cl by *M. extorquens* CM4 during growth with CH_3Cl as the sole carbon and energy source. (a) Measured $\delta^{37}Cl(CH_3Cl)$ values (filled triangles) versus remaining fraction of CH_3Cl (open triangles). The data obtained for one representative replicate are shown. Error bars of $\delta^{37}Cl(CH_3Cl)$ values indicate the standard deviation (SD) of the mean of three measurements (most error bars are smaller than the symbol). Error bars of the remaining fraction *f* show the uncertainty for CH_3Cl concentration measurements. (b) Rayleigh plots (equation 2) from CH_3Cl degradation

experiments. Data for one representative replicate are shown. (same as in (a)). Error bars were calculated by error propagation, including uncertainties in $\delta^{37}Cl(CH_3Cl)$ values and the remaining fraction *f*. Dashed lines represent 95% confidence intervals of linear regressions (bold lines).

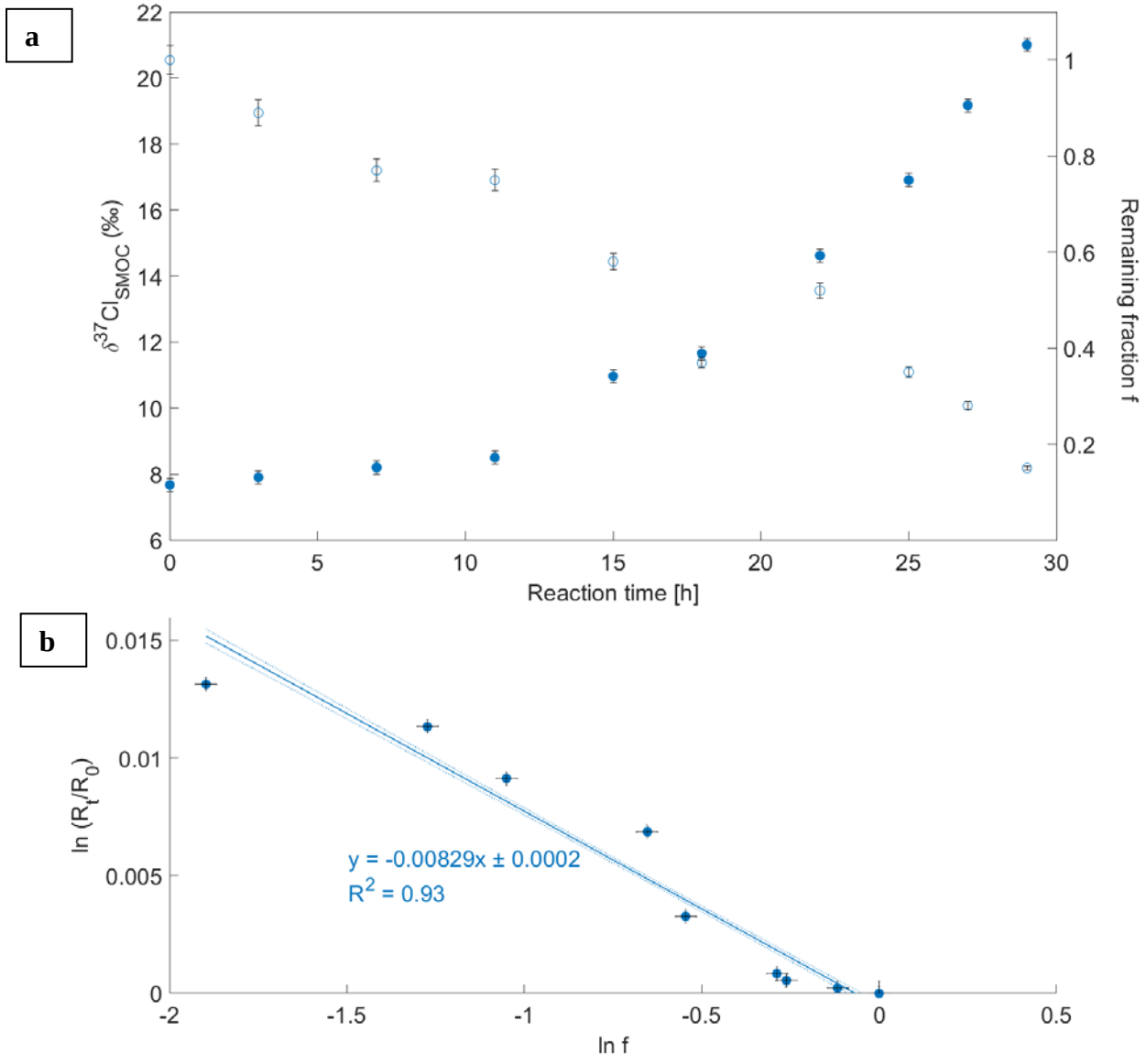


Figure 2: Chlorine isotope fractionation for degradation of CH_3Cl by *L. methylohalidivorans* MB2 during growth with CH_3Cl as the sole carbon and energy source. (a) Measured $\delta^{37}Cl(CH_3Cl)$ values (filled dots) versus remaining fraction (open dots) of CH_3Cl. The data obtained for one representative replicate are shown. Error bars of $\delta^{37}Cl(CH_3Cl)$ values

indicate SD of the mean of three measurements (most error bars lie within the symbol). Error bars of the remaining fraction *f* show the uncertainty for CH_3Cl concentration measurements. (b) Rayleigh plots from CH_3Cl degradation experiments. Data for one representative replicate are shown. (same as in (a)). Error bars were calculated by error propagation including uncertainties in $\delta^{37}Cl(CH_3Cl)$ values and the remaining fraction *f*. Dashed lines represent 95% confidence intervals of linear regression (bold lines).

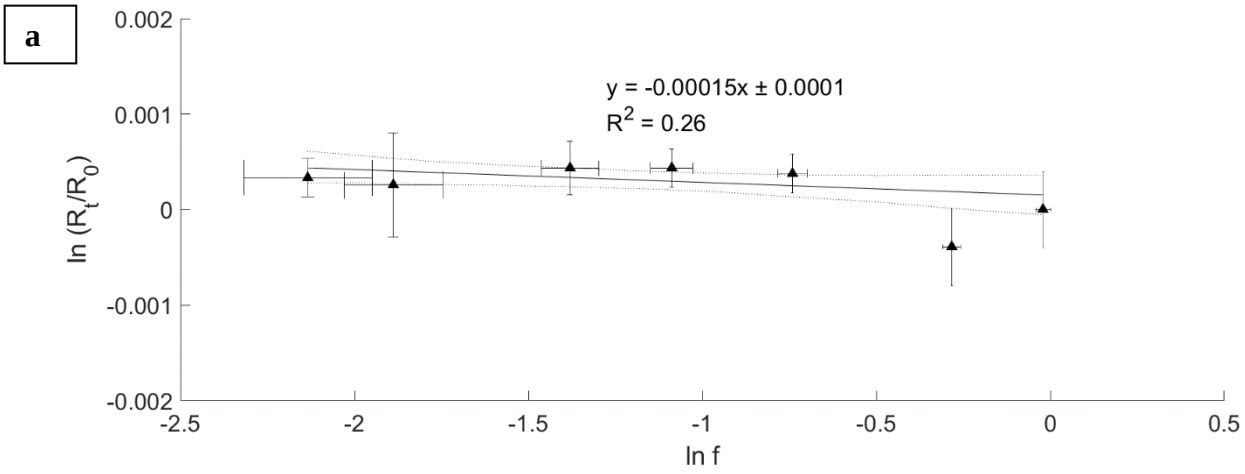
The new chlorine isotopic fractionation data obtained in this work provide further evidence that strains CM4 and MB2 use different pathways for CH_3Cl degradation[41]. So far, the molecular details of aerobic CH_3Cl utilization have been elucidated in detail for one pathway only, the *cmu* pathway found in many taxonomically diverse chloromethane-degradation strains[62 63], and discovered and characterised in detail for strain CM4. Random mutagenesis of strain CM4 allowed to identify genes essential for growth with CH_3Cl[64] and the corresponding CH_3Cl dehalogenase[65, 66]. Dehalogenation of CH_3Cl by the cmu pathway (for chloromethane utilization) involves the two-domain methyltransferase/corrinoid-binding protein CmuA which catalyzes the transfer of the CH_3Cl methyl group to a corrinoid cofactor[64, 66]. Methylcobalamin:H_4F methyltransferase (CmuB) effects transfer of the methyl group from the corrinoid to tetrahydrofolate (H_4F)[65]. *Leisingera methylohalidivorans* MB2, in contrast, grows with CH_3Cl with an as yet unknown pathway[41]. The genome sequence of the strain has been determined[53], and confirms it to be a methylotrophic bacterium capable of assimilating C1 compounds for growth, but the strain lacks *cmu* genes and thus the corresponding dehalogenase[41].

Nevertheless, the substantial chlorine isotope fractionation (ε_{Cl} ~-10 ‰) observed during growth of both CM4 and MB2 strains with CH_3Cl (with *M. extorquens* CM4 showing a slightly more negative mean ε_{Cl} value) suggests that CH_3Cl degradation starts with the

dehalogenation reaction in both strains. This is in agreement with Streitwieser's semi-classical limit for chlorine isotope effects for C-Cl bond breakage at 25°C, calculated to be -13 ‰[60]. Westaway et al.[67] also reported isotope effects for S_N2 reactions of para substituted benzylchlorides with cyanide, a chlorine leaving group effect of ~-5.3 ‰, and larger isotope effects of up to -10 ‰ for other nucleophiles. Potential differences in enzymatic dehalogenation mechanisms and associated isotopic fractionation patterns in the two bacterial CH_3Cl-degrading strains investigated here are discussed below, in the section 'Triple element isotope effects and mechanisms of CH_3Cl degradation'.

Chlorine isotope fractionation of CH_3Cl associated with ·OH and ·Cl reactions in the atmosphere

Strikingly, radical-driven degradation of CH_3Cl by ·OH and ·Cl does not result in detectable chlorine isotope fractionation, basing on smog chamber experiments (Fig. 3). In other words, the unreacted remaining CH_3Cl fraction did not vary in $\delta^{37}Cl(CH_3Cl)$ i.e. obtained regression coefficients were not significant, with p-values of 0.14 and 0.66 for reaction with ·OH and ·Cl, respectively.



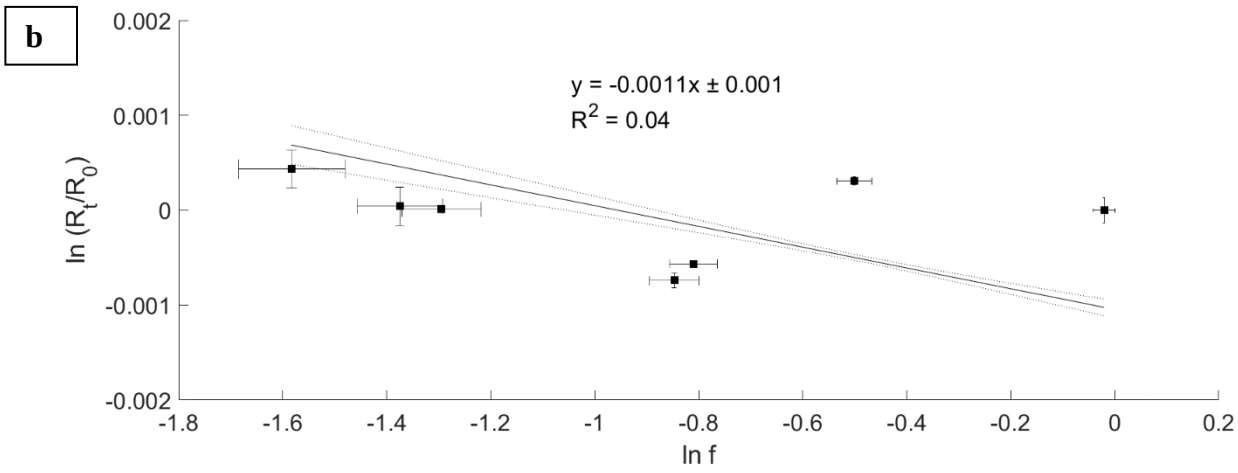


Figure 3: Rayleigh plots for gas phase reactions of CH_3Cl with ·OH (a) and ·Cl (b) radicals. Error bars include the SD of 2-3 replicate isotope measurements. Error bars were calculated by error propagation including uncertainties in $\delta^{37}Cl(CH_3Cl)$ values and the remaining fraction *f*. Uncertainty in quantification of *f* was usually better than 2%. Dashed lines represent 95% confidence intervals of the linear regressions (bold lines).

In addition to GC-MC-ICPMS analysis, stable chlorine isotopes were also analysed by GC-MS during smog chamber experiments (see supporting information, Method S1 and Figs. S1-S4), and confirmed non-significant isotope fractionation upon degradation of CH_3Cl with ·OH and ·Cl radicals. In both ·OH and ·Cl radical reactions with CH_3Cl, the first step is abstraction of a hydrogen atom to yield $·CH_2Cl$ and H_2O or HCl, respectively[27, 68]. Hence, the chlorine atom of CH_3Cl is not directly involved in these processes, and thus only contributes to a small secondary isotope effect for this type of reaction[60].

Triple element isotope effects and mechanisms of CH_3Cl degradation

CH_3Cl degradation through cleavage of the C-Cl bond by bacteria in terrestrial and marine environments

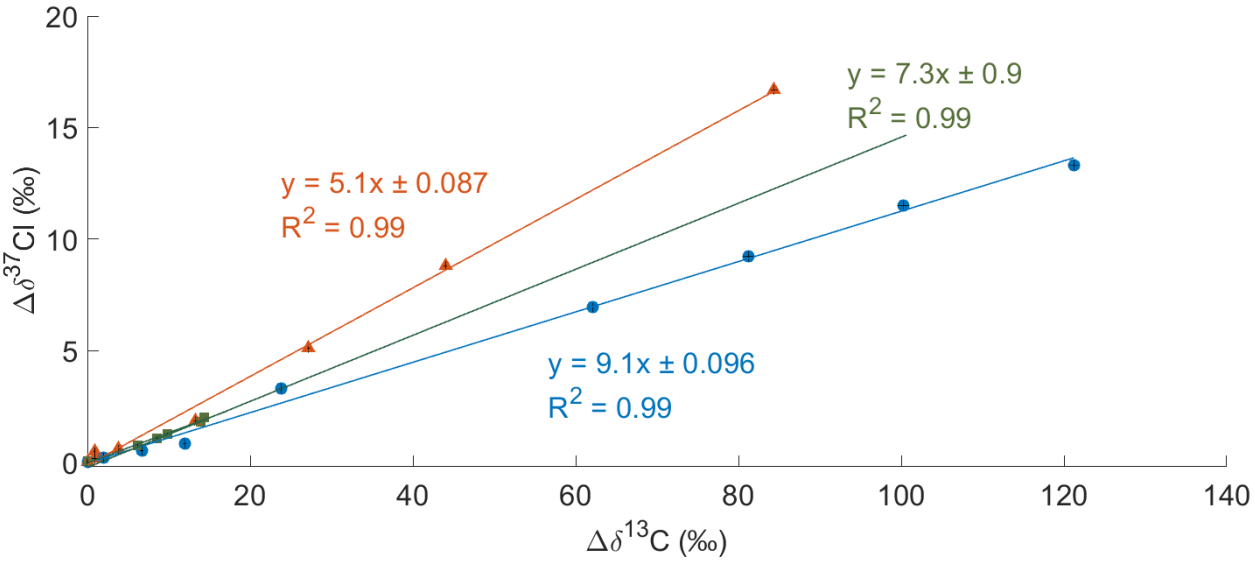
Bacterial degradation of CH_3Cl caused relatively large chlorine isotope fractionation, likely due to initial C-Cl bond cleavage as discussed above. However, the mechanistic details of degradation of CH_3Cl are not yet known for any microbial system. Nevertheless, in the case of the *cmu* degradation pathway[62], as well as in other still incompletely characterised systems[69], the types of proteins that are likely involved give strong hints. It seems clear that corrinoid and folate cofactors usually play a major role in CH_3Cl degradation. This strongly suggests nucleophilic attack with chloride as a leaving group, and thus the involvement of S_N2-type reactions. Alternative dehalogenation mechanisms, such as by direct nucleophilic attack on the chlorine substituent, have been reported for aromatic halogenated compounds[70]. However, this seems highly unlikely in the case of CH_3Cl, since this would involve a methyl anion or radical as the intermediate. Indeed, homolytic cleavage of C-Cl or C-H bonds, or initial abstraction of hydrogen, has to our knowledge not been proposed or documented to date for growth-supporting degradation of CH_3Cl.

Nucleophilic substitution reactions (S_N2) were also reported for abiotic degradation of CH_3Cl, with H_2O being the most relevant nucleophile in typical environmental settings[70, 71]. In these cases, chlorine isotope fractionation of -5.3 ‰ were reported[44] which is about half the value of biotic bacterial degradation found in the present study. Furthermore, a large carbon isotope fractionation of ~-42 ‰, and a secondary inverse hydrogen isotope fractionation of +25‰, were reported, in agreement with previous theoretic and experimental studies[72-74].

To further define isotope effects due to bacterial degradation of CH_3Cl, gas samples from bacterial growth experiments were also analysed for their corresponding $\delta^2H(CH_3Cl)$ and

399 $\delta^{13}C(CH_3Cl)$ values (Figs. S8 and S9, Table 1). For carbon, all samples of the three biological
400 replicate growth experiments of the two investigated bacterial strains were measured, and
401 samples from one biological replicate was analysed for hydrogen. Obtained results were in
402 general agreement with previous results by Nadalig et al.[41].
403 Although differences in observed hydrogen and chlorine isotope fractionation for the two
404 bacterial strains of different CH_3Cl degradation pathways are relatively modest (Table 1), the
405 two strains are most readily distinguished by their carbon isotope fractionation pattern. In
406 addition, combining the new isotope analysis of chlorine with that of carbon and hydrogen
407 clearly allows for better determination of the distinct modes of CH_3Cl degradation operating
408 in the two strains (Table 1). This is readily apparent, for example, from significantly different
409 plots of changes of chlorine and carbon isotope values for CH_3Cl upon its degradation by the
410 two strains (Fig. 4). Lambda ($\Lambda^{C/Cl}$) values expressing the slope of changing carbon and
411 chlorine stable isotope ratios[61] in the course of CH_3Cl transformation reactions were
412 calculated to be 5.1 and 9.1 for *M. extorquens* CM4 and *L. methylohalidivorans* MB2,
413 respectively.

414 Comparison of isotope fractionation of biotic degradation with previous results on abiotic
415 degradation thus demonstrates the power of triple-element isotope analysis. Indeed, both
416 abiotic hydrolytic and biotic degradation reactions follow S_N2 type reaction mechanisms.
417 However, the magnitude of ε_C and ε_{Cl} alone would not be sufficient to distinguish abiotic
418 from biotic degradation processes. Moreover, $\Lambda^{C/Cl}$ for abiotic hydrolysis of CH_3Cl was 7.3[44],
419 within the range of values obtained for bacterial degradation in the current study. If isotopic
420 fractionation for hydrogen is also considered, then abiotic and biotic mechanisms can be
421 clearly distinguished. Bacterial degradation yielded a normal isotope effect, in contrast to
422 abiotic hydrolysis (Fig. 5): $\Lambda^{H/C}$ ranged here between 0.6 and 0.9 for bacterial degradation, in
423 contrast to the negative $\Lambda^{H/C}$ value of -0.6[44] observed for abiotic degradation (Table 1).



424

425 **Figure 4**: Comparison of changes (Δ) in stable carbon and chlorine isotope values during
426 degradation of CH_3Cl by bacterial strains *M. extorquens* CM4 (triangles) and *L.*
427 *methylohalidivorans* MB2 (circles) and for abiotic hydrolysis (squares). Error bars in
428 $\delta^{13}C(CH_3Cl)$ and $\delta^{37}Cl(CH_3Cl)$ values of bacterial strains reflect analytical uncertainty in
429 replicate measurements (n=3-4; most error bars lie within the symbols). Confidence intervals
430 of the linear regressions are not displayed as they overlap with the line of linear regression.
431 Values for hydrolysis are from a previous study by Horst et al.[44].

432

433 ***Cleavage of the C-H bond of CH_3Cl - tropospheric degradation of CH_3Cl by ·OH and ·Cl***
434 The gas samples investigated here for chlorine isotope effects were previously analysed for
435 hydrogen and carbon[4, 38]. In these smog chamber experiments, no significant chlorine kinetic
436 isotope effects associated with degradation of CH_3Cl by ·OH and ·Cl radicals were detected
437 (Fig. 3, Table 1). However, a very large hydrogen isotope fractionation (-264 ± 45 ‰ and -
438 280 ± 11 ‰) was observed for reaction of CH_3Cl with hydroxyl and chlorine radicals,
439 respectively[38]. Thus large hydrogen isotope fractionation suggests a primary isotope effect
440 involving hydrogen in the reacting bond, and thus initial cleavage of the C-H bond in both

441 ·OH and ·Cl driven reactions. With regard to isotopic fractionation of carbon in the same
442 experiments, values of -11.2 ± 0.8 ‰ and -10.2 ± 0.5 ‰ respectively, were obtained[4], i.e. 5 to
443 6-fold smaller than previously reported[39]. Streitwieser's semi-classical limit for isotope
444 effects associated with C-H bond cleavage is -21 ‰[60], and an ε value of -15‰ had been
445 reported for reactions involving hydrogen radical transfer[75]. This suggests much lower carbon
446 fractionation values for abiotic radical-driven reactions than those previously reported[39]. For
447 a more detailed discussion regarding differences of carbon isotope fractionation by ·OH and
448 ·Cl radicals we would like to refer to the study by Bahlmann et al.[4].

449

450

451 **Application of stable chlorine isotopes and triple element isotope analysis: towards**
452 **resolving the global CH_3Cl budget**

453 The observed large differences in chlorine fractionation of the major CH_3Cl sinks by ·OH and
454 ·Cl radicals in the troposphere, and by bacteria from terrestrial and marine environments of
455 around ~-10 ‰ are ideally suited to apportion the two known major sinks into their relative
456 contribution and strength for balancing the global annual release of CH_3Cl of around 4 to 5
457 Tg yr^{-1}. The global magnitude of microbial CH_3Cl degradation currently still remains highly
458 uncertain, ranging from 0.1 to 1.6 Tg yr^{-1} in soils for example[3, 4, 31, 38] but even could be
459 higher if vegetation will be confirmed as another major sink of CH_3Cl[43]. For constraining the
460 global budget based on their stable isotope values, we may use equation 1, and Table 1 (also
461 see associated text in the introduction). In so doing, it becomes clear that once $\delta^{37}Cl(CH_3Cl)$
462 values for atmospheric CH_3Cl and the weighted average isotopic signature of all major
463 sources becomes available, it might be straightforward to calculate the relative contribution
464 of the two major sinks. Notably, should the difference between $\delta^{37}Cl(CH_3Cl)$ values of

atmospheric CH_3Cl and the weighted average isotopic signature of all major sources become larger, this will increase the relative importance of bacterial degradation relative to the tropospheric sink, and vice versa. Along the same lines, small deviations of tropospheric $\delta^{37}Cl(CH_3Cl)$ values from the weighted average isotopic signature of the sources will support radical-driven degradation processes in the atmosphere as the major CH_3Cl sinks in the environment. For instance, a change of ~1‰ of $\delta^{37}Cl(CH_3Cl)$ values between the tropospheric and the weighted average isotopic signature of major sources will roughly shift (increase/decrease) the relative contribution of each of the sinks by around 0.4 to 0.5 Tg yr^{-1}. However, much more advanced modelling tools will be necessary to disentangle the contribution of each degradation process and to take into consideration a constant emission of fresh CH_3Cl from sources to the tropospheric burden. Such a mass balance model was recently presented for stable carbon isotopes of CH_3Cl[4]. The findings of the current study may be particularly useful in triple-element isotopic approaches to develop more powerful models for better quantification of degradation processes. As shown above, the mechanisms and resulting isotope fractionations are highly different which bodes well for a successful application in such isotopic mass balance approaches.

Taken together, our results (Fig. 5) show that in the case of CH_3Cl, the combination of chlorine, carbon and hydrogen isotopic analysis provides insights that would be missed by analysis of only one or even two elements only. First, substantial chlorine isotope fractionation was observed for bacterial degradation of CH_3Cl as well as for hydrolysis[44], whereas no chlorine isotope fractionation was observed for photochemical degradation by ·OH and ·Cl radicals. This indicates that any change in $\delta^{37}Cl$ values in the atmosphere is either due to source emissions or degradation in water or soil. Second, the opposite was found for hydrogen, for which large isotope fractionation was observed for CH_3Cl destruction by ·OH and ·Cl radicals (ε_H values of around -250‰ to -300‰)[38], and only minor fractionation

by bacterial degradation (ε_H values of around 0 to -50‰)[41, 42]. Hence, large variabilities of δ^2H values may be indicative for atmospheric degradation providing the possibility, under certain conditions, to estimate the contribution by this sink directly from tropospheric samples. The small but inverse secondary hydrogen isotope fractionation was reported for hydrolysis of CH_3Cl[44] provides a valuable tool to distinguish abiotic processes from microbial reactions where hydrogen fractionation is normal. Finally, moderate carbon isotope fractionation was measured for abiotic decomposition by ·OH and ·Cl radicals (ε_C values of around -11‰), whereas large isotope effects (ε_C >-50‰) were determined for CH_3Cl consumption by the two bacterial strains *M. extorquens* CM4 and *L. methylohalidivorans* MB2, as well as for abiotic hydrolytic degradation (-42‰)[44]. This suggest that, even though carbon is involved in all reactions, significant differences in fractionations are found which may further help to disentangle sink processes in atmospheric samples.

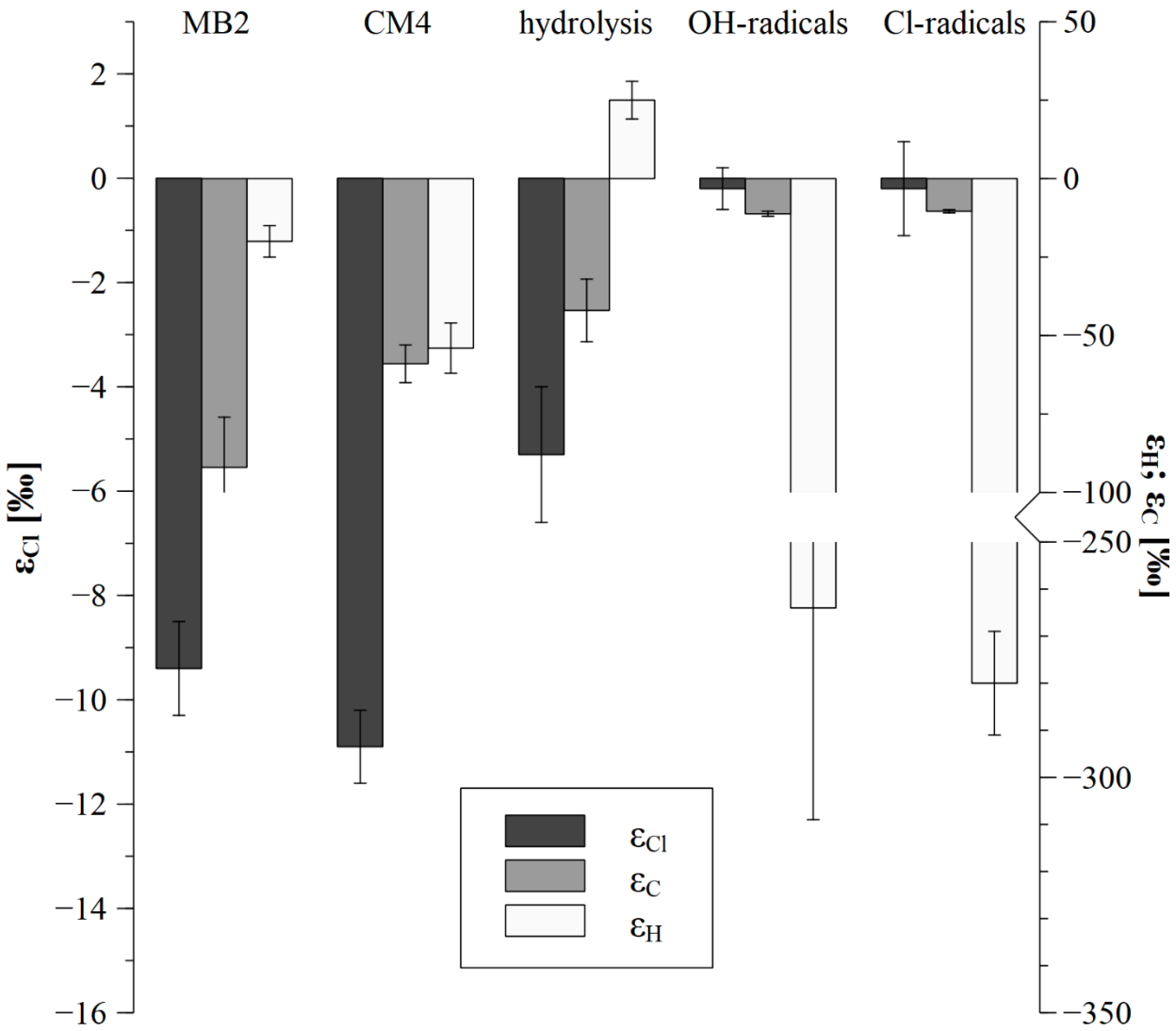


Figure 5: Comparison of hydrogen, carbon and chlorine isotope fractionation for known biotic and abiotic CH_3Cl degradation processes in the environment. Errors bars for the two bacterial strains *M. extorquens* CM4 and *L. methylohalidivorans* MB2 show SD of three independent experiments. Values of ε_C and ε_H for photochemical degradation of CH_3Cl by ·OH and ·Cl radicals and ε_C, $\varepsilon_{H \text{ and}}$ ε_{Cl} for hydrolysis are from previous studies by Keppler et al.[38], Bahlmann et al.[4] and Horst et al.[41], respectively.

Analysing the obtained data in more detail, the extremely large ε_C of around -80 ‰ to 90‰ associated with *L. methylohalidivorans* MB2 consumption of CH_3Cl also suggests that its still

512 unknown pathway for growth with CH_3Cl can be better distinguished from the *cmu* pathway
513 using triple element isotope analysis. Contributions to the atmospheric CH_3Cl budget from
514 bacteria living in marine and terrestrial environments with either the *cmu* pathway or the yet
515 unknown pathway of *L. methylohalidivorans* MB2 may thus be better teased apart in the
516 future, and their relative specific contributions better defined. The data obtained here on
517 strain CM4 and MB2 as two key and distinct terrestrial and marine reference bacterial
518 systems for biotic degradation of CH_3Cl pave the way for future work on other
519 chloromethane-degrading microorganisms capable of growth with CH_3Cl by other still
520 uncharacterised CH_3Cl degradation pathways, in particular under anaerobic conditions[69, 76],
521 as well as on ecosystems in which CH_3Cl degradation occurs. Recent evidence, also in part
522 from our own work, suggests that yet to be discovered processes of CH_3Cl degradation
523 beyond that of the only characterised *cmu* degradation pathway may prevail in the
524 environment, e.g. in forests[77], and perhaps also in specific ecosystems such as saline caves[78].
525 With respect to abiotic degradation, it becomes obvious that this process may be readily
526 identified and distinguished from biotic degradation via its unusual inverse fractionation
527 pattern for hydrogen (Table 1, Fig. 5).
528 Finally, the application of stable chlorine isotopes including its use for triple element isotopic
529 analysis approach may provide unique opportunities to refine our understanding of natural
530 CH_3Cl dynamics from process to global scale. However, a prerequisite for further detailed
531 analysis will be the determination of the average tropospheric variability of the isotopic
532 composition of CH_3Cl as well as $\delta^{37}Cl(CH_3Cl)$ signatures of the major sources, which are not
533 yet available. Although measuring tropospheric $\delta^{37}Cl(CH_3Cl)$ values represents a massive
534 analytical challenge due to the relatively low atmospheric abundance of ~550 pptv of CH_3Cl,
535 obtaining this information now appears crucial for a refined, improved isotopic mass balance
536 of atmospheric CH_3Cl, and thus to advance our understanding of the global CH_3Cl budget.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Method details and results of stable carbon and hydrogen isotopes of CH_3Cl measurements from bacterial degradation experiments as well as $\delta^{37}Cl(CH_3Cl)$ measurements from degradation of CH_3Cl by ·OH and ·Cl radicals.

AUTHOR INFORMATION

Corresponding Author

EMAIL: frank.keppler@geow.uni-heidelberg.de

ORCID

Frank Keppler: 0000-0003-2766-8812

Author Contributions

F.K., E.B. and S.V. conceived the study. J.L. and T.N. carried out bacterial degradation experiments and analyzed the data together with S.V. J.B. measured $\delta^{37}Cl(CH_3Cl)$ values of gas samples from bacterial degradation using CF-IRMS. E.B. performed smog chamber experiments and measured $\delta^{37}Cl(CH_3Cl)$ values using GC-MS. A.H. conducted $\delta^{37}Cl(CH_3Cl)$ analysis of smog chamber samples using GC-MC-ICPMS. S.C.H and M.G. performed $\delta^{2}H(CH_3Cl)$ and $\delta^{13}C(CH_3Cl)$ measurements and analyzed the data together with F.K.. The manuscript was written under the lead of F.K., with contributions of all authors. All authors have given approval to the final version of the manuscript.

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Table 1. Known sinks of tropospheric CH_3Cl, and corresponding values for chlorine, hydrogen and carbon isotope fractionation ε, $\Lambda^{C/Cl}$ and $\Lambda^{H/C}$ from the literature and obtained in this study.

Sinks	Sink (best estimate)[a] (Gg yr^{-1})	Sink (full range)[a] (Gg yr^{-1})	$\varepsilon_{chlorine}$ ‰	$\varepsilon_{hydrogen}$ ‰	ε_{carbon} ‰	$\Lambda^{C/Cl}$	$\Lambda^{H/C}$
Reaction with ·OH in troposphere	2832	2470 to 3420	-0.1±0.1[b]	-264±45[c] -410±50[e]	-11.2±0.8[d] -58±10[f]	112±8[b,d]	24±4[c,d]
Reaction with ·Cl in marine boundary layer	370	180 to 550	-1.1±1[b]	-280±11[c] -420±40[e]	-10.4±0.5[d] -70±10[f]	9.5±0.6[b,d]	27±0.6[c,d]
Loss to stratosphere	146	n.a.	0[g]	0[g]	0[g]	n.a.	n.a.
Microbial degradation in soil	1058	200 to 1482	-10.9±0.7[b]	-29 to -54[b,h]	-37 to -59[b,d,h,i,,j]	5.1±0.1[b]	0.6±0.1[b]
Loss in ocean - microbial	370	296 to 445	-9.4±0.9[b]	0 to -20[b,i]	-76 to 92[b,i]	9.1±0.1[b]	0.9±0.04[b]
and hydrolysis			-5.3±1.3[k]	+25±6[k]	-42±10[k]	7.3±0.9[k]	-0.6±0.3[k]
Microbial degradation in plants	n.a.	n.a.	n.a.	-8±19[l]	−39±3[l]	n.a.	n.a.
Total sinks	4406 (4776)	3292 to 6043					

[a] Values for the magnitude of sinks were mainly taken from Carpenter et al.[3], except for the reaction with ·Cl radical in the marine boundary layer, microbial degradation in soil, and for total sinks shown in brackets which includes the sink strength by chlorine radical in marine boundary layer[2]. The ocean sink was calculated as gross deposition fluxes to undersaturated regions of the global ocean. Isotopic fractionation values given for CH_3Cl net loss to oceans are based on both biological (microbial) and abiotic (hydrolysis) degradation processes. Due to possible intrinsic production, microbial degradation of CH_3Cl may be substantially larger. Microbial degradation in plants has been recently proposed[43], but estimates of the corresponding sink strength have not been reported so far.

[b] this study

[c] Keppler et al.[38]

[d] Bahlmann et al.[4]

[e] Sellevåg et al.[79]

[f] Gola et al.[39]

[g] Thompson et al.[36]

[h] Jaeger et al.[33]

[i] Nadalig et al.[41]

[j] Miller et al.[32]

[k] Horst et al.[44]

[l] Jaeger et al.[43]

n.a. (not available) indicates that no value has been reported