Investigating the origin of chloroform in soils and groundwater using carbon and chlorine stable isotopes analysis

by

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"Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand."

Albert Einstein

New York Times, March 8th 1931

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Summary

Chloroform ($CHCl_3$) has been considered for a long time as an anthropogenic contaminant which is known to be possibly carcinogenic to humans. Chloroform is also an important contributor to gaseous chlorine in the atmosphere and may catalyze some chemical reactions in the lower atmosphere. The presence of chloroform in aquatic and terrestrial environments has been widely demonstrated. The frequent detection of chloroform in forest soils, peatland and groundwater in absence of other anthropogenic contaminants suggests that chloroform may be produced naturally by biogeochemical processes. Numerous studies on natural organohalogens have suggested that enzymes such as haloperoxidases (e.g. chloroperoxidase from *Caldariomyces fumago*) and halogenases excreted by some fungi, plants and bacteria could play an important role in biosynthesis of chlorinated organic compounds in soils. Although the natural formation of chloroform in some forest soils and peatlands is rather well establish, it is difficult to demonstrate a natural origin of chloroform in groundwater as chloroform is widely used as solvent or chemical reagent and can be transported over extended distances from anthropogenic sources. For risk assessment and to implement appropriate remediation measures, there is a need for methods to differentiate between natural and anthropogenic chloroform. Moreover, little is known about the mechanisms leading to the formation of natural chloroform in the terrestrial environment.

This PhD thesis investigates if stable isotope methods can be used to distinguish between different sources of chloroform and provide additional insight into the mechanism of natural chloroform formation. It is expected that anthropogenic and natural chloroform have a different isotope signature due to the differences in the origin of the carbon and formation mechanisms. To evaluate the feasibility of the method, the isotope signature of chloroform was characterized at forested sites where chloroform is likely produced naturally and compared to the isotope signature in underlying groundwater. In addition, the isotope composition of industrial chloroform and chloroform at sites with known anthropogenic contamination was evaluated. Based on previous studies, chloroform formation might proceed via the formation of trichloroacetyl-containing compounds (TCAc) from which chloroform is released by hydrolysis. To confirm this hypothesis, the concentration and isotope ratio of TCAc was quantified as well at the sites where natural chloroform production was observed. The mechanism of chloroform formation and factors that control the isotope ratio of chloroform were investigated in more detailed using laboratory chlorination experiments. In addition to NOM, simple model compounds were used which make it easier to relate observed isotope trends to underlying reaction mechanisms. Chlorination was induced by using chloroperoxidase (CPO) and HOCl, which is expected to be the active compound also during enzymatic chlorination. A first set of studies explored the role of TCAc in chloroform formation in more detail. In these studies, the isotope composition of chloroform and TCAc containing compounds from laboratory incubation studies were compared to field observations and to predictions from an analytical model. In order to evaluate the contribution of different functional groups to chloroform formation, isotope effects associated with chlorination of model compounds

with specific function groupds were compared with isotope effects during chlorination of NOM. Finally a series of experiments were carried out that focues on chlorine isotope analysis to explore potential rate-limiting steps in chlorination in more detailed. These experiments also provided insight into the potential chlorine isotope composition of naturally formed chloroform.

In order to demonstrate that carbon isotope analysis can be used to determine the origin of chloroform in groundwater, the $\delta^{13}C$ of chloroform was determined in soil gas and groundwater at five different sites including three forested areas, one urban site and one landfill. The $\delta^{13}C$ of chloroform at the water table (-22.0‰) of forest sites corresponded well to the $\delta^{13}C$ of soil gas chloroform (-22.8 and -26.2‰) demonstrating that chloroform maintains its characteristic isotope signature during transport through the unsaturated zone. At the three forested sites, the $\delta^{13}C$ of groundwater ($\delta^{13}C$ from -22‰ to -27 ‰) was close to the values of soil gas chloroform indicating a natural origin of chloroform. This conclusion is plausible as the three sampling sites were located within spruce and pine forest which are associated with soils that are favorable for chloroform production. In contrast, chloroform from the landfill (-42.1‰) and urban (-47.0‰) sampling sites have clearly an anthropogenic signature ($\delta^{13}C$ from -43‰ to -63‰) consistent with a concentration higher than at the three forested sites. Chloroform was detected in groundwater samples as old as 35 years demonstrating that chloroform can persist over extended periods in oxic groundwater. The isotope analysis of groundwater samples collected in spruce forest indicates that the origin of chloroform can still be determined based on isotope composition even if chloroform was transported over an extensive distance. The $\delta^{13}C$ of chloroform sampled down gradient of a spruce plantation with an average groundwater age of 30 years had a $\delta^{13}C$ of -24‰, which is still within the range of the $\delta^{13}C$ in soil gas. Thus, the strong difference in $\delta^{13}C$ between natural and industrial chloroform makes it possible to unmistakably identify the origin of chloroform even if the some changes of the isotope composition occur during transport.

The TCAc in some forest soils are strongly enriched in ^{13}C (-10‰) compared to natural chloroform. Chlorination experiments combined with a mathematical model have revealed that TCAc could play a fundamental role in the formation of chloroform. Indeed, the laboratory experiments have shown that in addition to chloroform ^{13}C-enriched TCAc are also formed during the chlorination of NOM and humic substances. The large isotope fractionations measured experimentally (kinetic isotope effect from 1.014 to 1.018) for the hydrolysis of TCAc were comparable with those observed for field samples and for the hydrolysis of trichloropropanone. The kinetic isotope effect (KIE) corresponds to the difference in reactions due to the presence of a heavy carbon isotope at the position where hydrolysis takes place. The strong enrichment of TCAc in ^{13}C indicates that a fraction of the trichloromethyl groups is released as chloroform by hydrolysis. Using a mathematical model combined with the fractionation factors determined experimentally, it was shown that when the formation of TCAc and hydrolysis reach a steady state, the isotope composition of chloroform is expected to correspond to isotope ratio of NOM while TCAc should be enriched in ^{13}C. This study confirms that TCAc are reaction intermediates which subsequently release chloroform by hydrolysis, and explains why natural chloroform has a similar isotope signature as NOM despite a large carbon isotope fractionation during its release.

In order to assess the role of selected NOM functional groups in the chloroform formation process as function of pH, the isotopic trends of chloroform produced at different pH (4, 7 and 8) by chemical chlorination of model compounds, humic acid and SOM were measured. As phenolic and ketone functional groups are among the most abundant reactive NOM moieties, phenol and 2-propanone were chosen as model compounds. The isotopic trends of chloroform formed by chlorination of humic acid and NOM were compared with those measured for the formation of chloroform from model compounds to explore which functional groups in NOM might contribute to chloroform formation. These chlorination experiments have demonstrated that the apparent kinetic isotope effects for chloroform formation from model compounds representing NOM functional groups are strongly pH-dependent. Chloroform production from phenol displays a normal KIE (KIE>1), at pH 4 and 7, whereas at pH 8 the process gives rise to an inverse KIE (KIE<1). For chloroform production from 2-propanone, an opposite pH-dependence is observed with a normal KIE at higher pH (7 and 8) and an inverse KIE at lower pH (4). These results indicate that for both chloroform precursors the reaction mechanism and/or the rate limiting step in the sequence of reactions leading to chloroform changes as a function of pH. The comparison of KIE associated with the chlorination of model compounds and NOM suggest that phenolic and ketone groups might be responsible for chloroform formation upon chlorination of NOM in forest soils.

Chlorine isotopes analysis of chloroform sources might contribute to better discriminate natural and industrial chloroform in the terrestrial environment. To estimate the expected range of δ^{37}Cl values for chloroform naturally formed in forest soils and to gain a better understanding of the mechanisms involved in the CPO-catalyzed chlorination of humic substances, abiotic and enzymatic chlorination experiments were carried out. The chlorine isotope analysis of chloroform formed by CPO-catalyzed chlorination at different pH suggested that the formation of an HOCl−ferriprotoporphyrin(IX) intermediate is likely rate-limiting in forest soils (KIE between 1.006 and 1.007). Therefore, in the case where natural chloroform would be produced in forest soils by microorganism excreting extracellular iron-containing CPO and with chloride from atmospheric deposition with δ^{37}Cl value between -1‰ and 1‰, the δ^{37}Cl value of natural chloroform should ranges between -5‰ and -8‰. Thus, natural and industrial chloroform sources might be distinguished from the chlorine isotope composition since the δ^{37}Cl values of industrial chloroform range between 0.32 and -5.4‰ while natural chloroform is expected to be more depleted in ^{37}Cl. The analysis of the KIEs associated with the abiotic and enzyme catalyzed chlorination suggests also that the enzymatic chlorination of NOM occurs via the formation of free hypochlorous acid. These results support the hypothesis according to the CPO-catalyzed chlorination occur via chemo-enzymatic pathway where transiently formed hypochlorous acid diffuse out of the enzyme and react with an organic substrate.

This thesis demonstrates that carbon and chlorine isotope analysis constitute a method of choice to determine the origin of chloroform in soils and groundwater and to study the formation mechanisms. However, the fate of chloroform and related compounds in the studied forest soils and groundwater remains unclear. The biodegradation of anthropogenic chloroform might produces δ^{13}C values in the same range as natural chloroform, underscoring the need to evaluate the isotope signature at contaminated sites with care. Although compound-specific isotope analysis is a valuable approach for source fingerprinting, the δ^{13}C values of chloroform must be interpreted within the detailed hydrogeological and geochemical context of the site. The isotope analysis has also provided valuable information about the role of TCAc in the chloroform formation mechanism and the reactivities of the some functional groups representative of NOM.

Keywords: chloroform, trichloroacetyl-containing compounds, trichloroacetic acid, chlorination, hydrolysis, compound-specific isotope analysis, stable isotope, kinetic isotope effect, chloroperoxidase, forest soils, groundwater.

List of publications

This thesis is based on the following submitted or published articles. These papers are referred throughout the text by their roman numbers:

- **I.** Demonstrating a natural origin of chloroform in groundwater using stable carbon isotopes. *Environmental Science and Technology*, 2012, 46, 6096−6101
- **II.** Assessing the role of trichloroacetyl-containing compounds in the natural formation of chloroform using carbon stable isotopes analysis, *Chemosphere*, 2013, 90, 441-448.
- **III.** Mechanistic insights into the formation of chloroform from natural organic matter using carbon stable isotopes analysis, *submitted* to *Geochimica et Cosmochimica Acta*
- **IV.** Investigating chloroperoxidase-catalyzed formation of chloroform from humic substances using chlorine stable isotope, *submitted* to *Environmental Science and Technology*
- **V.** Position-specific carbon isotope analysis of trichloroacetic acid by gas chromatography/isotope ratio mass spectrometry. *Rapid Communication in Mass Spectrometry*, 2011, 25, 3659−3665.

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Introduction

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1 Chloroform in the terrestrial environment

Organohalogens are generally considered as man-made compounds and the myths considering that organohalogens do not occur naturally still persist. However, forty years ago around 200 organohalogens produced naturally had been already documented (Siuda and Debernar, 1973). Currently, approximately 2200 organochlorines, 1900 organobromines, 100 organoiodines and 30 organofluorides are known to be produced naturally in the environment (Gribble, 2003). The recent analytical developments (multidimensional NMR, LC-MS, GC-MS, etc.) and the recent exploration of oceans and other extreme environments (e.g. volcano, polar environments and black smokers) have yielded large number of novel natural organohalogens. Although most of biogeogenic organohalgens have been discovered in marine environment, different biological organisms (e.g. plants, fungi, lichens, bacteria and insects) and chemical processes producing organohalogens have been identified in terrestrial environment. Terrestrial environment constitute only 29% of the Earth's surface, nevertheless it is an important source of halogen-containing compounds.

Chloroform ($CHCl_3$) is an anthropogenic contaminant and one of most frequently detected chlorinated volatile organic compound in soil, surface water and groundwater. The presence of chloroform in numerous soils and groundwater in absence of other anthropogenic contaminants suggests that chloroform may be produced naturally by biogeochemical processes. Although the natural origin of chloroform in some forest soils and groundwater was rather well establish, no direct evidence has ever been brought. Indeed, it is difficult to directly demonstrate the natural origin of chloroform given its widespread use as an industrial solvent. Moreover, little is known about the mechanisms leading to the formation of natural chloroform. Therefore the aims of the present thesis are (*i*) to evaluate if carbon isotope analysis can be used to distinguish between natural and anthropogenic chloroform and trichloroacetic acid [**I** and **V**], (*ii*) to gain better understanding of the mechanism leading to the formation of chloroform [**II**], (*iii*) to assess the role of trichloroacetyl-containing compounds in the formation of natural chloroform [**III**] and (i*v*) to gain insight into the mechanisms associated with the abiotic and chloroperoxidase-catalyzed chlorination of the natural organic matter [**IV**]. The following sections will review the current knowledge on the origin and fate of chloroform and its related precursor in the environment, with an emphasis put on the biotic and abiotic chlorination processes possibly occurring in the environment and on the reaction mechanisms leading to chloroform. This review should serve as a background to articles **I** to **V** which deal with the identification of natural chloroform and trichloroacetic acid using carbon and chlorine stable isotopes and with the formation mechanisms of chloroform in the terrestrial environment.

Anthropogenic sources and fluxes

The industrial production of chloroform in USA, European Union and Japan exceeded 500 Gg/year at the end of 1990s (McCulloch, 2003). Before the implementation of the Montreal Protocol in 1989, industrial chloroform was essentially used in chemical manufacturing as reactant to produce HCFC-22 (Aucott et al., 1999; McCulloch, 2003). Now, chloroform is still used in the chemical industry but as a solvent or as an extracting agent. About half of this industrial chloroform is expected to be released to the atmosphere by volatilization. In 1999, the global release of industrial chloroform was ~11 Gg/year (Aucott et al., 1999). Anthropogenic chloroform is also produced during the delignification of wood and other cellulose pulps and the bleaching of paper by chlorine. The use of chlorine-containing oxidants in pulp and paper bleaching lead to significant chloroform emission of about 33 Gg/year (Aucott et al., 1999). Chloroform is also formed during treatment of drinking-water and wastewater containing natural organic matter and organic contaminants by Cl_2, ClO_2 or HOCl. The average concentration of chloroform in US drinking-water that has been treated by chlorination is approximately 13 $\mu g\ L^{-1}$ (Krasner et al., 1989). In Europe, the concentrations of chloroform in sewerage effluent vary from 1 $\mu g\ L^{-1}$ to 20 $\mu g\ L^{-1}$ (McCulloch, 2003). In USA, the survey of 50 wastewater treatment plan that used chlorination has shown that chloroform concentration ranged up to 1.10 mg L^{-1} in the influent and up to 1.06 mg L^{-1} in the effluent (Burns and Roe, 1982). The chloroform produced globally by wastewater chlorination amounts to 3 Gg/year. Including drinking-water treatment these sources amount to 12 Gg/year (Aucott et al., 1999). Khalil et al have reported that biogas generators and landfills which convert aerobically organic waste into methane also produced chloroform (Khalil et al., 1999). Chloroform is also supposed to be emitted during combustion processes. Howard et al have reported the presence of chloroform in motor vehicle exhaust (Howard, 1990). The combined emissions of chloroform from landfills, biogas generators and combustion processes correspond approximately to 2 Gg/year (Aucott et al., 1999). The analyses of chloroform presents in firn air suggest that the anthropogenic input to the atmosphere has diminished from 193-226 Gg/year in 1990 to 91-93 Gg/year in 2001 (Worton et al., 2006). This decrease is likely the consequence of changes in the paper bleaching procedures during 1990s. The estimations of the anthropogenic emissions of chloroform of Worton et al. are somewhat higher than the sum of all known sources (Table 1), indicating either large uncertainties in the calculation or the existence of unknown sources (Worton et al., 2006). On the basis of a global atmospheric lifetime of 6 months, Khalil and Rasmussen, and Keene et al. have estimated the total emission of chloroform to the atmosphere of 350-600 Gg/year (Keene et al., 1999; Khalil et al., 1999; Khalil and Rasmussen, 1999). This means that the anthropogenic emissions of chloroform are likely contributing in a range of 10 to 25% of the total atmospheric emissions. Therefore, the major fraction of chloroform emission seems to originate from marine and terrestrial natural sources.

Natural sources and fluxes

1.1.1 Abiotic natural sources and fluxes of chloroform

Forest fires, agriculture, volcanoes and other geothermal systems produce a wide range of organohalogens (Gribble, 1992, 1998, 2003). Biomass burning is a major source of atmospheric trace contaminants. It is estimated that about 90% of the biomass burning is human induced and only a small part is caused by natural phenomena such as lightning or lava (Öberg et al., 2003). According to the Global Emissions Inventory Activity (GEIA) conducted under the auspices of the International Global Atmospheric Chemistry (IGAC), the estimated global emission is 1.8×10^3 ton Cl_{org}/year for chloroform. Chloroform produced by biomass burning results of radical reactions of organic molecules in the presence of chloride at elevated temperature (Scholer et al. 2003). The degassing processes of the Earth's interior through the geothermal systems generate as well a large quantity of gases including halogenated compounds via radical chemistry starting from methane, ethane and ethyne in presence of chloride (Jordan et al., 2000; Frische et al., 2006). Isidorov et al. have shown that gases vented from solfataric volcanoes present concentration of chloroform at least 1000 times over the ambient concentration (Isidorov et al., 1990). However, the total amount of chloroform emitted from volcanic vents is relatively small compared to others natural and anthropogenic sources. Therefore the global contribution of the volcanic activity to the global flux does not appear to be significant. From thermodynamic considerations the radical chemistry of organic materials in the presence of chloride and redox-sensitive elements such as iron is as well possible at ambient temperature. Numerous studies have shown that such halogenations processes might occur in soils and sediment and produce various halogenated organic compound such as chloromethane (Keppler et al., 2000; Fahimi et al., 2003; Keppler and Biester, 2003; Scholer and Keppler, 2003; Huber et al., 2009). Huber et al., have recently demonstrated that chloroform could as well be formed abiotically in soils by Fenton-like reaction in presence of Fe(III) and chloride (Huber et al., 2009) (Figure 1).

1.1.2 Biogenic sources and fluxes of chloroform

Most of the natural chloroform is produced by biogenic sources such as plants, insects and probably fungi. Among the biogenic sources, the terrestrial and oceanic environments appear to be equal sources of natural chloroform (Laturnus et al., 2002). About half of the annual emission of chloroform in the atmosphere comes from oceanic sources. So far, micro (e.g. *Oscillatoria* sp., *Chlamydomonas* sp., *Nitzschia* sp.) and macroalgae (e.g. *Ulva lactuca*, *Cladophora albida*, *Saccharina latissima*) are the only biogenic sources known to release chloroform into oceans (Moore and Tokarczyk, 1993; Nightingale et al., 1995; Scarratt and Moore, 1999; Hong et al., 2008). However the annual production of chloroform form macroalgae is negligible compared to the contribution of microalgae. The emission of chloroform from macroalgae measured by Nightigale et al. range between 5×10^{-4} and 2.5×10^{-1} nmol per gram of wet tissue per day, whereas Scarrate and Moore reported emission rates from microalgae between 1.3×10^2 and 7.8×10^2 nmol per gram of chlorophyll A per day (Nightingale et al., 1995; Scarratt and Moore, 1999; Laturnus et al., 2002).

Although covering only 29% of the Earth's surface, the terrestrial ecosystem may also be an important source of natural chloroform. The data from many independent measurements of the latitudinal distribution of chloroform in the atmosphere show clearly the presence of a concentration peak in the middle northern latitudes (Khalil and Rasmussen, 1999) (Table 2). This heterogeneity in the latitudinal distribution of chloroform is likely due to land-based sources. On the basis of the data obtained from direct flux measurements, Khalil et al. have estimated the average annual emission of chloroform from terrestrial ecosystems excluding the polar regions to 200 Gg/year (100−400 Gg/year) (Khalil et al., 1999; Khalil and Rasmussen, 1999). So far, the terrestrial biogenic sources of chloroform are still poorly investigated. Different studies have shown that chloroform is emitted from various ecosystems such as rice fields, peatlands and forest soils (Khalil et al., 1998; Dimmer et al., 2001; Rhew et al., 2008) [**I**, **II**]. Khalil et al., have shown that six different species of termites are able to produce chloroform and the concentration of chloroform inside the termites mounds can be up to 1000 times higher than ambient levels (Khalil et al., 1990). According to this study the flux of chloroform from termite mounds is 2×10^{-3} pmol cm^{-2} sec^{-1} and the annual emissions range between 10 and100 Gg/year (Khalil et al., 1990). Khalil et al. have also measured fluxes between 1.2×10^{2} and 8×10^{2} nmol m^{-2} day^{-1} of chloroform in rice fields (Khalil et al., 1998). The annual emissions of chloroform from rice fields are estimated to range between 7.7 and 50 Gg/year. More recently, Dimmer et al. have demonstrated that peatland ecosystems also constitute an important source of chloroform (Dimmer et al., 2001). According to Dimmer, the emissions of chloroform from peatland ecosystems have been estimated to range between 0.1 and 151.9 Gg/year. Several studies have reported chloroform concentration in forest soil-air ranging from 3.4 to 100 pmol L^{-1} (Frank et al., 1991; Haselmann et al., 2000a; Laturnus et al., 2000; Hoekstra et al., 2001; Haselmann et al., 2002; Laturnus et al., 2002; Hellen et al., 2006; Albers et al., 2008). The typical relative concentration ratios between chloroform in ambient air and in soil-air range between 1/4 and 1/36 with an average value of ~1/10 (Laturnus et al., 2002). In general spruce forests present the lowest ratios compared to deciduous forests (Laturnus et al., 2002). According to Laturnus et al. the annual emission of chloroform from Northern temperate forests range between 0.4 and 24 Gg/year (Laturnus et al., 2002). The maximum chloroform concentration is usually located in the top organic rich soil layers (Hoekstra et al., 1998a; Haselmann et al., 2000a; Laturnus et al., 2002; Albers et al., 2008; Albers, 2010b; Albers et al., 2010b) [**I**]. In these studies no sources of contamination were reported in the sampling areas, therefore the upward diffusion of chloroform from contaminated groundwater can be excluded. Recently Haselmann et al. and Albers et al. have shown clear seasonal variations of the chloroform concentration in soil-air and in groundwater, and a very heterogeneous spatial distribution of chloroform suggesting a biogenic formation of chloroform in the upper layers of the forest soils (Haselmann et al., 2002; Albers et al., 2011). Moreover Asplund et al. and Laturnus et al. have shown that the halogenating activities in the upper soil layers is probably due to the presence of halogenating-enzymes (e.g. haloperoxidase, halogenase) (Asplund et al., 1993; Laturnus et al., 1995). Haloperoxidases such as chloroperoxidase are known to catalyze the formation of organohalogens in presence of chloride and organic matter [**III**, **IV**]. Walter & Ballschmiter and Hoekstra et al. demonstrated that organohalogens like chloroform are produced by incubating organic material (e.g. humic substances) with chloride, hydrogen peroxide and chloroperoxidase from *Caldariomyces fumago* (Walter and Ballschmiter, 1992; Hoekstra et al., 1998b) [**III**, **IV**]. These results suggest that natural chloroform present in numerous forest soils is likely of microbial or fungal origin. Such microbial or fungal production of chloroform may take place inside organisms or directly in the soil in the case where haloperoxidase would be excreted by microorganims.

Table 1. Reported sinks and sources of anthropogenic and biogenic chloroform.

	Annual average emission [Gg]	Range [Gg]	Reference
Biogenic sources			
macroalgae [a]	0.84	9×10^{-3}-3.1	Nightingale et al. 1995
microalgae [b]	23	7.9-49	Scarratt & Moore 1999
oceanic flux [c]	340	-	Khalil et al. 1999
termites	100	10-100	Khalil et al. 1990
rice field [d]	23	7.7-50	Khalil et al. 1998
peatland	4.7	0.1-150	Dimmer et al. 2001
soil [e]	200	100-400	Khalil et al. 1999
temp. coniferous forest soil	4.9	0.4-24	Haselmann et al. 2000
boreal coniferous forest soil	48	-	Hellén et al. 2006
tundra soil	3.9	-	Rhew et al. 2008
trop/subtrop. forest soil	-	7-120	Khalil & Rasmussen, 2000
Anthropogenic sources			
pulp & paper manufacturing	34	25-43	Aucott et al. 1999
drinking-water treatment	9	3-15	Aucott et al. 1999
waste water treatment	3	2-4	Aucott et al. 1999
other water treatment	9	3-15	Aucott et al. 1999
biomass burning	2	-	Lobert et al. 1999
biogas pits	1.5×10^{-5}	6×10^{-6}-2×10^{-5}	Khalil et al. 1990
others	15	9-20	Aucott et al. 1999
Sinks			
chemical reaction with OH radicals	560	-	Keene et al. 1999
transport to the stratosphere	2	-	Keene et al. 1999

[a] calculated with estimated macroalgae biomass of 2.8×10^{14} g (Carpenter and Liss, 2000).

[b] calculated with estimated microalgae biomass of 1.44×10^{12} g chlorophyll A (Behrenfeld et al., 2001).

[c] oceanic emission minus flux from macro- and microalgae.

[d] calculated with estimated global rice field area of 1.45×10^{12} m^2 (Redeker et al., 2000).

[e] global land area excluding polar areas.

Table 2. Average atmospheric concentrations and fluxes of chloroform reported by Khalil & Rasmussen (1999) for the period 1985 – 1996.

	Northern latitude			Southern latitude		
	Polar	Middle	Tropical	Tropical	Middle	Polar
	90° – 60°	60° – 30°	30° – 0°	0° – -30°	-30° – -60°	-60° – -90°
C (pptv)	28.3	32.8	17.3	9.7	12.9	17.6
mass (Gg)	24	75	57	33	33	14
flux (Gg/year)	5±2	280±40	50±50	50±30	70±10	10±1
variability (%)[a]	26	32	23	46	52	49

[a] Variability is the standard deviation of monthly average concentrations as a percent of the mean concentration C. The standard deviations are derived after subtracting seasonal cycles and trends from the data.

Chloroform in groundwater

Chloroform is widely distributed in the hydrologic system and is one of the most frequently detected volatile organic compounds (VOCs) in groundwater (Squillace and Moran, 2007). Because of its relatively low affinity with natural organic matter, chloroform is also quite persistent under oxic conditions and therefore it can migrate substantial distance through the subsurface, particularly in aquifers with low organic carbon content (McCarty et al., 1981; Ivahnenko and Barbash, 2004). Chloroform is monitored in groundwater by numerous regional and national environmental agencies. Chloroform is usually detected in groundwater beneath a broad range of land-use including urban and industrial areas as well as forested and agricultural areas (Moran et al., 2002; Juhler and Felding, 2003; Squillace and Moran, 2007; OFEV, 2009) [**I**] (Table 3). Between 1985 and 1995 the National Water-Quality Assessment Program of the U.S. Geological Survey has monitored sixty VOCs in 2948 wells (Squillace and Moran, 2007). This long term monitoring program has revealed that 26.4% of wells in urban areas and 5.1% of the well rural areas in the United States are contaminated with chloroform (Squillace and Moran, 2007) (Table 3). Similarly the National Groundwater Monitoring program of the Swiss Federal Office for the Environment has shown that between 2004 and 2006 more than 10% of the aquifers monitored are contaminated with chloroform (OFEV, 2009). The presence of chloroform in numerous aquifers located in urban and suburban areas likely originate from anthropogenic sources like chlorinated public-supply water, leakage from water and wastewater distribution systems, pools and industrial sites (Ivahnenko and Barbash, 2004). The reasons for detection of chloroform in aquifers beneath some agricultural areas remain more elusive. The occurrence of chloroform may be attributable to the use of chlorinated effluent from wastewater treatment plants for irrigation (Cordy et al., 2000), the proximity of urban areas (Stackelberg et al., 1997) or the application of commercial pesticide formulations containing chloroform as an adjuvant compounds (Ivahnenko and Barbash, 2004). The reasons for frequent detection of chloroform in groundwater beneath some forested areas are as well not completely elucidated. So far the main hypothesis is that chloroform would be naturally produced in forest soils by enzymatic chlorination of the NOM (Laturnus et al., 1995; Laturnus et al., 2000; Albers et al., 2010c).

Table 3. Monitoring of chloroform concentration in groundwater beneath different rural and urban areas.

location	land-use	number of sites sampled	frequency of chloroform dectection [%]	maximum chloroform concentration [µg/L]	reference
USA	Rural	2538	5.1	210	Squillace and Moran, 1999
USA	Urban	405	26	400	Squillace and Moran, 1999
USA	Rural	1926	4.3	74	Moran et al., 2002
USA-AZ	Rural	77	16	99.9	Cordy et al., 2000
Denmark	urb./rur.	1067	9.5	11.0	Juhler and Felding, 2003
Switzerland	urb./rur.	195	6	0.55	OFEV, 2002
Switzerland	urb./rur.	356	12	0.73	OFEV, 2003

Biotic and abiotic degradation processes

Although, chloroform can be hydrolyzed under alkaline medium, under the condition prevailing in natural water its half-lives times range from 1850 to 3650 years, therefore hydrolysis is likely not a significant sink (Hine and Dowell, 1954; Mabey and Mill, 1978; Jeffers et al., 1989; McCulloch, 2003). The main removal process in soil and groundwater is probably volatilization because of the relatively high volatility (K_{Henry}=0.15) and low soil adsorption (K_{OC}=45−80) of chloroform (Wilson et al., 1981; Sabljic, 1984). Recently Albers et al. have demonstrated that chloroform may be removed from soils by biotic degradation, evaporation and leaching to groundwater (Albers et al., 2010c). Several studies have shown that chloroform can be dechlorinated anaerobically by methanogens (e.g. *Methanosarcina thermophila* and *Methanosaeta concillii*) in presence of elemental iron (Novak et al., 1998b, a; Koons et al., 2001). Moreover, Gupta et al. have demonstrated that sulphate-reducing bacteria are able to degrade chloroform by reductive dechlorination (Gupta et al., 1996a; Gupta et al., 1996b). The reductive dechlorination by sulfate-reducing bacterial culture is an incomplete mineralization process and leads to the formation of dichloromethane (CH_2Cl_2) which can be further degraded. As aerobic conditions prevail in most soils, except in anoxic microniches that might occur under wet condition, the anaerobic degradation of chloroform in soils is very unlikely (van der Lee et al., 1999). Chloroform can be also co-metabolized in soil under aerobic condition by mono-oxygenase expressing bacteria (e.g. *Methylosinus trichosporium*) (Strand and Shippert, 1986; Arciero et al., 1989; Vannelli et al., 1990; Alvarez-Cohen and McCarty, 1991; Chang and Alvarez-Cohen, 1996). The aerobic mono-oxygenase oxidation of chloroform likely leads to the formation of phosgene ($COCl_2$) which is then decomposed into HCl and CO_2 by hydrolysis (Alvarez-Cohen and McCarty, 1991). Atmospheric removal of chloroform is likely the most efficient sink of chloroform. Hydroxyl radicals present naturally at a concentration of 9.5×10^5 mol cm^{-3} can oxidize chloroform (Keene et al., 1999; Prinn et al., 2001). According to the estimations reported in the literature about 410 Gg/year of chloroform is degraded by atmospheric oxidation which is in good agreement with the global emissions (Khalil et al., 1999).

Ecotoxicity and effects on human health

So far, no major ecotoxicological issues have been documented for chloroform in the environment. The lowest reliable acute ecotoxicity values as the EC_{50} reported in the literature is 13.3 mg L^{-1} for green algae (Brack and Rottler, 1994). A security factor of 1000 was applied to this value to calculate a predicted no-effect concentration (PNEC) of 13 μg L^{-1} for aquatic biota. On the basis of the results of 10 sets of toxicological data on algae, 17 on aquatic invertebrates and 23 on fish, Zok et al. have calculated a PNEC value of 72 μg L^{-1} (including a security factor of 50) (Zok et al., 1998). The environmental levels in surface and groundwater are currently very much lower than the calculated PNEC values. The most important ingestion pathway of chloroform for humans is the consumption of contaminated water and chlorinated drinking water. To date there is not sufficient evidence in humans for the carcinogenicity of chloroform. As chloroform is one of main by-product formed during water chlorination, numerous studies have attempted to assess the possible relationship between bladder and renal cancers and chlorinated drinking-water ingestion. Although some studies have found increased risks of bladder cancers associated with long term consumption of chlorinated drinking-water, results of these studies were inconsistent between men and women (IPCS, 2000). Nevertheless, some experiments on mice and rats have clearly demonstrated that chloroform may induce renal tubular tumors and hepatocellular tumors (WHO-IARC, 1999). According to the International Agency for Research on Cancer (IARC), chloroform is considered to be possibly carcinogenic to humans and is classified as a Group 2B chemical compound (WHO-IARC, 1999). To date, human data are very limited, but liver and kidney seems to be the key target organs in laboratory animals and likely to be so in humans (WHO, 2004).

Water-quality guidelines

The trihalomethanes and haloacetic acids concentrations in the hydrologic systems are monitored from the local scale to the national scale by several countries (Ivahnenko and Barbash, 2004; Squillace and Moran, 2007). Most of these monitoring studies have shown that chloroform is one of the most frequently detected VOCs in surface and groundwater (Ivahnenko and Barbash, 2004). The World Health Organization has established a guideline value of 300 μg L^{-1} for chloroform (WHO, 2007), whereas the United States Environmental Protection Agency has set a Maximum Contaminant Level (MCL) of 80 μg/L for all trihalomethanes and 70 μg L^{-1} if only a single compound is present in water (Ivahnenko and Barbash, 2004). As the health risks from exposure to trihalomethanes at levels up to 100 μg/L is not expected to be significant, the Canadian Federal-Provincial-Territorial Committee on Drinking water has established a Maximum Acceptable Concentration (MAC) of 100 μg L^{-1} for all trihalomethanes in drinking water including chloroform (Health Canada). Recently the French regulation on the quality of drinking-water has been modified. The maximum concentration of trihalomethanes in drinking-water previously set to 150 μg L^{-1} was changed to 100 μg L^{-1}. Among the European countries, Denmark and Switzerland have the most restrictive regulations concerning the drinking-water quality. According to the Swiss legislation on chemical substances in food (OSEC), the maximum concentration of chloroform in drinking water is set to 40 μg L^{-1}. However, groundwater used to produce drinking water should contain less than 1μg L^{-1}. In Denmark the Danish Environmental Protection Agency has also established a concentration limit at 1 μg L^{-1}. Contrary to natural chloroform, the anthropogenic sources of chloroform are often associated with other contaminants. Therefore the limit of chloroform in groundwater to be used for drinking-water has been recently changed in Denmark from 1 to 10 μg L^{-1} if chloroform is of natural origin. In this context the stable isotopes analysis of chloroform could plays an important role to distinguish natural sources from anthropogenic sources [**I**].

2 Formation mechanisms of natural chloroform

Chlorination mechanisms in the environment

2.1.1 Abiotic chlorination

Chlorination processes taking place in the terrestrial and aquatic environments are mostly attributed to enzymatic reactions. There is, however, some evidence for the formation of certain chlorinated compounds by purely abiotic processes. From a thermodynamic point of view, it is possible for chloride ions to form covalent bonds with an organic substrate by pure chemical processes.

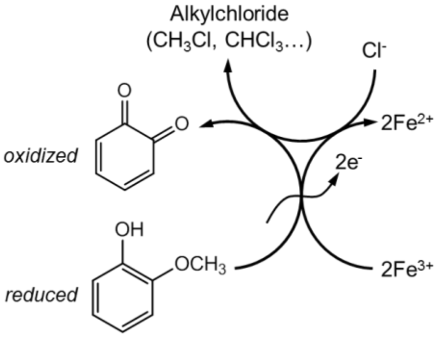

Figure 1. Abiotic formation of alkylchloride by the reaction of Fe(III) and guaiacol as a model compounds in the presence of chloride.

A new abiotic chlorination reaction was reported by Keppler *et al*., which forms aliphatic chlorinated organic compounds in aerobic soils (Keppler et al., 2000). The phenolic moieties of the natural organic matter containing alkoxy group (e.g. 2-methyoxyphenol, 1,2-dihydroxybenzene, hydroquinone) might be oxidized and the redox partner Fe(III) is reduced to Fe(II). During this reaction the chloride present in soil is alkylated and forms chlorinated aliphatic compounds which represent the degradation products of the natural organic matter. Recently, Huber *et al*. have demonstrated that chloroform can be also produced by Fenton-like reaction in presence of chloride, H_2O_2 and Fe(III) (Scholer and Keppler, 2003; Huber et al., 2009). The proposed reaction mechanism is initiated by the formation of hydroxyl radicals which are produced by the reaction between Fe(III) and H_2O_2. The hydroxyl radicals can then oxidize chloride from rock weathering and via precipitation to HOCl or Cl_2 and react with organic substrate to form chloroform.

2.1.2 Biotic chlorination

Numerous studies on natural organohalogens have suggested that halogenases and haloperoxidase excreted by some fungi and plants could play an important role in biosynthesis of chlorinated organic compounds in various terrestrial environments (Asplund et al., 1993; Gribble, 1998; Hoekstra et al., 1998a; Hoekstra et al., 1998b; Gribble, 2003). Asplund et al. and Hoekstra et al., have shown that chloroperoxidase (CPO) from *caldariomyces fumago* can catalyze the chlorination of fulvic and humic acids, and lead to the formation of chlorinated

VOCs and chlorinated carboxylic acids (Asplund et al., 1993; Hoekstra et al., 1998a) [**IV**, **V**]. There are several microorganisms especially fungi and bacteria that are known to produce halogenase and haloperoxidase. As example, it has been shown that chloroform can be produced in pure cultures of *Caldariomyces fumago, Mycena metata, Peniophora pseudopini* grown on SOM-glucose mixture and SOM (Hoekstra et al., 1998a). It can be speculated that biological halogenations is used by organisms as a defense against infections or for the degradation of biomacromolecules such as lignin and humic substances (van Pee and Unversucht, 2003). Iron-containing chloroperoxidase (CPO) is an extracellular heme glycoenzyme containing ferriprotoporphyrin IX as the prosthetic group that is produced by the fungus *Caldariomyces fumago* (Figure 2a) (Sundaramoorthy et al., 1995a; Sundaramoorthy et al., 1995b). CPO, like all heme-dependent haloperoxidases, follows a reaction cycle that involves initial reaction of H_2O_2 and heme-Fe(III) to produce Fe(IV)=O species, called compound I (Blasiak and Drennan, 2009). Compound I can oxidize chloride to produce an enzyme-bound heme-Fe(III)-OCl intermediate (Figure 2). Two hypothesis have been postulated on how this intermediate could promote the chlorination of organic substrates. One hypothesis is that CPO expresses a chlorinating activity by the direct reaction of the enzyme-bound heme-Fe(III)-OCl intermediate with organic substrates (Figure 2b). Recent studies have shown that certain substrates are chlorinated at higher rates than others and much higher reaction rates are obtained with CPO when compared to the chlorination with HOCl (Murali Manoj, 2006). These observations support that chlorination mechanism involved in the CPO-catalyzed chlorination is different of those of the abiotic process.

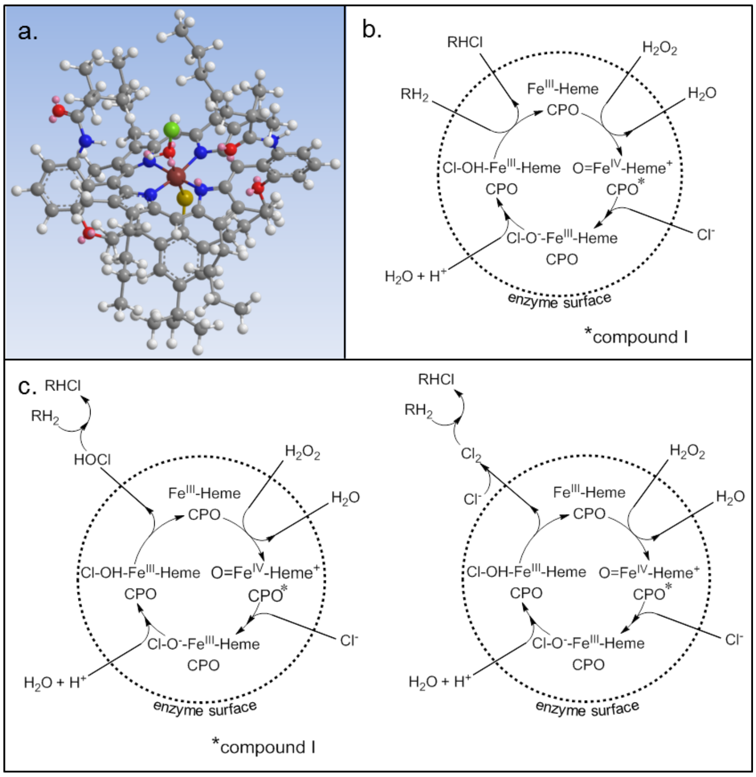

Figure 2. (a) Structure of the ferriprotoporphyrin of CPO from *Caldariomyces fumago*, (b) formation of organochlorine by reaction at the reactive site of CPO, (c) formation of organochlorine via enzymatic production of hypochlorite and molecular chlorine.

The second hypothesis is that HOCl or Cl_2 species are formed at the active site of CPO and diffuse out of CPO trough channels to chlorinate organic substrates (Figure 2c). This latter hypothesis is supported by the following observations: (*i*) free HOCl and Cl_2 species were observed in CPO chlorination experiments, and (*ii*) no enantiomeric excess was seen for chlorination reaction products of many substrates (Griffin, 1983). Thus, so far there is no clear evidence whether free HOCl or Cl_2 are formed, or if the chlorination of organic substrates occurs directly at the catalytic site of CPO via a hypochlorite intermediate.

Reaction mechanisms leading to the formation of chloroform

In the following sections, the speciation and reactivity of different chlorinating agents will be discussed. Moreover these sections will review the current knowledge on chemical mechanisms associated with the formation of chloroform from hydroxyaromatic and aliphatic functional groups and humic-like substances. These sections should serve as a background for the papers **II, III** and **IV** which deal with the mechanisms of formation of chloroform in terrestrial environments.

2.1.3 Chlorinating agent: speciation, reactivity and selectivity

As discussed previously, HOCl is likely formed during chloroperoxidase-promoted chlorination and Fenton-like abiotic chlorination. HOCl is a weak acid which dissociates in aqueous solution ($K_{\mathrm{HOCl\ at\ 25°C}}=2.98\times10^{-8}$) (Morris, 1966; Deborde and von Gunten, 2008; Sivey et al., 2010):

$$HOCl \leftrightharpoons H^+ + ClO^-$$

Under natural water conditions and for pH ranging from 5 to 9, hypochlorous acid and hypochlorite are the main chlorine species. Depending on the pH conditions, chloride concentration and temperature different distribution of chlorine species are observed. In presence of chloride and under acidic condition, molecular chlorine (Cl_2) is formed from HOCl.

$$HOCl + Cl^- + H^+ \leftrightharpoons Cl_2 + H_2O$$

In addition to major chlorine species, other chlorine species such as chlorine hemioxide (Cl_2O) can also be formed at pH<4 via dehydration of 2 equivalent of HOCl (Sivey et al., 2010; Sivey and Roberts, 2012):

$$2\ HOCl \leftrightharpoons Cl_2O + H_2O$$

However the quantity of Cl_2O formed under typical environmental condition is very limited. In aqueous solution the distribution of HOCl, ClO^-, Cl_2 and Cl_2O is a function of temperature, pH and the chloride concentration (Cherney et al., 2006; Deborde and von Gunten, 2008; Sivey et al., 2010). Figure 3 shows the distribution of main chlorination agents in aqueous solution for different pH and chloride concentration. Under typical environmental conditions (i.e. 5<pH<9) the chlorine species are dominated by HOCl and ClO^- whereas under strong acidic condition the main chlorine species in solution are HOCl, Cl_2 and Cl_2O (Figure 3).

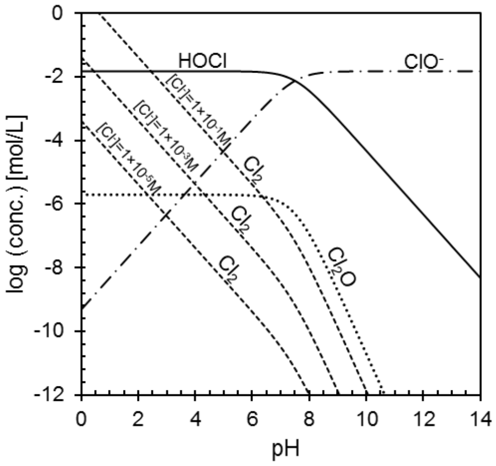

Figure 3. Aqueous free chlorine equilibrium speciation for [free available chlorine]=1.5×10^{-2} M and $[Cl^-]=1\times10^{-1}$ M, 1×10^{-3} M and 1×10^{-5} M at 25°C.

Due to its oxidizing power and its polarized $Cl^{\delta+}-O^{\delta-}$ bond HOCl is a chlorination agent which can react with the majority of organic compounds (Deborde and von Gunten, 2008). Three kinds of reactions between HOCl and organic substances are possible: (*i*) addition reactions to unsaturated bonds, (*ii*) electrophilic substitution reactions at nucleophilic sites and (*iii*) oxidation reactions (Deborde and von Gunten, 2008). Hypochlorous acid can reacts with numerous organic molecules; nevertheless its reactivity is restricted to unsaturated sites. In general, the reaction of HOCl with organic substances induces only small structural changes of the parent compound. Usually, one chlorine atom is added or a double bond is oxidized. Some studies have suggested that the very reactive chlorination agent Cl^+ (chlorinium ion) is formed from HOCl during reaction with aromatic and unsaturated aliphatic compounds (Delamare et al., 1954). Although the formation of Cl^+ can explain the kinetics of chlorination of aromatic compounds by HOCl in aqueous solution, thermodynamic arguments show that Cl^+ is highly unstable and therefore its concentration is likely too low to be kinetically significant (Gardner and Crist, 1972). The chlorination reaction is presumably initiated by the conversion of HOCl into more reactive chlorine species such as H_2OCl^+ (hypochlorous acidium ion), Cl_2 or Cl_2O (Gardner and Crist, 1972). After the addition of chlorine to the double bond, the reaction is generally followed by the addition of HO^- or the elimination of H^+ (Deborde and von Gunten, 2008). Several authors have reported that Cl_2O is also a very good chlorinating agent of organic compounds (Snider and Alley, 1979; Voudrias and Reinhard, 1988; Sivey et al., 2010; Sivey and Roberts, 2012). As HOCl, Cl_2O can react with electron-rich molecules (including aromatic activated structures) by electrophilic substitution (Israel et al., 1950; Marsh et al., 1982; Sivey et al., 2010). Cl_2 and Cl_2O may react primarily with the less reactive organic structures of NOM which are responsible of about 70% of trihalomethane formation potential (Gallard and von Gunten, 2002a; Sivey and Roberts, 2012)

2.1.4 Reaction with humic-like substances

Numerous studies have demonstrated that chlorination of humic substances produce chloroform. It is known that humic substances are the major precursors for the formation of chloroform and chloroacetic acids during wastewater chlorination (Gallard and Von Gunten, 2002b, a). Since humic substances have very complex chemical structures it is impossible to propose a unique reaction mechanism. Therefore, most studies have focused on the chlorination mechanism of simple model compounds such as phenol, substituted phenols, aliphatic β-dicarbonyl acids, pyrole nitrogen derivatives (e.g. tryptophan) and glycosides (Rook, 1977; Boyce and Hornig, 1983; Gallard and Von Gunten, 2002b, a; Dickenson et al., 2008) [**III**]. Recently Weber et al. have shown by converting enzymatically phenolic and anilic moieties of fulvic acids into ether and secondary amine that the removal of those functional groups led to diminished formation of chloroform and chloroacetic acids (Weber et al., 2005). Furthermore, it has been shown that the potential of forming trihalomethanes is correlated with the UV absorbance at 254 nm which corresponds to the absorption band of aromatic moieties in NOM (Reckhow et al., 1990). These results suggest that molecules containing phenolic, carbonyl and anilic groups might be reasonable to use as model compounds and are the likely the mains precursors of chloroform and chloroacetic acids.

2.1.5 Reaction with hydroxy- and dihydroxyaromatic groups

The chlorination and the formation of chloroform from hydroxy- and dihydroxyaromatic precursors are probably the best documented reaction mechanisms (Rook, 1977; Boyce and Hornig, 1983; Deleer et al., 1985) [**IV**]. Boyce and Horning have proposed a very detailed reaction mechanism for the formation of chloroform from dihydroxyaromatic compounds (Figure 4) (Boyce and Hornig, 1983). The reaction is initiated by an electrophilic substitution leading to the formation of tetrachloro-1,3-dihydroxybenzene (I). Further addition of chlorine leads to the formation of chlorinated cyclohexenedione intermediate (Figure 4, II) followed by the hydrolysis of and the oxidative bond cleavage of the C_2–C_3 and C_3–C_4 bonds of the aromatic ring via a complex series of steps to yield carboxyenolate species (Figure 4, III). After a complex sequence of decarboxylation and chlorination steps, chlorinated keto-carboxylic acids are formed (Figure 4, IV). As the carbonyl group is part of a conjugated double bond system, the hydrolysis of these trichloroacetyl-containing compounds to chloroform is hampered by resonance stabilization which makes these compounds fairly stable under acidic pH conditions (Deleer et al., 1985). However, under neutral and alkaline conditions the hydrolysis takes place and chloroform is released. The pH dependence for the chloroform formation from dihydroxy aromatic precursors is relatively complex since the reaction depends on the speciation of both chlorine and hydroxy aromatic compounds (Boyce and Hornig, 1983; Gallard and Von Gunten, 2002b). Boyce and Horning have demonstrated that the maximum conversion of hydroxyl aromatic substrate to chloroform occurs at pH8-10 (Boyce and Hornig, 1983). At higher pH the chloroform formation tends to decrease. This decline of chloroform yields suggests that other reaction pathways may compete with the haloform mechanism at high pH. The yields of chloroform formation depend also on the type and position of the substituents and may vary between 2 and 95% based on the concentration of the phenol (Gallard et al. 2005). Gallard and von Gunten have shown that the rate constants for the chlorination of monohydroxybenzenes are 10^2–10^4 and 5×10^2–5×10^3 fold smaller than for *m*-dihydroxybenzenes and *o*-dihydroxybenzenes, respectively (Gallard and Von Gunten, 2002b). The ring cleavage of dihydroxybenzenes leads to chlorinated keto-carboxylic acids whereas for monohydroxybenzenes the ring cleavage leads to chlorinated ketones. The enol form of keto-carboxylic acids can be stabilized by carboxylate groups which induce a high reactivity with chlorine and therefore high yields of chloroform (Larson and Rockwell, 1979). In the case of monohydroxybenzenes such stabilization of the chlorinated ketone intermediates is not possible. Therefore, the rate of chlorination of chlorinated ketone intermediates should be lower than the keto-carboxylic acids. The keto-enol hypothesis may explain the slower chlorination rates and chloroform formation from monohydroxybenzenes compared to dihydroxybenzenes (Larson and Rockwell, 1979). In addition, the rate constants of chloroform formation for monohydroxybenzenes and dihydroxybenzenes depend on the nature of the other substituent. The formation of the intermediate products which control the rate-determining step leading to chloroform depends of the type of substituent of the parent compounds. For the deactivating para substituent, rate constants of chloroform formation decrease with increasing the withdrawing effect of substituent in the order −H > −I > −CN > $-NO_2$ (Gallard and Von Gunten, 2002b).

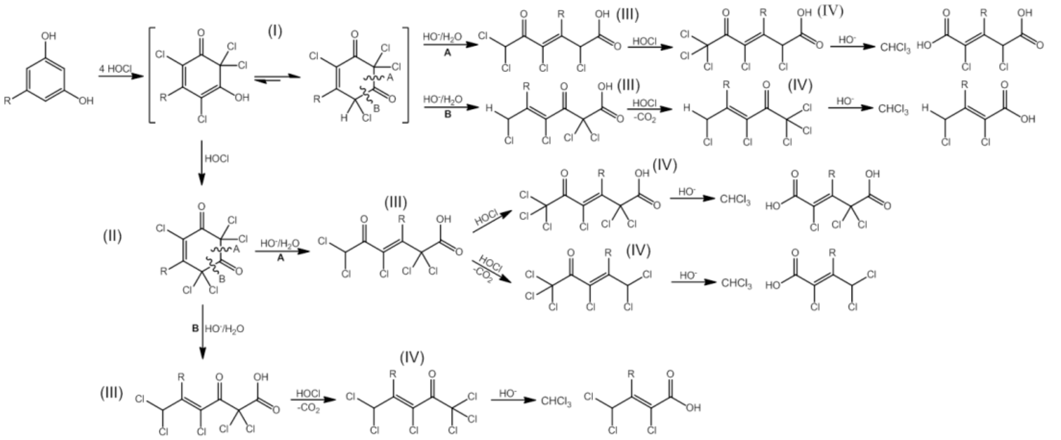

Figure 4. Reaction mechanism for the formation of chloroform from dihydroxybenzens (Boyce and Hornig, 1983).

2.1.6 Reaction with aliphatic groups

Aliphatic structures containing functional groups with heteroatoms present in NOM are also susceptible to lead to the formation of chloroform and other chlorinated carboxylic acids. The chlorination of aldehydes and ketones is initiated by the α substitution of the carbonyl group. This step induced the replacement of hydrogen by a chlorine atom. The keto-enolization enables to pursue chlorination until the complete chlorination of the α-carbon atom leading to the formation of trichlorinated ketones (Figure 5). There are two forms of this substitution reaction: acid catalyzed and base-catalyzed chlorination. However, the base catalyzed chlorination is likely the predominant mechanisms in aqueous solutions at pH>5 (Deborde and von Gunten, 2008). After the complete chlorination of the α-carbon atom, the trichloroacetyl-containing intermediate is hydrolyzed with H_2O or OH^- and release chloroform and carboxylic acids. As previously shown for hydroxyaromatic precursors, the nature of the substituents may strongly influences the reaction rate. The presence of an electron-withdrawing group makes the hydrogen atoms of the methyl group more acidic and therefore increasing chlorine substitution. Inversely, electron-donor substituents decrease the acidity of the methyl groups and therefore inhibit the chlorine substitution (Smith and March, 2007). In the case of aliphatic compounds with β-dicarbonyl moieties consisting of either two ketones groups (e.g. 2,4-pentanedione) or one ketone and one carboxylic group (e.g. 3-oxo-butanedioic acid), one possible pathway is the double chlorination of the carbon atom between the two carbonyl functions and followed by hydrolysis leading to the formation of dichlorinated ketones. Then, a third chlorination would occur at the α-carbon followed by hydrolysis to yield chloroform (Dickenson et al., 2008). In this case the hydrogen atom of the methyl group between the two carbonyl groups can be very easily dissociated since the enol forms are stabilized by resonance assisted-hydrogen bonding (Gilli et al., 1989). The second possible pathway would be that chlorination occurs at the terminal methyl group leading to the formation of trichloroacetyl-containing compounds followed by hydrolysis to yield chloroform (Dickenson et al., 2008).

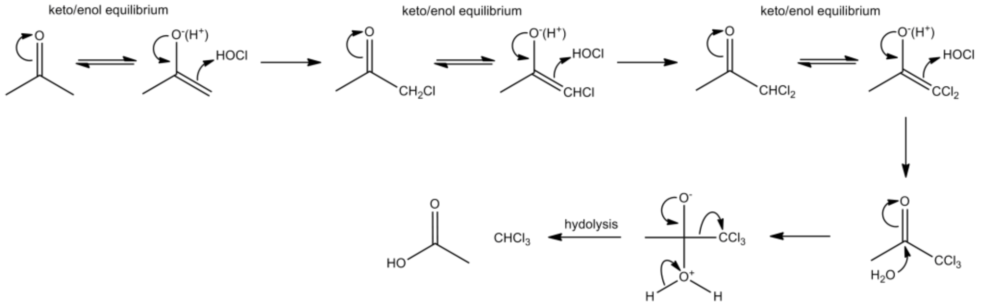

Figure 5. Reaction mechanisms for the formation of chloroform from acetone.

3 Naturally occurring trichloromethyl compounds

As discussed in the previous sections, trichloroacetyl-containing compounds are intermediates produced during the chlorination of phenolic and ketones moieties of natural organic matter. These compounds which contain a trichloromethyl group are likely the main precursors of chloroform. The following sections will review the current knowledge on the sources apportionment and the fate of trichloroacetic acid and trichloroacetyl-containing compounds sources in the environment. These sections serve as a background for the papers **II, III** and **V** which deal with the formation of trichloroacetic acid and the role of trichloroacetyl-containing compounds in the formation of chloroform.

Trichloroacetic acid

Trichloroacetic acid is an important environmental contaminant widely distributed around the world (Hoekstra, 2003; Scholer et al., 2003). Because of its accumulation in conifer needles, trichloroacetic acid has been widely associated with forest decline in Northern hemisphere

(McCulloch, 2002; Hoekstra, 2003). Trichloroacetic acid has also been found in remote locations such as in 100-year-old glaciers and firn ice in Antarctica deposited before the industrial development (Haiber et al., 1996; von Sydow et al., 1999; von Sydow et al., 2000). These findings suggest that trichloroacetic acid may be produced naturally in soils and in the atmosphere. The debate on its natural occurrence is still open, since the origin of some sources of trichloroacetic acid is not completely elucidated.

3.1.1 Anthropogenic sources

Apart from small quantities used as antiseptic, trichloroacetic acid was essentially used in Europe and USA as an herbicide to control monocotyledons (e.g. weeds) and as polymerization catalyst in the chemical industry (US-EPA, 2001; McCulloch, 2002) until the last decade of the 20th century. Trichloroacetic acid was banned in the European Union during the 1990's because more effective herbicides that required much lower rates of application became available (McCulloch, 2002). Trichloroacetic acid is also known as a degradation product of two organophosphate acetylcholinesterase inhibitors (trichlorofon and chlorethoxyfos) used as insecticides from 1950 to 1990's (Albers, 2010a). Water chlorination and pulp bleaching are among the major contemporary sources of trichloroacetic acid. Similarly ass chloroform, it is a by-products formed during disinfection of water with chlorine and paper manufacturing (McCulloch, 2002). The concentration of trichloroacetic acid in chlorinated drinking-water can reach hundreds of μg/L (Albers, 2010a). However, Benanou et al. have shown that small quantity of trichloroacetic acid can be also found in water treated by ozonation (Benanou et al., 1998). It is likely that during ozonation chloride ions are converted into Cl_2 or HOCl, as in chloroperoxidase-catalyzed chlorination. The atmospheric oxidation of 1,1,1-trichloroethane (1,1,1-TCA) and tetrachloroethene (PCE) which are widespread trace contaminants has also been considered to be a major source of trichloroacetic acid (McCulloch, 2002). The tropospheric decomposition of 1,1,1-trichloroethane is initiated by the reaction with $OH^{\bullet}$ radicals leading to the formation trichloroacetaldehyde (chloral) as an intermediate (Figure 6). A small fraction of the trichloroacetaldehyde (from ~1.6% to 14%) can form trichloroacetic acid after its oxidation by O_2 and $OH^{\bullet}$ radicals in could (Jordan and Frank, 1999a, b; McCulloch, 2002). To date this potential formation pathways has not been demonstrated, nevertheless it has been estimated that ~1.3% of 1,1,1-trichloroethan released into the atmosphere could be converted into trichloroacetic acid (McCulloch, 2002). Like 1,1,1-trichloroethane, the tropospheric degradation of tetrachloroethene is initiated by the reaction with radical species such as $OH^{\bullet}$ or $Cl^{\bullet}$ (McCulloch, 2002; Hoekstra, 2003). The reaction with $OH^{\bullet}$ radicals leads to formation of carbon dichloride oxide (phosgene) with little possibility of forming trichloroacetic acid or its precursors. The reaction of tetrachloroethene with $Cl^{\bullet}$ radicals leads to the formation of pentochloroethoxy radical which is in turn converted into carbon dichloride oxide (15%) and trichloroacetyl chloride (85%). The yield of 1,1,1-trichloroacetic acid formed by decomposition of trichloroacetyl chloride is estimated to be ~45% (McCulloch, 2002; Hoekstra, 2003). According to Franklin and Sidebottom, approximately 5% of tetrachloroethene released into the atmosphere could be converted into trichloroacetic acid (Sidebottom and Franklin, 1996). 1,1,1-trichloroethane and tetrachloroethene can be also transformed into trichloroacetic acid in biota via a mechanism involving the P450 monooxygenase (Henschler, 1977a, b). Numerous papers have also shown that trichloroacetic acid is produced from 1,1,1-trichloroethane and tetrachloroethene in the chloroplast of some coniferous and deciduous tree species, however the mechanism of this biotic oxidation of 1,1,1-trichloroethane and tetrachloroethene has still not been elucidated (Plumacher and Schroder, 1994; Weissflog et al., 2007; Strycharz and Newman, 2009a, b).

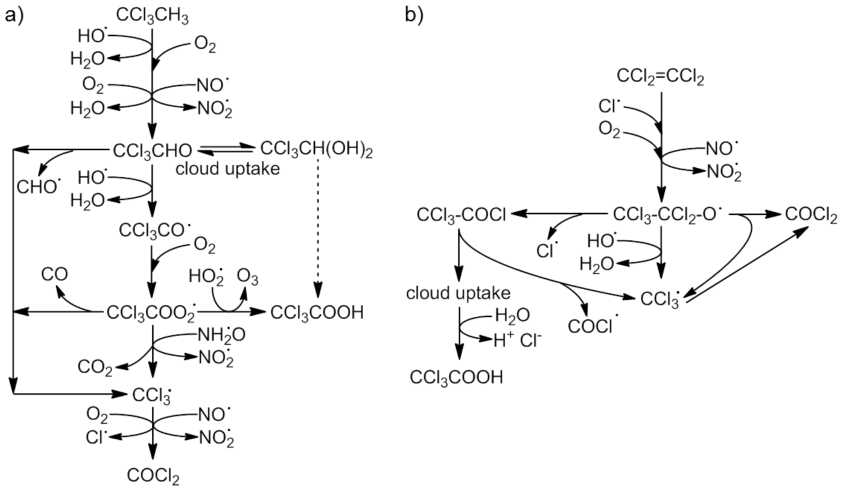

Figure 6 a) Degradation mechanism of 1,1,1-trichloroethane. b) Degradation mechanism of tetrachloroethene (Sidebottom and Franklin, 1996; Hoekstra, 2003).

3.1.2 Natural sources

Some papers have suggested that a significant fraction of trichloroacetic acid present in the environment may be of natural origin (Haiber et al., 1996; Hoekstra, 2003). However, this hypothesis is still controversial. Indeed, the widespread occurrence of trichloroacetic acid in some organisms such as lichens, pine and spruce species, may be an indication that trichloroacetic acid is naturally formed (Juuti et al., 1996; Weissflog et al., 2003). However it is currently impossible to discriminated natural trichloroacetic acid from trichloroacetic acid produced by biotic oxidation of C_2-chlorocarbons. Hoekstra et al. have reported a positive relationship between trichloroacetic acid in soils and the concentration of chloroform in soil-air (Hoekstra et al., 1999; Hoekstra and Juuti, 1999). More recently Albers et al. have shown a positive correlation between trichloroacetic acid in spruce needles and chloroform in soil next to the tree trunk, supporting the observations of Hoekstra et al. (Albers et al., 2010a). To date, the only direct evidence of the natural formation of trichloroacetic acid was given by Matucha et al. who showed the formation of ^{36}Cl labeled trichloroacetic acid in forest soil by adding $^{36}Cl^-$ to forest soils rich in organic matter (Matucha et al., 2007b). It has also been shown that trichloroacetic acid can be produced abiotically when soil organic matter, humic acids or phenols are incubated with Fe(II)/Fe(III), Cl^- and H_2O_2 (Fahimi et al., 2003). The reaction likely involve a Fenton-like mechanism where $OH^\bullet$ radicals are produced by the reaction between Fe(III) and H_2O_2, $OH^\bullet$ radicals which in turn oxidize Cl^- to reactive chlorine species such as Cl_2 or HOCl. Further experiments are still needed to confirm the occurrence of such abiotic mechanism in terrestrial environment.

3.1.3 Trichloroacetic acid in the terrestrial environment

The presence of trichloroacetic acid in precipitations water has been reported in Arctic, Antarctic, Canada, Malawi, Chile, central and northern-Europe (McCulloch, 2002). The concentration of trichloroacetic acid in rainwater and snow is highly variable. As example Berg et al. have measured concentrations of trichloroacetic acid ranging from 37 ng L^{-1} up to 2064 ng L^{-1} in precipitations (rain and snow) collected in Dübendorf, Switzerland between July 1996 and June 1997 (Berg et al., 2000). Nevertheless similar average concentrations of trichloroacetic acid were found at very different geographic location which suggest that trichloroacetic acid in precipitation is likely derived from long-lived precursors in the atmosphere (Berg et al., 2000). The concentration of trichloroacetic acid in surface waters in Switzerland (average of rivers and lakes) is approximately 2.3 times lower than in precipitation and is well correlated with the chloride concentrations in rivers, indicating that wastewater are an important source of trichloroacetic acid in surface waters (Berg et al., 2000). The concentration of trichloroacetic acid in soils is very variable. Although the concentrations in soils range from 0.5 to 150 µg Kg^{-1}, about 60% of the determinations reported by McCulloch were less than 0.5 µg Kg^{-1} (McCulloch, 2002). Dickey et al. and Albers et al. have shown that soils in coniferous forests contain high amounts of trichloroacetic acid (Dickey et al., 2005; Albers et al., 2010a, b). Numerous studies have found fund trichloroacetic acid in different plants such as *Pinus sylvestris* (Scots pine), *Picea abies* (spruce) and *Usneaceae* (lichens) (McCulloch, 2002). Recently, Matchua et al. have suggested that the main translocation path for trichloroacetic acid into *Picea abies* is uptake from soil into the roots, then to the needles (Matucha et al., 2001).

3.1.4 Removal processes

Recent studies using ^{14}C labeled trichloroacetic acid have shown that some soils and organisms have a strong trichloroacetic acid degrading activity (Forczek et al., 2001; Matucha et al., 2003; Schroder et al., 2003; Forczek et al., 2004; Matucha et al., 2007a). Numerous soil microorganisms are known to use chlorinated aliphatic compounds such as trichloroacetic acid as source of energy. Among these microorganisms Foy et al. have identified several dehalogenating bacteria such as *Pseudomonas sp*., *Arthrobacer sp*., *Pseudomonas dehalogens*, fungi such as *Trichoderma viride, Clorostachys sp*., *Acrostalagmus sp*. and two actinomycetes (Foy, 1975). Matucha et al. have demonstrated that trichloroacetic acid become less labile and therefore less available by absorption on carbonaceous soil components such as old wood residues (Matucha et al., 2001). In water, trichloroacetic acid can be decarboxylated and form chloroform and CO_2. However the rate constant for decarboxylation is strongly temperature dependent. At 23°C, trichloroacetic acid has an half-life of 2190 days whereas at 50°C its half-life is only 13 days, therefore this degradation pathway is hardly relevant at ambient temperature (Verhoek, 1934; Zhang and Minear, 2002).

Trichloroacetyl-containing compounds

Several papers have shown that various compounds containing a trichloroacetyl group are formed during the chlorination of organic precursors at low pH (Boyce and Hornig, 1983; Deleer et al., 1985; Streicher et al., 1986; Reckhow et al., 1990) [**II**, **III**] (Figure 7). Hoekstra et al. and Albers et al. suggested that such compounds might be formed during the formation process of natural chloroform in some forest soils (Hoekstra et al., 1998a; Albers et al., 2010b) [**II**, **III**]. Albers et al. have recently detected water extractable and soil bound trichloroacetyl-containing compounds in the organic horizons of two Danish and two Swiss locations (Albers et al., 2010b). Such compounds have also been detected in spruce xylem, spruce needles, throughfall, soil, pore water and groundwater (Albers et al., 2010b). Trichloroacetyl-containing compounds are generally the dominating trichloromethyl-containing compounds in forest soils. It is likely that this class of compounds constitute an important part of the total absorbable organic halogen (AOX) generally measured in surface water and groundwater of non-industrial area. Albers et al. have shown that the concentration of this class of compounds is well correlated with chloroform concentration, indicating that these compounds are likely formed together during the chlorination of the natural organic matter (Albers et al., 2010b).

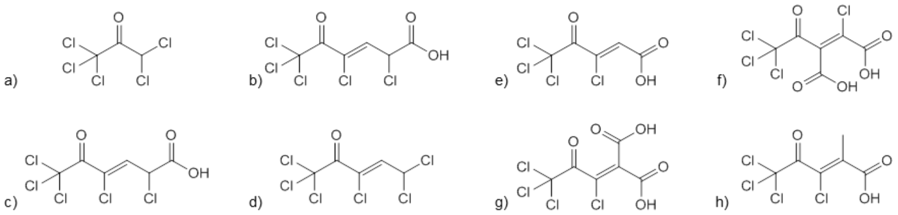

Figure 7. Structures of trichloroacetyl-containing compounds formed by chemical chlorination of dihydroxyaromatic model compounds (a–d) (Boyce and Horning, 1993) and humic acid (e–h) (De Leer et al., 1985).

Although trichloroacetyl groups detected in soils are more likely bound to macromolecular structures (e.g. humic substances), Albers et al. have shown that the leachable fraction detected in soil pore water and in groundwater is more likely constituted of smaller molecular structures containing a trichloroacetyl and carboxylic group (Albers et al., 2010b). Similar structures have been detected by Boyce and Horning and De Leer et al. during the chemical chlorination of resorcinol and humic substances in the laboratory (Boyce and Hornig, 1983; Deleer et al., 1985). Trichloroacetyl-containing compounds without carboxylic group have also been found in spruce needles and in throughfall samples (Albers et al., 2010b). The formation of such non-carboxylic compounds has also been shown for the chemical chlorination of resorcinol and citric acid (Boyce and Hornig, 1983; Streicher et al., 1986).

4 Isotopic methods in natural trace gases research

As this thesis is focuses on the carbon and chlorine stable isotopes analyses of natural organochlorine and its application to investigate the natural formation of chloroform and related compounds, the latest developments in compound-specific isotope analysis (CSIA) and their application to the study of natural trace gases and VOCs will be also reviewed here.

Isotope analysis of natural trace gases at ultra-trace level

So far ^{13}C-CSIA is mainly used to investigate the origin and the fate of anthropogenic contaminants such as chlorinated hydrocarbons (e.g. perchloroethene) (Abe et al., 2009), petroleum hydrocarbons (e.g. benzene) (Bouchard et al., 2008), and some herbicides (e.g. atrazine, isoproturon) (Meyer et al., 2008; Penning et al., 2010). Nevertheless ^{13}C-CSIA is also an emerging approach for studying origins, budgets and chemistry of numerous natural and anthropogenic traces gases (e.g. isoprene, CH_3Cl, C_2–C_5 alkanes, formic acid) (Goldstein and Shaw, 2003). However, due to the relatively high mass required for $^{13}C/^{12}C$ isotope analysis by gas-chromatography isotope ratio mass spectrometry (GC-C-IRMS), the pre-concentration of the analyte is usually needed to measure the $\delta^{13}C$ value of natural trace gases and VOCs (Goldstein and Shaw, 2003). The most common sampling method for gas samples is to collect large samples in gas canisters. The collected gas is then injected through a two-stage cryogenic concentration and focusing system connected with gas-sampling valve to a GC-C-IRMS. However off-line gas sampling with canister can place significant limitations on the volume of air or the number of samples which can be collected and brought to the laboratory. Alternative approaches include the cryogenic pre-concentration of atmospheric or soil-air samples and the sorption of the analyte in gas phase on solid-phase adsorbents (Goldstein and Shaw, 2003). However a lot of care must be taken during these pre-concentration steps to avoid isotopic fractionation, which could affect the measured isotopic composition. Sorption on tubes filled with different sorbents combined to a thermal desorption system is frequently used to analyze non-methane hydrocarbon in the atmosphere (Redeker et al., 2007; Bechara et al., 2008). Von Eckstaedt et al. have recently extended this technique to ^{13}C and ^{2}H isotope analysis of atmospheric VOCs (C_6–C_{10}) produced during biomass combustion and by gasoline and diesel car engines (von Eckstaedt et al., 2011a; von Eckstaedt et al., 2011b). These studies clearly demonstrated that breakthrough has only minor impact on δ^2H and $\delta^{13}C$ values. Mead et al. have used similar sampling technique to analyze the $\delta^{13}C$ values of a suite of atmospheric halocarbons in the City of Bristol (Mead et al., 2008). Using an automated thermal desorber coupled with a GC-C-IRMS Mead et al. were able to measure the $\delta^{13}C$ value of atmospheric chloroform at pptv level with a precision of ±0.3‰ (Mead et al., 2008). In framework of the present thesis this thermal desorption method has been applied to measure the ^{13}C-isotopic signature of natural chloroform present in soil-air at trace level [**II**].

The analysis of trace gases and VOCs in water at ultra-trace levels is challenging as well since large samples of water cannot be compressed like gas samples. Amaral et al. have developed a pre-concentration method consisting of vacuum extraction followed by cryogenic trapping (Amaral et al., 2010) and reached quantification limits ranging from 0.03 to 0.15 $\mu g\ L^{-1}$

(accuracy ±1‰) for ^{13}C in petroleum hydrocarbons and from 0.21 to 1.34 μg L^{-1} (accuracy ±1‰) for ^{13}C in chlorinated ethenes. Although this method is between 5 to 10 times more efficient as the Purge &Trap method typically used to analyze VOCs present in water samples, this method requires the use of liquid nitrogen. The application of this method is limited to water presenting relatively high concentrations of trace gases. Alternative methods like partial vaporization of the analyte through porous or non-porous membrane (known as pervaporation) or by vacuum in a spray chamber are very promising approaches to reach even lower quantification limits, but there is no measurements published to date utilizing these methods.

Sources discrimination using stable isotopes analysis

4.1.1 Stable carbon and chlorine isotopes source signatures

Knowledge of the isotopic signature of a variety of water and atmospheric contaminants such as chlorinated hydrocarbons has proven to be useful to fingerprint contamination sources (Aelion M.C., 2010). The carbon and chlorine isotopic composition of organic contaminant depends of several factors such as (*i*) the origin of carbon and chlorine atoms involved in the formation of a chlorinated compound, (*ii*) the isotope fractionation associated with formation and degradation processes, (*iii*) isotope sensitive branching of competing reaction pathways and (*iv*) transport processes such as diffusion (Hayes, 1993; Schmidt, 2003; Elsner, 2010). So far the variability of the carbon and chlorine isotopic composition of chloroform in the atmosphere and in hydrologic systems has received little attention (Holt et al., 1997; Bergamaschi et al., 1999; Jendrzejewski et al., 2001) [**I**]. Holt et al. and Jendrzejewski et al. have reported $\delta^{13}C$ values equal to -43.3‰ and -51.7‰ respectively for industrial chloroform (Holt et al., 1997; Jendrzejewski et al., 2001). Bergamashi et al. have used carbon stable isotopes to determine the origin of carbon in chloroform formed by chlorination of agricultural drainage waters (Bergamaschi et al., 1999). This study has shown that the carbon isotopic composition of chloroform depends of the chemical alterations of plant materials during diagenesis and whether dissolved organic matter originates from C3 or C4 plants. More recently Redeker et al. and Mead et al. have monitored the $\delta^{13}C$ values of selected halocarbons including chloroform in the atmosphere of urban locations (Redeker et al., 2007; Mead et al., 2008). The studies have shown that chloroform present in the atmospheres of Belfast and Bristol is likely emitted from mixed biogenic and anthropogenic sources.

Chlorine stable isotopes constitute as well a powerful tool to discriminate chlorinated contaminant sources. However to date only three $\delta^{37}Cl$ values of industrially produced chloroform have been reported in the literature (Holt et al., 1997; Jendrzejewski et al., 2001). The $\delta^{37}Cl$ values reported by Holt et al. and Jendrzejewski et al. suggest that industrial chloroform sources should have an isotope composition close to oceanic chloride. Nevertheless further measurements are still needed to evaluate the variability of the chlorine isotope composition of anthropogenic chloroform and to better constrain chloroform sources. In the present thesis the carbon isotopic composition of industrial and natural chloroform

[**I**−**III**] as well as the chlorine isotopic signatures of chloroform produced by abiotic and chloroperoxidase-catalyzed chlorination have been measured [**IV**].

Elucidation of reaction mechanisms using kinetic isotope effects

4.1.2 Deriving formation mechanism from isotope fractionation

Compounds-specific isotope analysis constitutes a powerful tool to gain insights into the mechanisms leading to the formation of chloroform and related compounds from natural organic matter. The changes of C, H or Cl isotope composition of an organic molecule during its formation or degradation can be attributed to a kinetic isotope effect (KIE) at the reacting bond(s). The KIE is defined as the ratio between the (pseudo) first-order rate constants for light (^{12}k) and heavy (^{13}k) substituted molecules. KIEs arise when at the reactive bond(s) a light isotope (e.g. ^{12}C, ^{35}Cl) is substituted by a heavy isotope (e.g. ^{13}C, ^{37}Cl). If the molecules with reacting position containing a light isotope have lower activation energy, these molecules will react faster than the heavy isotope substituted molecules and thus induce a *normal isotope effect* (KIE >1). In this case, the reactant becomes enriched in heavy isotope over time. An *inverse isotope effect* (KIE <1) can be observed in the rare cases where the activation energy is lower for the heavy isotope substituted molecules. Such inverse isotope effects may be observed in cases in which the reacting bond(s) are strengthened or formed in the rate limiting step(s) (Elsner, 2010). The analysis of isotope enrichment or depletion in reactant(s) or the product(s) can provide significant information about the reaction mechanism. Recently Arnold et al. have used carbon stable isotopes analysis to evaluate the role of selected NOM functional groups in the chloroform formation process (Arnold et al., 2008). In this study Arnold et al have monitored the $\delta^{13}C$ value of chloroform produced by chlorination of different models compounds and NOM samples, and used the KIEs as proxies for chloroform formation reactions. The qualitative comparison of the apparent KIE (AKIE) of chloroform produced by chlorination of NOM with those of NOM model compounds suggests that the phenolic moieties may be responsible for chloroform formation (Arnold et al., 2008). However, the significant difference observed between the AKIE measured for the formation of chloroform from phenol ($AKIE_{CHCl_3}=0.980$) and NOM ($AKIE_{CHCl_3}=0.997$) may be an indication for the simultaneous implication of other slowly reacting chloroform precursor with normal AKIEs such as ketone-containing functional groups (Arnold et al., 2008). Arnold et al. have shown that the $\delta^{13}C$ values at steady-state of chloroform produced from NOM model compounds are sometimes more enriched or more depleted in ^{13}C compared to the initial $\delta^{13}C$ values of the precursors (Arnold et al., 2008). This deviation of the isotope signature of chloroform compared to the precursors may be indicative for an isotope sensitive branching of competing reaction pathways (Melander and Saunders, 1987; Schmidt, 2003; Arnold et al., 2008) [**III**]. In parallel to the formation of chloroform, the precursors can be transformed into other chlorinated compounds. The rate and isotope fractionation induced by these parallel reactions determine the concentration and isotopic composition of the reaction intermediate(s) leading to chloroform. Further studies are still needed to gain additional insight into the mechanisms of chloroform formation, rate limiting and factors that control isotope fraction.

The recent developments in stable chlorine isotopes analysis (Sakaguchi-Soder et al., 2007; Aeppli et al., 2010; Bernstein et al., 2011) and in computational isotope chemistry (Sicinska et al., 2005; Swiderek and Paneth, 2012) has opened new perspectives in the study of abiotic and enzymatic (de)chlorination processes. Recently Reddy et al. have used chlorine stable isotopes to investigate the abiotic and the chloroperoxidase-catalyzed chlorination of 1,3,5-trimethoxybenzene (TMB) and 3,5-dimethoxyphenol (DMP) (Reddy et al., 2002). Very distinct AKIEs values for the abiotic ($AKIE_{TMB}$=1.0037; $AKIE_{DMP}$=1.0039) and the enzymatic chlorination ($AKIE_{TMB}$=1.012; $AKIE_{DMP}$=1.011) have been reported by Reddy et al. These differences of AKIEs observed between both chlorination processes remain unexplained. On the basis of molecular modeling, Sicinska et al. and Swiderek and Paneth have developed hypotheses to explain the large AKIE value measured for the chloroperoxidase-catalyzed chlorination (Sicinska et al., 2005; Swiderek and Paneth, 2012). Swiderek and Paneth have shown that reactions in which chlorine is passed between two heavy atoms and is shielded from interactions with solvent molecules can induce large KIEs (Swiderek and Paneth, 2012). In the case of an incoming chlorine atom, the observed KIE results from the combination of the effect of a temperature-independent factor (TIF) which reflect to the motion along the minimum energy pathway and a temperature-dependent factor (TDF) which reflect changes in bond strengths and the interactions between the entering isotopic atom with solvent molecules as the reactant approach the transition-state (Cromartie and Swain, 1976; Kohen and Limbach, 2006; Swiderek and Paneth, 2012). Swiderek and Paneth have demonstrate that for reactions in which chlorine atom is transferred between two heavy atoms the main contribution to the Cl-AKIE essentially results from TIF (Swiderek and Paneth, 2012). One of the most striking result of this study is the possibility of finding secondary chlorine KIEs that can be as large as typical primary Cl-KIEs in most reactions studied thus far (i.e.1.005 per atom) (Swiderek and Paneth, 2012). Complementary experiments are still required to interpret the large AKIEs values measured by Reddy al. In the present thesis chlorine isotope signature of chloroform produced in laboratory by abiotic and enzymatic-catalyzed chlorination has been investigated to gain a deeper understanding of the chlorination mechanisms involved into formation of chloroform [**IV**].

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Chapter I

Demonstrating a Natural Origin of Chloroform in Groundwater Using Stable Carbon Isotopes

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ABSTRACT Chloroform has been for a long time considered only as an anthropogenic contaminant. The presence of chloroform in forest soil and groundwater has been widely demonstrated. The frequent detection of chloroform in groundwater in absence of other contaminants suggests that chloroform is likely produced naturally. Compound-specific isotope analysis of chloroform was performed on soil-gas and groundwater samples to elucidate whether its source is natural or anthropogenic. The $\delta^{13}C$ values of chloroform (−22.8 to −26.2‰) present in soil gas collected in a forested area are within the same range as the soil organic matter (−22.6 to −28.2‰) but are more enriched in ^{13}C compared to industrial chloroform (−43.2 to −63.6‰). The $\delta^{13}C$ values of chloroform at the water table (−22.0‰) corresponded well to the $\delta^{13}C$ of soil gas chloroform, demonstrating that the isotope signature of chloroform is maintained during transport through the unsaturated zone. Generally, the isotope signature of chloroform is conserved also during longer range transport in the aquifer. These $\delta^{13}C$ data support the hypothesis that chloroform is naturally formed in some forest soils. These results may be particularly relevant for authorities regulation of chloroform which in the case of Denmark was very strict for groundwater (<1 μg/L).

▪ Introduction

Chlorinated hydrocarbons are among the most frequently detected contaminants in groundwater worldwide. For example in the U.S., chloroform ($CHCl_3$) was detected (>0.2 µg/L) in 26.4% of urban and 5.1% of rural wells, trichloroethene (TCE) in 11.6% of urban and 1.6% of rural wells and tetrachloroethene (PCE) in 16.6% of urban and 2.5% of rural wells (Squillace et al., 2002; Squillace and Moran, 2007). In the Danish national groundwater quality monitoring program, chloroform was detected in 8% of the wells, TCE in 9% and PCE in 6.2% (Juhler and Felding, 2003). Chlorinated hydrocarbons in groundwater are generally considered to be of anthropogenic origin only. However, it has been demonstrated that chlorinated hydrocarbons can also be naturally produced and more than 3800 naturally occurring halogenated compounds have been identified (Gribble, 2003). Among the frequently detected chlorinated groundwater contaminants, chloroform has been shown to be produced naturally, while a widespread natural production of TCE and PCE seems less likely (Laturnus et al., 2002). Natural chloroform production in forest soils has been demonstrated using spatial and temporal concentration patterns, laboratory incubation experiments and in situ field incubations with ^{37}Cl. (Hoekstra et al., 1998a; Haselmann et al., 2000b; Laturnus et al., 2000; Hoekstra et al., 2001; Haselmann et al., 2002; Laturnus et al., 2002). Different pathways have been proposed for natural chloroform production. When incubating organic compounds (organic acids, glucose) with chloroperoxidase and hydrogen peroxide, chloroform was detected as the main reaction product (Walter and Ballschmiter, 1992). The reaction was postulated to proceed via the formation of reactive chlorine species such as hypochlorous acid and thus is analogous to the formation of chloroform during chlorination of water (Boyce and Hornig, 1983; Hoekstra et al., 1998a). Since chloroperoxides likely originating from wood-rotting fungi were detected in forest soils, it was postulated that a similar production mechanism also occurs in soils (Asplund et al., 1993; Laturnus et al., 1995). Another proposed pathway is the formation of chloroform by decarboxylation of trichloroacetic acid that is itself product of chlorination of organic matter in soil or originates from atmospheric oxidation of chlorinated ethenes (Hoekstra et al., 1998a). Recently, it was demonstrated that chloroform can also be produced from organic model substance in a Fenton-like process in the absence of chloroperioxidases (Huber et al., 2009).

For groundwater management and remediation, it is important to know to what extent chloroform in groundwater originates from natural sources. In Denmark which rely heavily (>95%) on clean groundwater for its portable water supply this knowledge proved to be particularly urgent in recent years. A maximum of 1 µg/L of volatile halogenated hydrocarbons including chloroform is allowed for groundwater to be used for drinking water, although up to 25 µg/L is permitted if chlorination is needed for disinfection. The strict regulation for groundwater with respect to chloroform compared to drinking water is due to a general fear of pollution, assuming that chloroform is entirely anthropogenic, and that its presence may indicate presence of more toxic halocarbons as well. One major water work already had to seek for a dispensation from the strict rules and others may follow as water works tend to place new water wells in forested areas rather than in farmland in order to avoid problems with nitrate and pesticides. Here, we characterize the carbon isotope composition of natural and anthropogenic chloroform and compare it to chloroform sampled in different aquifers. We show that natural chloroform present in groundwater can be distinguished from anthropogenic chloroform based on its carbon isotope signature. Hence, carbon isotope analysis of chloroform can provide unique insight into the origin of chloroform in the environment and help to take appropriate measures when detecting chloroform in groundwater. While natural chloroform production in forest soils is well established, it is difficult to demonstrate a natural origin of chloroform in groundwater as chloroform can be transported over extended distances from anthropogenic sources. Compounds-specific isotope analysis is increasingly used to distinguish between different anthropogenic sources of chlorinated hydrocarbons in the subsurface because their isotopic signature frequently varies depending on the manufacturer (vanWarmerdam et al., 1995; Jendrzejewski et al., 2001; Shouakar-Stash et al., 2003; Hunkeler et al., 2004). A similar approach can potentially also be used to distinguish between natural and anthropogenic chloroform because they originate from a different carbon source and are produced by a different mechanism. Industrial chloroform is usually produced from methane which has a typical $\delta^{13}C$ between −40 and −60‰ (Holt et al., 1997; Hunkeler and Aravena, 2000; Jendrzejewski et al., 2001). In contrast, naturally produced chloroform originates from natural organic carbon which has $\delta^{13}C$ around −22 to −30‰. However, depending on the pathway, the isotope composition of chloroform may differ from this value due to isotope fractionation during production (Bergamaschi et al., 1999; Arnold et al., 2008). Furthermore, the isotopic signature of chloroform may be influenced by degradation of chloroform, for example in anoxic microniches of soil and unsaturated zone or in the saturated zone below the water table. To evaluate if carbon isotope analysis can be used to distinguish between natural and anthropogenic chloroform in groundwater, the $\delta^{13}C$ values of anthropogenic and natural chloroform was characterized and compared to chloroform detected in groundwater at four sites.

▪ Materials and Methods

Field Sites. Viborg Site. Viborg is located in Jutland, Denmark. The study area is situated in a coniferous plantation that has been in place since mid 1800. The study area is underlain by sandy glacial outwash deposits acting as an aquifer with a water table around 5 m below land surface. The soil type is an early podsolic profile. Groundwater flows toward the waterworks of Viborg located 2 km down gradient of the study site. At the Viborg waterworks, chloroform was detected since 1995 at a concentration around 1.5 μg/L. In this study, groundwater samples were taken in four multilevel groundwater monitoring wells where chloroform concentration above 0.1 μgL has been detected in previous studies. The groundwater wells consist of 63 mm diameter HDPE tubes with 1−2 m long screens. The soil gas samplers are 4 cm long brass screens situated in 5−7 m depths from 0.5 m bgs to just below the water table. The soil gas screens are situated few meters from the well no. 66.1797, Figure 1.

Tisvilde Site. Tisvilde is located at approximately 60km in north of Copenhagen in Zealand, Denmark. The study area is a forest far from industrial activity. This area was forested in the 19th century to prevent soil drift. The coniferous forest consists mainly of Scots Pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*). The topsoil is constituted of an organic horizon mainly composed of partly degraded needles and branches. The aquifer is constituted of sandy glacial outwash deposits with a water level at around 5 m below surface. The monitoring well has a screen in 11−14 m bgs.

Vellev Site. Vellev is located 27 km east of Viborg in an agricultural area with clayey soils and underlain sand gravel aquifer. The investigated well which has a screen between 31.5 and 37.5 m bgs and is situated adjacent to an industrial landfill for refrigerator waste. The groundwater contains beside chloroform (∼10 μg/L) high amounts of tetra chloromethane, trichloroethylene and other solvents.

Thisted City Site. The well is situated in the central part of Thisted city in a minor park and is a part of the public waterworks network. The well intake is between 20 and 35 m below surface and the water has a CFC-model-age of 5 years. The location is adjacent to several former small industries as a car repair and a dry cleaning shop.

Tved Site. Tved Plantation is situated 5 km from the coast 9 km north of Thisted and consists mainly of coniferous forest of spruce and pine planted 110 years ago. The top soil is Aeolian sand which is widespread with shifting sand deposits caused by the drifting sand. The sand is underlain by glacial weathered until and the aquifer top about 16 m bgs coincides with the Prequaternary surface of Cretaceous white chalk. Two abstraction wells are equipped with screens with screens from 12 to 18 and 24 to 40 m bgs, respectively.

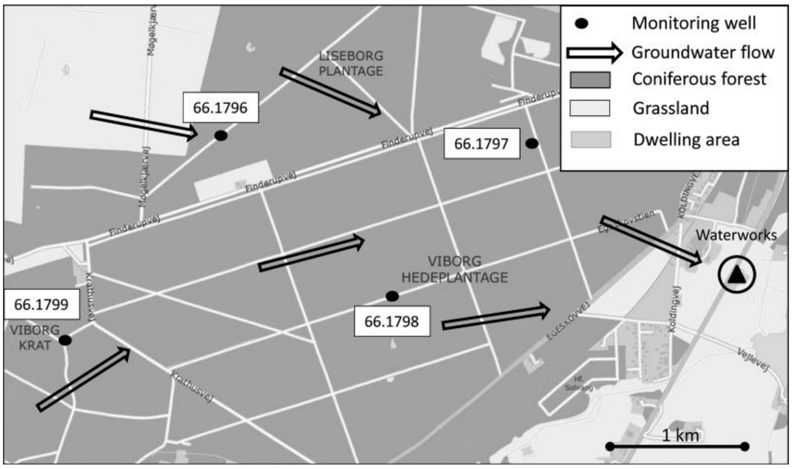

Figure 7. Map of Viborg study site with groundwater flow arrows illustrating the location of the coniferous plantation, the multilevel groundwater wells, the soil gas sampler, and the municipal waterworks.

Groundwater Sampling and Concentration Analysis. Groundwater samples were obtained using a submersible MP1 pump (Grundfos, Bjerringbro, Denmark) with variable flow rate. For the brass filters a peristaltic pump (Ole Dich , type 111LMT, Hvidovre, Denmark) operated at a flow rate of from 100 to 300 mL/min. After purging the internal volume of the sampling system at least 3 times, 125 mL vials were filled for concentration analysis and 2 L redcap bottles (Schott, Mainz,Germany) for stable carbon isotope analysis. Since groundwater was oxic and chloroform is known to be stable under oxic conditions, no preservatives were added and the samples were stored at 4 °C until analysis. The soil gas samples were collected in 300 and 500 mL stainless steel gas cylinders with valves at both ends using a membrane pump (model 5002, Thomas, Sheboygan, WI). After purging the internal volume of the sampling system and cylinder at least three times, the outlet of the gas cylinder was closed and the cylinders pressurized to around 3 bar to increase the amount of sampling gas.

Chloroform Analysis and CFC Age-Dating. Air samples were analyzed for chloroform, CFCs and other C1-organohalogens on a gas chromatograph equipped with an ECD detector (GC-8A, Shimadzu, Kyoto, Japan). The analytical procedure was similar to that described by Busenberg and Plummer for chlorofluorocarbons (CFC) in age-dating of young groundwater. Briefly, the halocarbons were trapped at 30 °C on a precolumn, which was then heated to 95 °C. Separation of gas constituents was done on a 1.7 m packed column (Poracil-C, Supelco, Bellefonte, PA) at 70 °C. Precolumn back-flush technique was used to complete the analysis of each sample within 11 min. 50 mL of gas sample was used for analysis. The limit of quantification (LOQ) for chloroform was ∼10 pptv.

Carbon Isotope Analysis. Since chloroform may occur at low concentration in groundwater, a sensitive analytical method is required. The most sensitive commercial method usually used in groundwater stable isotope studies of VOCs is purge and trap coupled to gas chromatography isotope-ratio mass spectrometry with an estimated detection limit of 2.3 μg/L for chloroform (Zwank et al., 2003). However, such a detection limit is not sufficient to evaluate if low levels chloroform represent a plume fringe or correspond to a natural background. Therefore, a method using a large scale online purge and trap is validated by evaluating the linearity and reproducibility of the method. Stable carbon isotope analysis was carried out with a Thermo Trace gas chromatograph (GC) coupled to a Thermo Delta Plus XP isotope-ratio mass spectrometer (IRMS) via a GC II interface (all from Thermo Fisher Scientific, San Jose, CA) set to 940 °C. Chloroform in aqueous and gaseous samples was preconcentrated using a modified Velocity XPT Purge & Trap (P&T) system (Teledyne Tekmar Dohrmann, Mason, OH) equipped with a VOCARB 3000 trap (Supelco, Bellefonte, PA), a forward focusing cell and a dry trap. The P&T system was coupled to the GC via an injector with a cryotrap (Optic 3, ATAS-GL, Vedhoven, The Netherlands) to cool down the initial portion of the capillary column. The GC was equipped with a 60 m × 0.25 mm × 1.8 μm DB-VRX column (Agilent, Santa Clara, CA). For aqueous samples, the purge gas stream was redirected through a 2 L

Schott glass bottle equipped with a frit from a gas-washing bottle. Aqueous samples were purged for 20 min at 150 mL/min with leads to a removal of 20% of the dissolved chloroform considering a Henry coefficient of 0.13 (gas/water). The chloroform was trapped on a VOCARB 3000 trap at 30°C. After a dry purge period of 30 s at 200 mL/min, the chloroform was desorbed for 2 min at 250 °C, transferred to the cryotrap set at −100 °C in splitless mode, and then released to the GC column by rapidly heating the cryotrap to 180 °C. The GC was set to 40 °C for 3 min followed by a ramp of 10°C/min until a final temperature of 200 °C that was held for 4 min. The column flow was 1.7 mL/min. For gaseous samples, an analogous procedure was used; expect that a stainless steel gas cylinder was integrated into the purge gas circuit rather than a purge vessel. The sample gas was released from the pressurized cylinder at a rate of 150 mL/min to the VOCARB trap followed by purging two times the volume of the cylinder. The isotope ratios are reported in the delta notation relative to the V-PDB standard defined as follows:

$$\delta^{13}C_{CF} = \left(\frac{R}{R_{V-PDB}} - 1 \right) \times 1000 \qquad (1)$$

To validate the system, reference chloroform with a δ^{13}C of −47.9 ± 0.3‰was analyzed for a concentration range of 0.3−3 µg/L (see Supporting Information, Figure S1). The method detection limit (MDL) was calculated using the moving mean procedure. The samples were measured five times for each concentration. As the difference in δ^{13}C between anthropogenic and natural chloroform was expected to be large, a standard deviation (n = 5) of ±0.8‰ was considered as acceptable to reach lower detection limits. An interval of ±0.8‰ was set around the moving mean δ^{13}C value. The moving mean δ^{13}C value was calculated by including consecutively the δ^{13}C value of the next lower concentration as long as the standard deviation was smaller or equal to the specified level (±0.8‰). The MDL corresponds to the lowest concentration for which the mean δ^{13}C is within an interval given by the moving mean δ^{13}C value ±0.8‰. The total uncertainty was characterized by calculating the standard deviation of all δ^{13}C values at or above the MDL. Using this approach, a MDL of 0.6 µg/L and a standard deviation of ±0.6‰ (n = 25) was obtained.

▪Results and Discussion

The isotopic signature of industrial chloroform was characterized by analyzing chloroform from several suppliers and taking into account literature values (Table 1). The δ^{13}C of industrial chloroform was comprised between −43.2‰ and −63.6‰ (n = 1−6; 1σ = 0.08−0.39‰) consistent with its production from methane (Zwank et al., 2003). The similarities in δ^{13}C values between some chloroform suppliers suggest that the samples may come from the same carbon feedstock and similar production processes. It is also possible that chloroform from different suppliers is produced by the same manufacturer. The isotopic signature of natural chloroform produced in forest soils was constrained based on soil−air samples from a field site in Viborg, Denmark. At this site, a natural origin of chloroform in soils is well established based on spatial and temporal concentration patterns (Albers et al., 2010b, a; Albers et al., 2010c; Albers et al., 2011).

Table 1. ^{13}C isotope signature of different industrially produced chloroform

δ^{13}C (‰/ V-PDB)	±1σ	n	supplier	purity (%)	ref.
-43.17	-	1	-	-	Holt et al., 1997
-43.25	-	1	-	-	Holt et al., 1997
-47.07	0.20	5	Fluka	99.5	this study
-47.88	0.08	5	Alfa Aesar	99	this study
-48.56	0.30	5	Acros Organics	99.8	this study
-51.46	0.22	3	Fisher Scientifc	>99	this study
-51.66	0.39	2	-	-	Jendrzejewski et al., 2001
-63.43	0.22	6	Sigma-Aldrich	99.8	Hunkeler & Aravena, 2000
-63.62	0.14	4	Sigma-Aldrich	99.8	Hunkeler & Aravena, 2000

Chloroform concentrations and isotope ratios were measured in a soil-gas multilevel sampler reaching to 4 m depth located adjacent to well n° 66− 1797, where the highest chloroform concentrations occurred (Figure 1). Maximum chloroform concentrations were detected during summer and autumn at 0.5 m depth (Figure 2) consistent with a biological production of chloroform in the organic-rich soil zone during the warmer periods of the year (Albers et al., 2010a; Albers et al., 2010c; Albers et al., 2011).

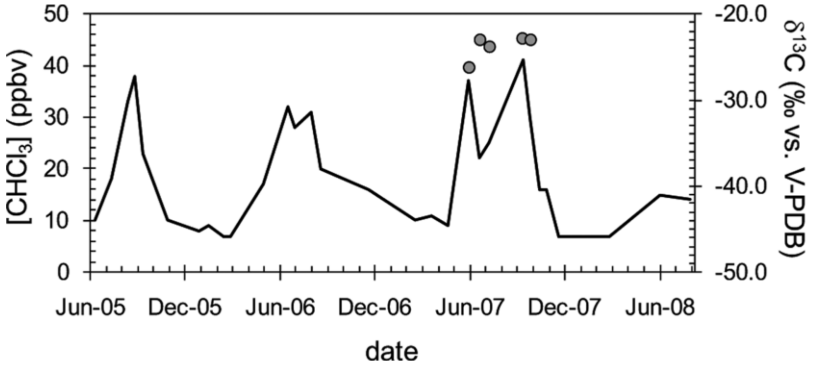

Figure 2.Concentration and carbon isotope signature of the natural chloroform source of Viborg. The black line corresponds to the evolution of the chloroform concentration between June 2005 and August 2008 (modified from Albers et al., 2010). The circles represent the isotope δ^{13}C ratios of chloroform.

The δ^{13}C of chloroform was within a narrow range from −22.8 to −26.2‰ (n = 2; 1σ=1.08−1.97‰, Figure 2), which is close to the typical isotope signature of soil organic carbon (Figure 3) and strongly enriched in ^{13}C compared to industrial chloroform (Figure 2). The somewhat higher standard deviation of these results is a consequence of a change in the P&T-method to use compressed soil air. However, these results confirm that the soil organic matter constitute the main carbon source of natural chloroform. To demonstrate that carbon isotope analysis can be used to determine the origin of chloroform in groundwater, the δ^{13}C of chloroform at five sites was determined using a new method that permits to measure δ^{13}C at low concentration levels. Groundwater was collected at the Viborg site where chloroform is known to be produced naturally in the soil organic horizon (see above) and down gradient of an industrial landfill at Vellev, Denmark, where chloroform is likely of anthropogenic origin. In addition, groundwater was also sampled at the Tisvilde and Tved sites with no identified anthropogenic chloroform sources. At Viborg, groundwater samples were taken in four multilevel monitoring wells in which chloroform was previously detected (Figure 1).

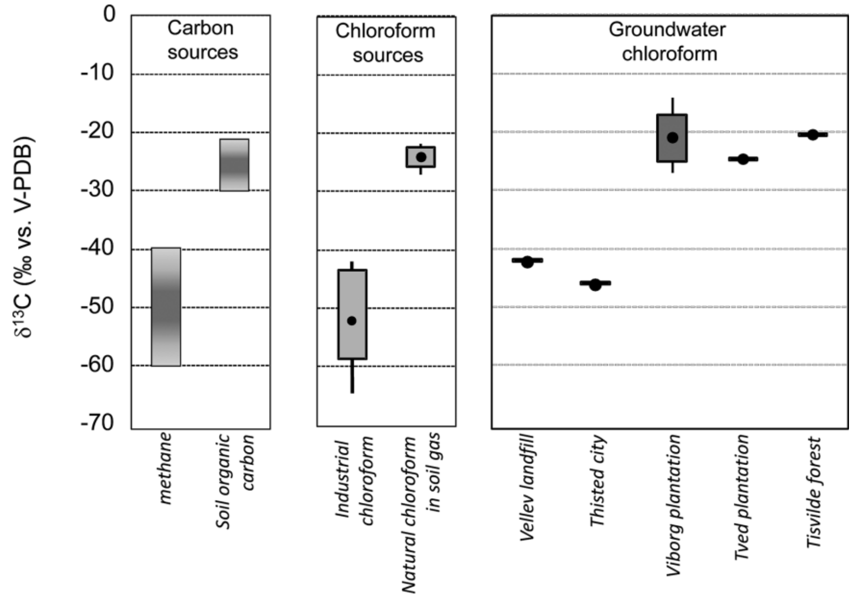

Figure 3.Carbon isotopic signature of carbon and chloroform sources and groundwater chloroform. The gray bars represent the standard deviation around the mean δ^{13}C value (black dots) and the vertical black lines correspond to the minimum and the maximum δ^{13}C values.

The highest chloroform concentrations were detected in 66−1797 ranging from 0.9 to 4.1 μg/L (n = 24) (Figure 4b). The CFC groundwater-age reached 35 years in the deepest sampling point (Figure 4c). The δ^{13}C of chloroform at the water table (−22.0‰) corresponded well to the δ^{13}C of soil gas chloroform (−22.8 and −26.2‰) demonstrating that chloroform maintains its characteristic isotope signature during transport through the unsaturated zone. The δ^{13}C values of chloroform in deeper zones varied between −16.7 and −26.8‰ (n = 5) (Figure 4a). The range in δ^{13}C chloroform of uppermost groundwater was found within −17 to −29‰ (unpublished data) and the nearly horizontal flowing groundwater from five screens reflect the different infiltration areas of chloroform formation in the forest. At the Tved site, the chloroform concentrations in groundwater between 12 and 18 m bgs. were 1.54−1.86 μg/L (n = 9), the δ^{13}C between −24.3 and −25.2‰ (n = 10), and CFC-groundwater ages approximately 20 years. At the Tisvilde site, the δ^{13}C of chloroform was −20.1‰, (Figure.3). At the Vellev site, the chloroform concentration in groundwater was 10.7 μg/L, which is 2 to 5 times higher than the concentration measured at the other sites in forested areas. The δ^{13}C of chloroform at the

Vellev site amounted to −42.1‰. At the Thisted site the chloroform concentration was minor than the Vellev site, <0.2 μg/L and the chloroform δ^{13}C was −47.0‰.

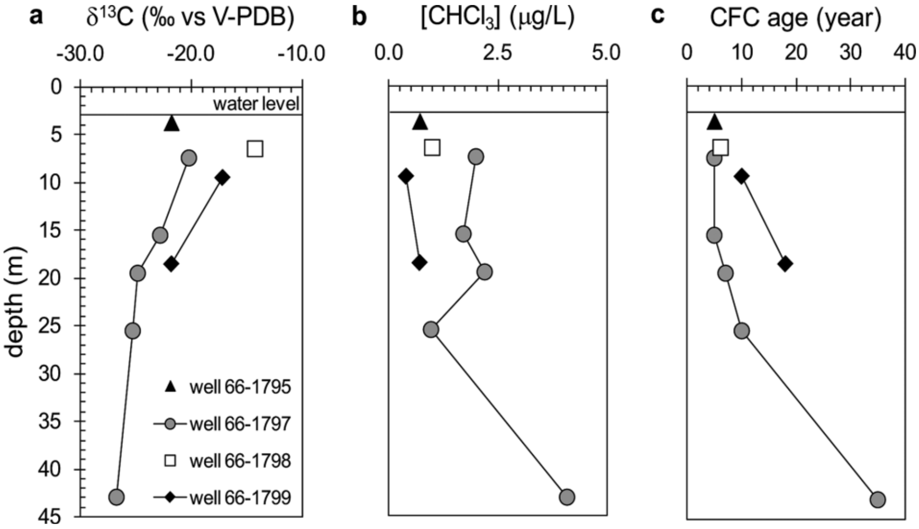

Figure 4.Profile of the δ^{13}C values, concentration and CFC age of groundwater at different depths at the Viborg plantation.

At three of the study sites (Viborg, Tved and Tisvilde), the δ^{13}C of groundwater chloroform was close to the values of Viborg soil-gas chloroform indicating a natural origin of chloroform (Figure 3a and 3c). This conclusion is plausible as the three sampling sites were located within spruce and pine plantations, which are associated with soils that are favorable for chloroform production. In contrast, chloroform from the Vellev site had clearly an anthropogenic signature (Figure 3) consistent with is likely origin from an industrial landfill and a concentration higher than at the other three sites. Chloroform was detected in groundwater samples as old as 35 years demonstrating that chloroform can persist over extended periods in oxic groundwater. The persistence of chloroform in groundwater and its high mobility makes it particularly difficult to relate chloroform in groundwater to its source based on concentration data alone as chloroform might be transported over several kilometers. However, the Viborg study indicates that the origin of chloroform can still be determined based on isotope data even if chloroform was transported over an extensive distance. The δ^{13}C of chloroform at the Viborg water works down gradient of the plantation with an average CFC-groundwater age of 30 years had a δ^{13}C of −24.2‰, which is still within the range of the δ^{13}C in soil gas at the Viborg plantation. In summary, the study demonstrates that natural and anthropogenic chloroform have a distinctly different carbon isotope signature that can be related to the carbon source from which chloroform originates, soil organic matter and methane, respectively. The strong difference in δ^{13}C makes it possible to clearly identify the origin of chloroform in groundwater even if some changes of the isotope ratios occur during transport. The study demonstrates that chloroform can be naturally present in groundwater at the low microgram level and persist over decades. The isotope method opens new possibilities for a comprehensive assessment of the natural occurrence of chloroform in groundwater. It also helps to take appropriate measures when detecting chloroform in groundwater. We have in this study demonstrated that it is possible to distinguish between the naturally formed chloroform and industrial produced chloroform using isotopic signature. This has led to the Danish EPA to change the limit of chloroform in groundwater to be used for drinking water from 1 to 10 µg/L when the origin of chloroform is a natural source.

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▪Supporting information

Additional materials regarding the validation of the analytical system are provided in Appendix 1.

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Chapter II

Assessing the Role of Trichloroacetyl-Containing Compounds in the Natural Formation of Chloroform using Stable Carbon Isotopes Analysis

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ABSTRACT Chloroform ($CHCl_3$) is an environmental contaminant widely distributed around world, as well as a natural compound formed in various aquatic and terrestrial environments. However, the chemical mechanisms leading to the natural formation of chloroform in soils are not completely understood. To assess the role of trichloroacetyl-containing compound (TCAc) in the natural formation of chloroform in forest soils, carbon stable isotope analyses of chloroform and TCAc in field samples and chlorination experiments were carried out. The isotope analysis of field samples have revealed that the $\delta^{13}C$ value of natural chloroform ($\delta^{13}C_{mean}$=-25.8‰) is in the same range as the natural organic matter ($\delta^{13}C_{mean}$ =-27.7‰), whereas trichloromethyl groups of TCAc are much more enriched in ^{13}C ($\delta^{13}C_{mean}$ =-9.8‰). A similar relationship was also observed for TCAc and chloroform produced by chlorination of natural organic matter with NaOCl. The strong depletion of ^{13}C in chloroform relative to TCAc can be explained by carbon isotope fractionation during TCAc hydrolysis. As shown using a mathematical model, when steady state between formation of TCAc and hydrolysis is reached, the isotope ratio of chloroform is expected to correspond to isotope composition of NOM while TCAc should be enriched in ^{13}C by about 18.3‰, which is in good agreement with field observations. Hence this study suggests that TCAc are likely precursors of chloroform and at the same time explains why natural chloroform has a similar isotope composition as NOM despite large carbon isotope fractionation during its release.

▪ Introduction

Chloroform ($CHCl_3$) has for a long time been considered as of anthropogenic origin only, classified as a Group B2 probable human carcinogen according to the World Health Organization classification scheme (WHO-IARC, 1999). Recently, the presence of chloroform in coniferous forest soil and groundwater has been demonstrated (Haselmann et al., 2000a; Haselmann et al., 2002; Albers et al., 2008). The frequent detection of chloroform in groundwater in absence of other anthropogenic contaminants suggests that chloroform may be formed naturally by biogeochemical processes (Laturnus et al., 2002). Numerous studies on natural organohalogens have suggested that enzymes such as chloroperoxidase (CPO) excreted by fungi could play an important role in biosynthesis of chlorinated organic compounds in soil (Hoekstra et al., 1998a; Hoekstra et al., 1998b; Urhahn and Ballschmiter, 1998; van Pee and Unversucht, 2003). The current hypothesis is that CPO expresses a chlorinating activity by forming HOCl or other oxidized chlorine species (Hoekstra et al., 1998a; Hoekstra et al., 1998b). Recently, Huber et al. (Huber et al., 2009) have demonstrated that chloroform can also be formed abiotically, when organic matter is incubated with Cl^-, Fe^{3+} and H_2O_2.

As CPO is able to chlorinate the natural organic matter (NOM) likely via the formation of an oxidized diffusible intermediate like HOCl (Griffin, 1983), the chemical chlorination with sodium hypochlorite constitutes a good model system to mimic the enzymatic chlorination of NOM. Since NOM has a very complex chemical structure it is impossible to propose a unique reaction mechanism. Therefore, most studies have focused on the chlorination mechanism of simple model compounds such as phenol, substituted phenols, aliphatic β-dicarbonyl acids and glycosides (Rook, 1977; Boyce and Hornig, 1983; Gallard and Von Gunten, 2002b, a; Dickenson et al., 2008). The chlorination of substituted aromatic compounds is presumed to take place through a halogenation by oxidized chlorine species on the activated aromatic carbon atoms. This step is followed by hydrolytic cleavage of the aromatic ring; further chlorination of aliphatic intermediates and finally the release of chloroform by hydrolysis. Boyce and Hornig have demonstrated that during the conversion of 1,3-dihydroxyaromatic substrates to chloroform the aromatic ring is broken and several chlorinated aliphatic intermediates are formed (Boyce and Hornig, 1983). Among these intermediates, several trichloroacetyl-containing compounds (TCAc) have been identified by GC/MS (Figure 1) (Boyce and Hornig, 1983; Deleer et al., 1985).

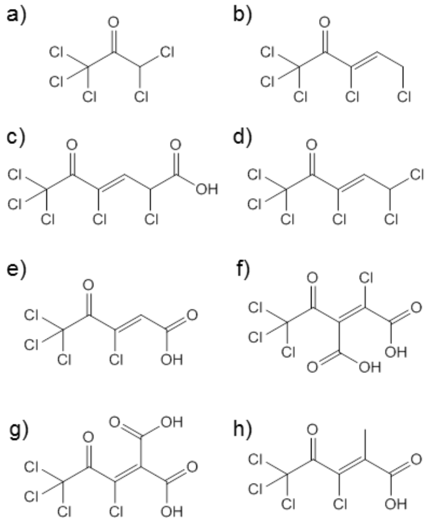

Figure 1. Structures of trichloroacetyl-containing compounds formed by chemical chlorination of dihydroxyaromatic model compounds (a–d) (Boyce and Horning, 1983) and humic acid (e–h) (de Leer et al., 1985).

TCAc can release chloroform either by nucleophilic substitution or by alkaline hydrolysis. Recently, Albers et al. have shown that TCAc are present in forest soils containing natural chloroform and the concentrations of TCAc and chloroform show a similar spatial variability(Albers et al., 2010b, a). They furthermore found the concentration of TCAc in soil profiles to decrease when the pH increases, suggesting natural TCAc to be stable only at acidic conditions.

Compound-specific isotope analysis (CSIA) constitutes a potential tool to assess the mechanisms leading to formation of chloroform during chlorination of organic matter. This technique has recently been used to distinguish natural and anthropogenic sources of contaminants and to gain insights into the mechanisms of degradation of various pollutants (Aelion et al., 2010). Changes of the carbon isotope composition of organic compounds during formation and transformation processes can be attributed to a kinetic isotope effect due to the presence of a heavy isotope in the reacting bond(s), which is characteristic for the underlying reaction mechanism. Recently Arnold et al. (Arnold et al., 2008), have used CSIA to investigate the apparent ^{13}C kinetic isotope effect of the formation of chloroform during chlorination of selected model compounds to evaluate the functional group(s) in NOM responsible of chloroform formation. They suggest that compounds containing 1,1,1-trichloropropanone-like functional groups could be intermediates leading to chloroform.

The aims of this study are to characterize the isotope signature of natural chloroform and trichloromethyl groups of the TCAc in order to (*i*) gain better understanding of the role of TCAc in the formation of natural chloroform and (*ii*) constrain the carbon isotope signature of TCAc and chloroform in the natural environment in view of using isotope data for distinguishing different sources of chloroform. If TCAc is the precursor of chloroform, the isotopic signature of trichloromethyl groups of TCAc should be offset from that of chloroform by an amount that depends on the kinetic isotope effect associated with chloroform release. This study included a field study at forested sites where natural chloroform production is well established (Albers et al., 2010c; Albers et al., 2011) and laboratory experiments to investigate the relationship between chloroform and trichloromethyl groups in TCAc in a well-defined closed system. In the field study, the isotope ratio of chloroform present in soil air was compared to the isotope ratio of trichloromethyl groups of TCAc in forest soil. The chlorination experiments were carried out under various pH (pH=4, 7, 8) conditions as the release of chloroform by hydrolysis is pH dependant using soil material from one of the field sites and humic substances. As for the field study, the carbon isotope ratio of chloroform and trichloromethyl-groups in TCAc was determined. Finally, a mathematical model of the carbon isotopic trends of chloroform and TCAc was established to assess the role of TCAc in the formation of chloroform and to better constrain the isotope signature of natural chloroform.

▪ Materials and Methods

Chemicals. The following chemicals were used as received: sodium hypochlorite (Sigma-Aldrich, available chlorine ≥4%), phosphoric acid (Fluka, 85%), sodium dihydrogenphosphate monohydrate (Merck, >99%), disodium hydrogenphosphate dodecahydrate (Fluka, >99%), sodium sulfite (Sigma-Aldrich, >98%). Ultrapurified water (18.2 MΩ cm at 25°C, Direct-Q UV-3 Millipore) was used to prepare the phosphate buffer solutions. Chloroform from Fluka (99.5%) and Acros Organics (99.8%) were used to prepare concentration and isotope standards. Humic substances used for chlorination experiments were obtained from the International Humic Substance Society: Suwannee river NOM (SRNOM), Nordic reservoir NOM (NRNOM), Pahokee peat humic acid (PPHA), Elliot soil humic acid (ESHA). Soil organic matter collected in the H and F horizons of a forest soil from Tisvilde Hegn (Denmark) and humic acids from the same forest NOM (FOHA) were also used for chlorination experiments. Humic acids were extracted by alkaline extraction with aqueous NaOH, followed by precipitation of humic acid at low pH and a desalting steps involving dialysis (Albers et al., 2008). Information regarding the chemical properties of humic substances and NOM are available in Appendix 3.The use of sodium hypochlorite to mimic CPO-catalyzed chlorination of NOM is based on the following arguments. Recently Breider et al. have shown that abiotic and CPO-catalyzed chlorination of humic substance produce trichloroacetic acid with very similar carbon isotope composition suggesting that the formation mechanisms of organochlorine from humic substances by abiotic and enzymatic chlorination are likely very similar [**V**]. Moreover, Kühnel et al. have shown using high resolution X-ray synchrotron diffractometry that only a narrow channels (Ø~4 Å) connect the protein surface with the heme of CPO (Kuehnel et al., 2006). However, humic substances have molecular weights typically between ~10 kDa up to ~300 kDa and a diameter between ~100 Å and ~2000 Å (Christl et al., 2000). It can therefore be excluded that humic substances can reach the heme of CPO and react directly at the catalytic site. Hence we can conclude that the chlorination of humic substances can only occurs via the formation and diffusion of free HOCl from the enzyme.

Field sites and sampling. Field sampling campaigns were conducted in Denmark in June 2009 and July 2010 in two mixed Spruce (*Picea abies*) and Scots Pine (*Pinus sylvestris*) forests at the Tisvilde Hegn (THN) (56° 02’ N − 12° 04’ E) and Viborg Hedeplantage (VBH) (56° 25’ N − 9° 22’ E) where natural chloroform production occurs (Albers et al., 2011). At these sites, chloroform production in soil varies spatially with hotspots of high production of limited spatial extent (20 to 400 m^2). In this study, soil-air samples were taken at one of the hotspots of each site that are equipped with a multilevel wells throughout the unsaturated zone (Albers et al., 2010c), denoted as THN and VBH hotspots. The top soil at the THN and VBH hotspots is constituted of an organic horizon mainly composed of partly degraded needles and branches. Soil-air samples for chloroform analysis were collected at 0.5 m depth using sorption tubes filled with 100 mg of Tenax TA (Supelco, Bellefonte, USA) connected to a membrane pump NMP05L (KNF, Balterswil, Switzerland) (Mead et al., 2008). Before sampling, the sorption tubes were conditioned at 200°C during 120 min under N2 flow of 40 mL min^{-1}, and sealed with a Teflon septum. After purging the internal volume of the sampling system at least two times, 3 L of soil-air were sampled at ~200 mL min^{-1}. During sampling, soil-air was dried using a stainless steel cartridge filled with anhydrous sodium sulfate and the sorption tubes were cooled at ~15°C below ambient temperature using a Peltier device.

Preparation and analysis of field samples. Concentrations of chloroform in soil-air and TCAc in soil were measured by gas chromatography electron capture detector (8A, Shimadzu, Kyoto, Japan) according to the procedure described by Busenberg and Plummer (Busenberg and Plummer, 1992). These analyses were carried out in Denmark directly after field sampling. The samples preparation for TCAc concentration analysis was done as described previously by Albers et al. (Albers et al., 2010b; Albers et al., 2010c). Briefly, the soil samples were amended with chloroform free water in gas tight vials. Then, the soil-bound and water soluble TCAc present in soils were hydrolyzed by adding concentrated NaOH ($pH \geq 12$) solution and incubated during 24h. The chloroform released by the hydrolysis of TCAc was analyzed by gas chromatography electron capture detector.

The concentrations and the carbon isotope measurements were conducted with subsamples of the same soils. For carbon isotope analysis of trichloromethyl groups of TCAc, the same procedure was used except 4 g of dried soil was mixed with ~500 mL of pure water in a 1 L gas-tight bottle (Schott, Mainz, Germany) which was connected to a purge-and-trap system (Velocity XPT, Teledyne Tekmar Dohrmann, Mason, USA). For selected sample, the hydrolysis process was repeated and no further chloroform was detected (detection limit=0.8 μg L^{-1}) indicating that the procedure leads to nearly complete hydrolysis. The purge gas stream of the purge-and-trap system was directed through the 1 L glass bottle equipped with a frit (Hunkeler et al., 2012). The sample was purged during 20 minutes at 150 mL min^{-1} and trapped at 30°C with a VOCARB 3000 trap (Supelco, Bellefonte, USA). In order to avoid saturation of the trap with water, the moisture was removed during the sample purge step with a Velocity XPT DryFlow trap heated at 100°C. The carbon isotope ratios of chloroform formed by hydrolysis of TCAc was measured using gas chromatography (Trace GC Ultra, Thermo Fisher Scientific, San Jose, USA) coupled to a combustion interface and an isotope ratio mass spectrometer (IRMS) (Delta XP plus, Thermo Fisher Scientific, San Jose, USA). After the extraction step, the VOCARB 300 trap was heated to 250°C for 3 min. Chloroform was thermally desorbed and transferred to the GC at 1.7 mL min^{-1} and trapped in a cryogenic focuser (Optic 3 ATAS-GL, Veldhoven, The Netherlands) held at -100°C during 3 min with liquid nitrogen before chromatographic separation. The chromatographic separation was carried out with a 60 m × 0.25 mm × 1.4 μm film thickness DB-VRX column (Agilent, Santa Clara, USA). The GC oven temperature program used was as follows: 6 min at 40°C, 10°C/min to 175°C, hold for 1 min. Oxidation and reduction reactors of the combustion interface were respectively maintained at 940 and 640°C.

To determine the $\delta^{13}C$ signature of natural gaseous chloroform, the sorption tubes containing chloroform from soil-air were analyzed using a thermal desorption system TDAS 2000 (CTC Analytics, Zwingen, Switzerland) with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) connected to the GC/C/IRMS. The sorption tubes were flushed at ambient temperature with N_2 for 15 sec and were desorbed at 200°C during 60 min. Then, carbon isotopic ratios of chloroform were analyzed using the GC/C/IRMS method described above. The thermal desorption was carried out in splitless mode with cryogenic focusing. This approach minimizes the possibility for isotope fractionation, as the totality of the desorbed analyte is transferred to the GC-C-IRMS. To confirm that no isotopic fractionation occurs, a standard of chloroform with known $\delta^{13}C$ value (-53.8±0.3‰, n=3) was measured with this technique in laboratory. Gaseous chloroform was sampled during 15min with sorption tubes at a flow rate of 200 ml min^{-1} corresponding to the flow rate used in the field. The obtained $\delta^{13}C$ value (-53.4±0.3‰, n=3). was not significantly different from the expected value (-53.8±0.3‰, n=3) according to a student’s t-test (p=0.18). The carbon isotopic composition of NOM was determined in triplicate using an elemental analyzer coupled with a Delta S stable isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany).

Chlorination experiments and analysis of laboratory samples. The different soil and humic substances were chlorinated with sodium hypochlorite using the following procedure: 4 mg of humic substance or 40 mg of soil was added in 42 mL vial containing 40.8 mL of 100 mM phosphate buffer and the reaction was initiated by adding 1 mL of 0.02 M aqueous solution of NaOCl. The vials were sealed with Teflon septum caps and agitated during 24h at room temperature (~25°C). After 24h, samples were quenched with 200 μL of a 100 g L^{-1} aqueous solution of sodium sulfite to stop the reaction. The reaction was carried out at pH 4, 7 and 8. To analyze the concentration and the isotope signature of the trichloromethyl groups in TCAc, the samples were purged for 30 min with pure N_2 to remove chloroform formed during the chlorination and the pH was adjusted to ≥12 with 6

M NaOH solution to hydrolyze TCAc and form chloroform. All samples were stored in the dark at 4°C until concentration and carbon isotopes analysis.

The concentration of chloroform formed during chlorination experiments and by hydrolysis of TCAc was analyzed using gas chromatography (Trace GC Ultra, Thermo Fisher Scientific, San Jose, USA) with a quadrupole mass spectrometer (DSQII, Thermo Fisher Scientific, San Jose, USA). The analyses were carried out with 20 ml headspace vials containing 15 ml of sample. After equilibrating with agitation at 60°C for 2 min, 500 μL of headspace from 20 ml vials containing 15 ml of sample were injected in split mode (1:10) by an autosampler (Combi-PAL, CTC Analytics, Zwingen, Switzerland) onto a 60 m × 0.32 mm × 1.8 μm film thickness Zebron ZB 625 column (Phenomenex, Torrance, USA). The injector temperature was 250°C and the ion source temperature was 200°C. The oven temperature was held at 150°C for 5 min. The analyses were carried out in single ion monitoring mode using the following *m*/*z*: 48, 50, 83, 85, 118 and 120.

For carbon isotope ratios analysis of chloroform formed during chlorination experiments and by hydrolysis of TCAc, the GC/C/IRMS system described above was used except for the purge volume and N_2 purge flow rate, which were 25 mL and 40 mL min^{-1} during 10 min, respectively. In order to maximize the accuracy of measured $\delta^{13}C$ values, the aqueous samples were diluted to obtain constant peak amplitudes (5000 mV).

The hydrolysis of TCAc is assumed to be much slower than the chlorination steps. Thus in approximation hydrolysis can be considered as a simple one step reaction and thus the AKIE can be approximated using a Rayleigh approach. Therefore the isotope fractionation factor, α, for the formation of chloroform from TCAc was estimated using the following equation:

$$\alpha = \frac{\ln\left(\dfrac{R_{TCAc}}{R_{TCAc}\,f + R_{CF}\,(1-f)}\right)}{\ln(f)} + 1 \tag{1}$$

Where RTCAc is the isotope ratio of the trichloromethyl position in TCAc, RCF is the isotope ratio of accumulated chloroform, and *f* is the remaining fraction of TCAc. Since only one carbon atom is present in chloroform, the fractionation factor α can be directly related to the apparent kinetic isotope effect (AKIE) according to:

$$AKIE = \frac{1}{\alpha} \tag{2}$$

▪Results and Discussion

Concentrations and δ¹³C of chloroform and TCAc in natural samples. The concentrations of chloroform in soil-air at 0.5 m depth in the THN and VBH hotspots varied within a range of 15 to 120 ppbv. The $\delta^{13}C$ values of chloroform measured in soil-air at a depth of 0.5 m in the THN and VBH hotspots ranged between -22.8 to -31.3‰ (Figure 2). The δ^{13}C values of NOM from THN and VBH (-27.2 to -27.7‰) are in the same range as the δ^{13}C values of chloroform (Figure 2). The total concentration of TCAc measured in four soil samples from THN and VBH ranged between 63 and 5565 µg CHCl$_3$ kg^{-1}. The δ^{13}C values of the trichloromethyl component of TCAc varied between -9.2 to -10.2‰ for soil samples (Figure 2). The variations of the δ^{13}C of chloroform in soil-air could be related to the combined isotope effect associated with diffusion in the unsaturated zone and the equilibration between soil-air and pore water. Modeling studies for CO$_2$ showed that in the unsaturated zone during transient conditions (e.g. related to rain events) isotope ratios of gaseous compounds can deviate from steady state values (Cerling et al., 1991; Nickerson and Risk, 2009). The δ^{13}C of chloroform is similar to that of NOM and distinctly different from the known range of anthropogenic chloroform (-43.2 to -63.6‰, Hunkeler et al., 2012) suggesting that chloroform in soil air originates from NOM. The trichloromethyl groups of TCAc present in upper soil horizons are considerably more enriched in ^{13}C compared to chloroform. Nevertheless, it is very likely that natural chloroform is released from TCAc during its decomposition in soils, since large carbon isotope fractionation is expected for the hydrolysis of TCAc (Arnold et al., 2008). In order to test this hypothesis and to better understand the mechanisms that could lead to the formation of chloroform from TCAc present in soils, chlorination experiments of NOM of different origins were carried out.

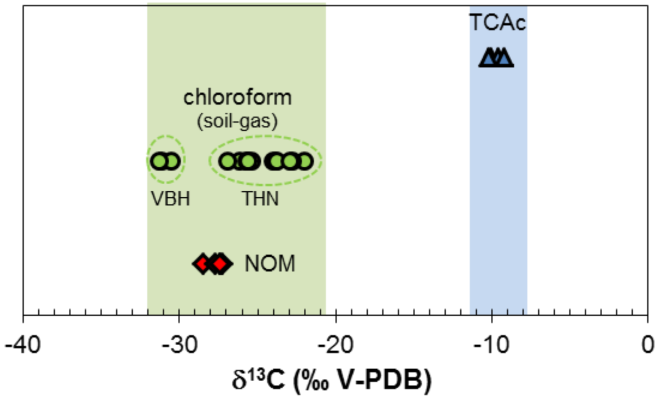

Figure 2. δ^{13}C values of natural chloroform in soil–gas measured at VBH hotspot (*n* = 3, δ^{13}C$_{mean}$ = -31.0‰) and THN hotspot (*n* = 12, δ^{13}C$_{mean}$ = -24.6‰) at 0.5 m depth (green circles), δ^{13}C values of trichloromethyl groups of TCAc in THN and VBH soils (*n* = 4, δ^{13}C$_{mean}$ = -9.8‰) (blue triangles), and NOM from THN and VBH hotspots (*n* = 5, δ^{13}C$_{mean}$ = -27.7‰) (red diamonds).

Chlorination of NOM with hypochlorite. For all materials, the concentrations of chloroform and the total concentration of trichloromethyl groups in chloroform and TCAc increase with increasing pH whereas the TCAc concentration decreases (Figure 3). This inverse correlation between chloroform and TCAc concentrations with pH could be due to the formation of chloroform by nucleophilic attack of the carbonyl C-atom of TCAc by OH^- or OCl^- , which are present at higher abundance at an elevated pH. At pH 4, the release of chloroform is more likely related to the nucleophilic attack of the carbonyl C-atom by H_2O. The increase of the total concentration of trichloromethyl groups with rising pH can be rationalized in terms of the reactivity of the ionized and un-ionized forms of functional groups in humic substances. Under alkaline conditions phenolic groups tend to be deprotonated. The electron donating character of the deprotonated O^- substituents tends to stabilize electron rich structures by resonance and thus activate the electrophilic substitution of the aromatic moieties in ortho and para positions (Rebenne et al., 1996; Gallard and Von Gunten, 2002b). The chlorination of ketone functional groups can also be activated under alkaline pH as the keto-enol equilibrium is shifted in the direction of the enolate isomer and thereby makes the carbon bond more susceptible to attacking electrophiles. The reactivity of HOCl depends of its speciation as function of pH. Therefore, the pH can indirectly strongly influence the chlorination rate and thus TCAc and chloroform production rates. The amount of chloroform released by chlorination of NOM of different origin at a given pH is likely determined by the relative amount of reactive functional groups in humic substances and the pH which controls the hydrolysis rate.

Carbon isotope signatures of chloroform and TCAc. The $\delta^{13}C$ values of chloroform and the trichloromethyl position of TCAc formed upon chlorination of NOM at pH 4, 7 and 8 are shown in Figure 4. For all samples, the $\delta^{13}C$ values of chloroform are more depleted in ^{13}C compared to NOM. Chloroform released upon chlorination of NOM tends to be gradually enriched in ^{13}C with rising pH. Similar offsets are observed independent on the type of organic matter. With increasing pH, the $\delta^{13}C$ values of chloroform approaches the carbon isotopic signature of the original NOM. The $\delta^{13}C$ values of the trichloromethyl position of TCAc were only measured at pH 4 since at pH≥7 there is not enough remaining TCAc for carbon isotope analysis. In contrast to chloroform, at pH 4 the trichloromethyl position of TCAc is enriched in 13C compared to NOM with a difference in $\delta^{13}C$ of about 30‰ between chloroform and TCAc. The carbon-weighted mean $\delta^{13}C$ values of trichloromethyl groups formed upon chlorination (chloroform and TCAc) at pH 4 range between -28.3 ‰ and -36.6‰ (Figure 4). The mean $\delta^{13}C$ values of trichloromethyl groups produced at pH 4 are similar to the $\delta^{13}C$ values of chloroform produced at pH 8 which suggest that at pH 8 almost all trichloromethyl groups formed upon chlorination have been converted to chloroform consistent with the concentration data. Furthermore, the mean $\delta^{13}C$ values of trichloromethyl groups are slightly depleted in ^{13}C compared to the $\delta^{13}C$ values of the natural organic precursors used for chlorination experiments. This small deviation of the $\delta^{13}C$ values may be due to (*i*) heterogeneities in ^{13}C distribution between the functional groups involved in the formation of trichloromethyl groups and the rest of the organic precursors (Galimov, 2006; Schmidt, 2003), (*ii*) an isotopic effect associated with a reaction step preceding the TCAc hydrolysis such as the cleavage of aromatic rings present in NOM, or (*iii*) an isotope sensitive branching of competing reaction pathways (Arnold et al., 2008)

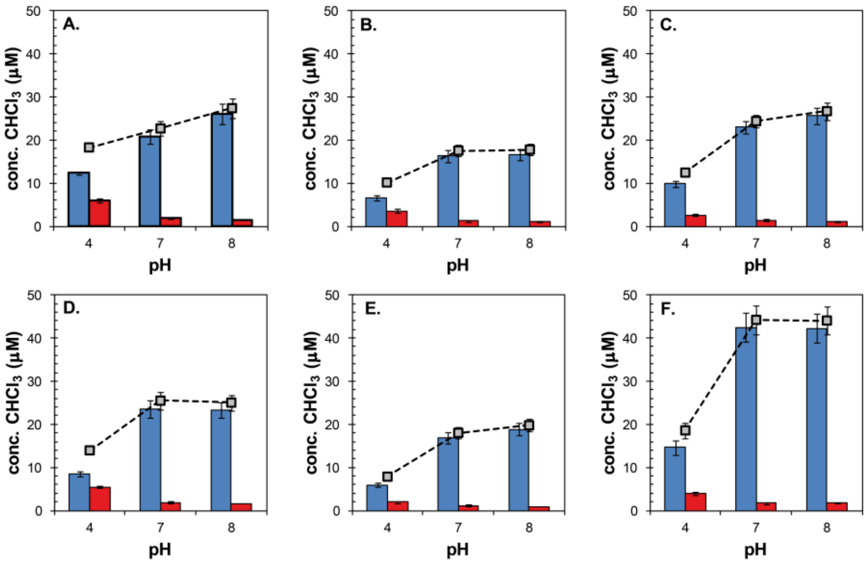

Figure 3. Concentrations in µM of chloroform (blue bars), chloroform produced by hydrolysis of TCAc (red bars) and total concentration of trichloromethyl groups from chloroform and TCAc (grey squares) formed at pH 4, 7 and 8 by chlorination of NOM. (A) Forest soil organic matter (THN); (B) Elliot soil humic acid; (C) Suwannee river NOM; (D) Humic acid from forest soil (THN); (E) Pahokee peat humic acid, and (F) Nordic lake NOM. The error bars correspond to the standard deviation (1σ) of the concentrations.

The gradual enrichment of chloroform in ^{13}C with rising pH strongly suggests that the isotopic fractionation between NOM and chloroform is likely due to a pH-dependent reaction. As previously discussed, the concentration of chloroform formed upon chlorination increase with pH, whereas the concentration of TCAc tends to decrease (Figure 3). Under alkaline conditions almost all trichloromethyl groups formed are converted into chloroform. Considering that the mean δ^{13}C value of the functional groups involved in the formation of trichloromethyl groups is close to the mean δ^{13}C value of NOM, the δ^{13}C value of chloroform must increase toward the δ^{13}C value of the precursor with the increasing extent of the hydrolysis. Therefore, the pH-effect observed on the δ^{13}C values of chloroform can likely result from different degree of hydrolysis of the trichloroacetyl groups of TCAc formed during the chlorination. The AKIEs calculated using equation 2 for the formation of chloroform from TCAc at pH 4 is 1.0183±0.0002 for the chlorination of forest NOM and varies between 1.0142 and 1.0187 for the chlorination of humic substances. These AKIEs are in the same range as the AKIE determined by Arnold et al. for the alkaline hydrolysis of 1,1,1-trichloropropanone (AKIE=1.014±0.002) (Arnold et al., 2008), which is compatible with the hypothesis of chloroform release by hydrolysis of TCAc.

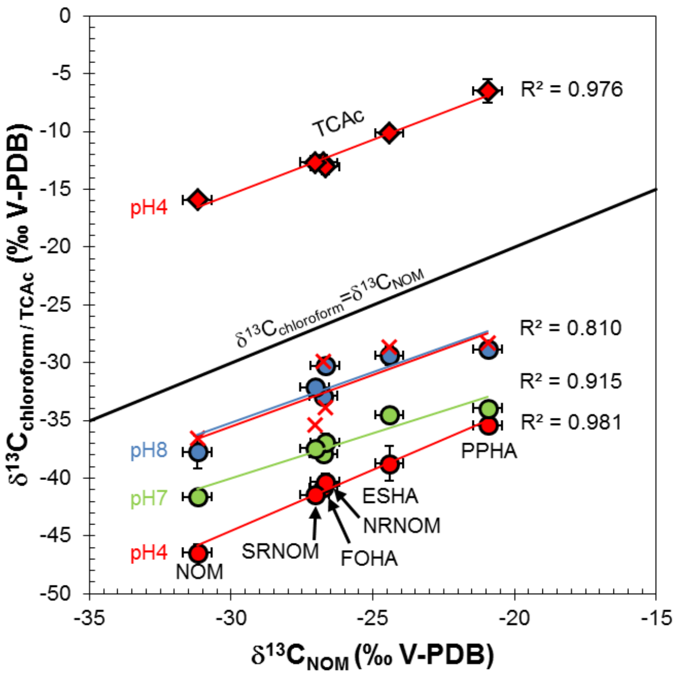

Figure 4. δ^{13}C values of chloroform formed at pH 4 (red circles), pH 7 (green circles), pH 8 (blue circles) and trichloromethyl groups in TCAc formed at pH 4 (red diamonds) by chlorination of forest soil organic matter from THN (NOM), Suwannee river NOM (SRNOM), humic acid from forest soil (FOHA), Nordic lake NOM (NRNOM), Elliot soil humic acid (ESHA), and Pahokee peat humic acid (PPHA). The red crosses correspond to the carbon-weighted average δ^{13}C values of trichloromethyl groups formed upon chlorination at pH 4. The error bars correspond to the standard deviation (1σ) of the d13C values (*n* = 3).

The chlorination experiments confirm that chloroform is released by hydrolysis of TCAc, which induces a large carbon isotopic fractionation. Contrary to the chlorination experiments, the δ^{13}C values of trichloromethyl groups in TCAc and chloroform in soils cannot be rationalized by a typical Rayleigh fractionation trend. Otherwise, both chloroform and trichloromethyl groups in TCAc should become increasingly enriched over time. Therefore, to better constrain the carbon isotope signature of chloroform and TCAc measured in soils, and to assess the combined effects of the simultaneous production and hydrolysis of TCAc, an isotopic model was developed in the following section.

Modeling of carbon isotopic trends of chloroform and TCAc. A mathematical model to assess the evolution of the δ^{13}C values of the trichloromethyl groups of TCAc and chloroform with time was established assuming a constant formation of TCAc in soil, the consumption of the trichloroacetyl groups by hydrolysis according to a first order rate law and no degradation of chloroform in soil. Here we hypothesize that the formation of TCAc follows a zero-order kinetic. In soils, NOM is present in excess and hence the reaction rate is not a function of the NOM concentration. Moreover, it can be assumed that HOCl is produced at a constant rate (for a given period within the year) and controls the rate of reaction. Thus, the TCAc concentration is governed by the following first order linear non-homogeneous differential equation:

$$\frac{\partial C}{\partial t} = P - k \cdot C \tag{3}$$

Where C is the concentration of TCAc, P is the rate of formation and k the first order rate coefficient of degradation of TCAc. Solving equation 3 for an initial concentration of zero leads to the following equations for TCAc with trichloromethyl groups containing a ^{12}C and ^{13}C atom:

$$^{12}C_t = \frac{^{12}P}{^{12}k}\left(1 - e^{-^{12}kt}\right) \text{ and } ^{13}C_t = \frac{^{13}P}{^{13}k}\left(1 - e^{-^{13}kt}\right) \tag{4}$$

^{12}P and ^{13}P are the rates of formation of TCAc with trichloromethyl groups containing a ^{12}C and ^{13}C atom, respectively. ^{12}k and ^{13}k are the first order rate coefficients of degradation of trichloroacetyl groups in TCAc containing a ^{12}C and ^{13}C atom at the trichloromethyl position, respectively. Hence the isotope ratio of TCAc ($R_{\mathrm{TCAc,\ t}}$) evolves as follows:

$$R_{TCAc,t} = \frac{^{13}C_t}{^{12}C_t} = \frac{^{12}k}{^{13}k} \cdot \frac{^{13}P}{^{12}P} \cdot \frac{1 - e^{-^{13}kt}}{1 - e^{-^{12}kt}} \tag{5}$$

Where,

$$\frac{^{12}k}{^{13}k} = AKIE \tag{6}$$

As

$$k = {}^{12}k + {}^{13}k \tag{7}$$

Therefore,

$$^{12}k = \frac{k}{1 + \frac{1}{AKIE}} \text{ and } ^{13}k = \frac{k}{1 + AKIE} \tag{8}$$

Thus,

$$R_{TCAc,t} = AKIE \cdot \frac{{}^{13}f \cdot P}{{}^{12}f \cdot P} \cdot \frac{1-e^{-\frac{k}{1+AKIE}\cdot t}}{1-e^{-\frac{k}{1+\frac{1}{AKIE}}\cdot t}} = AKIE \cdot \frac{{}^{13}f}{{}^{12}f} \cdot \frac{1-e^{-\frac{k}{1+AKIE}\cdot t}}{1-e^{-\frac{k}{1+\frac{1}{AKIE}}\cdot t}}$$

(9)

Where AKIE (AKIE=1.0183±0.0002) is the calculated isotope fractionation factor for TCAc hydrolysis of forest NOM, and ${}^{12}f$ and ${}^{13}f$ are the fractions of ${}^{12}C$ and ${}^{13}C$ atoms, respectively in NOM given by:

$$ {}^{12}f = 1 - \frac{R_{NOM}}{R_{NOM}+1} \text{ and } {}^{13}f = \frac{R_{NOM}}{R_{NOM}+1} \qquad (10)$$

Where R_{NOM} is the measured isotopic ratio of forest NOM (R_{NOM}=0.010925). The $\delta^{13}C$ values of TCAc and chloroform can be calculated using the following equations:

$$\delta^{13}C_{TCAc,t}\ (‰) = \left(\frac{R_{TCAc,t}}{R_{V-PDB}} - 1\right) \times 1000 \qquad (11)$$

And

$$\delta^{13}C_{CHCl_3,t}\ (‰) = \delta^{13}C_{TCAc,t}\ (‰) - 1000 \times (AKIE - 1)$$

(12)

The $\delta^{13}C$ values of TCAc and chloroform were plotted versus $[\mathrm{TCAc}]_t/[\mathrm{TCAc}]_{steady\text{-}state}$ which was calculated using:

$$\frac{[TCAc]_t}{[TCAc]_{steady-state}} = 1 - e^{-kt} \qquad (13)$$

Where $[\mathrm{TCAc}]_t$ and $[\mathrm{TCAc}]_{steady\text{-}state}$ are respectively the concentration of TCAc at time *t* and at steady-state.

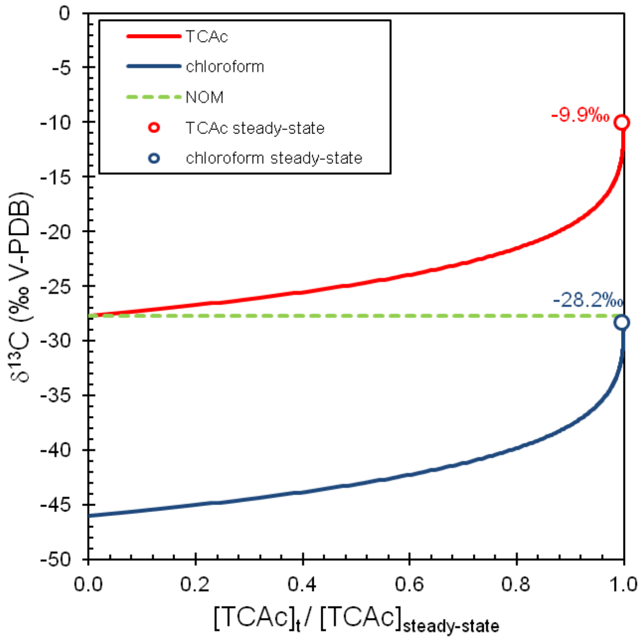

Figure 5. Model of the carbon isotopic trends of chloroform (blue line), trichloromethyl groups in TCAc (red line), and forest NOM (green dashed line) versus [TCAc]t/[TCAc]steady-state.

The results of the modeling show that chloroform and TCAc become progressively enriched in ^{13}C before reaching the steady-state ([TCAc]*t*/[TCAc]$_{steady\text{-}state}$=1) (Figure 5). The calculated carbon isotopic ratio of trichloromethyl groups of chloroform and TCAc at steady state are -28.2‰ and -9.9‰, respectively. These calculated δ^{13}C values agree well with the carbon isotopic ratios of chloroform and TCAc measured in the field and in the chlorination experiments, suggesting that TCAc likely plays an important role in the natural formation of chloroform. The simulation suggests that the natural chloroform evolves towards an isotopic signature close to the NOM from which it is derived despite substantial carbon isotope fractionation during release of chloroform thanks to the presence of ^{13}C-enriched precursors. Such isotope patterns have to be expected at a steady-state situation because each trichloromethyl group that enters in the TCAc pool has to have the same isotopic composition as the chloroform that leaves the pool if there are no other major entries and if the formation of TCAc is not associated with isotope fractionation. The isotopic fractionation associated with reaction step(s) preceding the TCAc hydrolysis (e.g. chlorination, ring opening) could induced a deviation of the isotope composition of chloroform compared to NOM. In the case where the reaction step(s) preceding the hydrolysis would induced a normal isotope effect (KIE>1), chloroform formed by hydrolysis would be depleted in ^{13}C compared to NOM. Indeed in the laboratory experiment at pH=8 when nearly complete hydrolysis of TCAc is observed (Figure 3), the CF is depleted by about 5‰ compared to NOM. Inversely, if the step preceding the hydrolysis of TCAc would involve an inverse isotope effect (KIE<1) chloroform would be slightly more enriched in ^{13}C. The steady-state situation between the formation and the hydrolysis of TCAc could be perturbed during some periods of the year which could also lead to some variations of the chloroform isotope ratio around the average value of biomass. Even if the produced CF deviates from NOM by several ‰, it will still be distinctly different from anthropogenic CF (-43.2 to -63.6‰, Hunkeler et al., 2012).

Conclusions. Although the carbon isotopic signatures of chloroform and the trichloromethyl group in TCAc are very distinct, the chlorination experiments combined with a mathematical model have revealed that TCAc could play a fundamental role in the formation of chloroform in the terrestrial environment. The strong isotopic enrichment of the trichloromethyl group in TCAc indicates that a fraction of the trichloromethyl groups is released as chloroform by hydrolysis which will then equilibrate into soil-air. Using a mathematical model combined with field data, the present study shows that when the formation of TCAc and hydrolysis reach a steady state, the isotope composition of chloroform is expected to correspond to isotope ratio of NOM while TCAc should be enriched in ^{13}C. This study confirms that TCAc are reaction intermediates which are subsequently degraded in soil into chloroform, and explains why natural chloroform has a similar isotope signature as NOM despite a large carbon isotope fractionation during its release.

▪Acknowledgements

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▪Supporting information

Additional information regarding the elemental composition, the carbon isotopic composition and the carbon distribution in humic substances and NOM are provided in the Appendix 3.

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Chapter III

Mechanistic Insights into the Formation of Chloroform from Natural Organic Matter using Carbon Stable Isotopes Analysis

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submitted to Geochimica et Cosmochimica Acta

ABSTRACT Chloroform can be naturally formed in terrestrial environments (e.g. forest soils) by chlorination of natural organic matter (NOM). In this study, compound specific isotope analysis was used to better understand the mechanisms controlling the formation and the carbon isotopic composition of natural chloroform. Thus, the carbon isotopic composition of chloroform formed at pH 4, 7 and 8 by chlorination of humic acid, soil organic matter (SOM) and model compounds (i.e. phenol and 2-propanone) representing NOM functional groups was measured. For each model compounds and pH, different apparent kinetic isotope effects (AKIE) were found for chloroform formation. At pH 4 a normal AKIE was found for phenol (1.0156±0.0012) while the AKIE of 2-propanone was inverse (0.9935±0.0007). Inversely, at pH 8 the chlorination of phenol and 2-propanone displayed respectively an inverse (0.9900±0.0007) and normal (1.0189±0.0016) AKIE. The chloroform formation from humic acid and SOM gives rise to small isotope effect. A comparison of the isotopic trends of chloroform formed from humic acid and SOM with those found for the model compounds suggest that opposed AKIEs associated with the chlorination of phenolic and ketone moieties of NOM partly compensate each other during chlorination of NOM indicating that different types of functional groups contribute to chloroform formation. Moreover, this study suggests that under acidic condition, as encountered in forest soil, the isotopic composition of chloroform formed by chlorination of NOM is partly controlled by (*i*) the chemical composition of NOM, (*ii*) the hydrolysis of trichloroacetyl-containing intermediates and (*iii*) the tautomerization of ketone groups.

▪Introduction

Chloroform is an anthropogenic contaminant widely used in the chemical industry as a solvent or as an extracting agent. Moreover chloroform is also a disinfection by-product resulting of the reaction of chlorine with natural organic matter (NOM) present in water (Deborde and von Gunten, 2008). The frequent detection of chloroform in groundwater in absence of other industrial pollutants suggests that chloroform may be also produced naturally by biogeochemical processes (Laturnus et al., 2002; Laturnus et al., 2000). Recently Hunkeler et al. have demonstrated that chloroform present in groundwater beneath some forested areas in Denmark is formed naturally from NOM using carbon stable isotopes analysis (Hunkeler et al., 2012). This study has shown that natural chloroform has δ^{13}C values in the same range as NOM whereas industrial chloroform is characterized by an isotopic composition similar to methane (Hunkeler et al., 2012). The current hypothesis is that natural chloroform formed in soils is produced by enzyme-catalyzed chlorination of NOM (Asplund et al., 1993; Ortiz-Bermudez et al., 2007). It has been demonstrated that in presence of chloride and H_2O_2, enzymes such as chloroperoxidase from *Caldariomyces fumago* produces hypochlorous acid (HOCl) which is a strong chlorination agent (Griffin, 1983; Murali Manoj, 2006). As the chemical structure of NOM is complex and constituted of various functional groups which could potentially be involved in the formation of chloroform, it is impossible to propose a unique formation mechanism. So far most studies have focused on the chlorination of simple molecular structures such as mono and poly-hydroxybenzenes, ketones, aliphatic β-dicarbonyl acids and some glycosides (Arnold et al., 2008; Boyce and Hornig, 1983; Dickenson et al., 2008; Rook, 1977). Some studies have suggested that trichloroacetyl-containing compounds produced during chlorination of model compounds and humic subtances might release chloroform by hydrolysis of the trichloroacetyl group (Boyce and Hornig, 1983; Dickenson et al., 2008, [**II**]). Recently Albers et al. have detected trichloroacetyl-containing compounds in some forest soils with natural chloroform (Albers et al., 2010; [**II**]). Such trichloroacetyl-containing compounds could be formed by successive chlorination of aliphatic groups or by chlorination followed by hydrolytic cleavage of the aromatic rings.

Compounds-specific isotope analysis (CSIA) is a powerful tool to determine the origin of contaminants and gain insight into the mechanisms leading to the formation and the degradation of various organic contaminants (Aelion M.C., 2010; Arnold et al., 2008; Bergamaschi et al., 2001; Bergamaschi et al., 1999; Hunkeler et al., 2012). The changes of carbon isotopic composition of an organic molecule during its formation can be attributed to a kinetic isotope effect (KIE) at the reacting bond(s). The KIE is defined as the ratio between the (pseudo) first-order rate constants for light (${}^{L}k$) and heavy (${}^{H}k$) substituted molecules.

$$KIE = \frac{{}^{L}k}{{}^{H}k} \qquad (1)$$

KIEs arise when at the reactive bond(s) a light isotope is substituted by a heavy isotope. A faster reaction of molecules with light isotopes in the reactive position (KIE>1) is denoted as *normal isotope effect*. In this case, the reactant becomes enriched in heavy isotope over time. An *inverse isotope effect* (KIE<1) can be observed in the rare cases where the activation energy is lower for the heavy isotope substituted molecules. Such inverse isotope effects may be observed in cases in which the reacting bond(s) are strengthened or formed in the rate limiting step(s) (Elsner, 2010). The analysis of isotope enrichment or depletion in reactant(s) or the product(s) can provide information about the reaction mechanism. In multistep processes the apparent kinetic isotope effect (AKIE) reflects the rate limiting step of the overall reaction (Elsner, 2010). Recently Arnold et al. have used carbon stable isotopes analysis to evaluate the role of selected NOM functional groups in the chloroform formation process (Arnold et al., 2008). In this study, Arnold et al. have monitored the $\delta^{13}C$ value of chloroform produced by chlorination of different models compounds and NOM samples, and used KIE values to identify specific mechanisms of chloroform formation. The qualitative comparison of the AKIE of chloroform produced by chlorination of NOM with those of NOM models compounds suggested that the phenolic moieties may be essentially responsible for chloroform formation (Arnold et al., 2008). However, the significant difference observed between the AKIE measured for the formation of chloroform from phenol (AKIE=0.980±0.004; Arnold et al., 2008) and NOM (AKIE=0.9971±0.0004; Arnold et al., 2008) may be an indication for the simultaneous implication of other slowly reacting chloroform precursor with normal AKIEs such as ketone-containing functional groups. While the study of Arnold et al. demonstrate the potential of isotope analysis to explore formation pathways, the results of the study are not directly applicable to forest soils as the experiments were carried out at pH 8, while the pH of forest soils ranges between 4 and 7. As the rate limiting step might vary as a function of pH, AKIE values might vary as well.

The aims of the present study are (*i*) to better understand the mechanisms controlling the formation and the carbon isotopic composition of chloroform produced a from forest soils organic matter (SOM) and (*ii*) to evaluate the effect of pH on the carbon isotopic composition of chloroform. In order to reach these objectives, chloroform was produced in laboratory by chemical chlorination of two model compounds, humic acid and SOM. As phenolic and ketone functional groups are among the most abundant reactive NOM moieties (Arnold et al., 2008; Sutton and Sposito, 2005)., phenol and 2-propanone were chosen as model compounds. For these experiments, the organic precursors in excess were chlorinated with hypochlorous acid to mimic the enzymatic-catalyzed chlorination. During the chlorination experiments the concentration and the $\delta^{13}C$ values of the chloroform were monitored. The isotopic trends of chloroform formed by chlorination of humic acid and NOM were compared with those measured for the formation of chloroform from model compounds to explore which functional groups in NOM might contribute to chloroform formation.

▪Materials and methods

Chemicals. The following chemicals were used as received: sodium hypochlorite (Sigma-Aldrich, available chlorine ≥4%), phosphoric acid (Fluka, 85%), sodium dihydrogenphosphate monohydrate (Merck, >99%), disodium hydrogenphosphate dodecahydrate (Fluka, >99%), sodium sulfite (Sigma-Aldrich, >98%), phenol (Sigma Aldrich, >99%), 2-propanone (Acros Organics, >99.97%). Ultrapurified water (18.2 MΩ cm-1 at 25°C, Direct-Q UV-3 Millipore) was used to prepare the solutions. Chloroform from Fluka (99.5%) was used to prepare concentration and isotope standards. SOM collected in H and F horizons of a forest soil from Denmark (spruce forest) was used for chlorination experiments. Humic acid also used for chlorination experiments were obtained by alkaline extraction of SOM with aqueous NaOH, followed by precipitation of humic acid at low pH and a desalting steps involving dialysis (Albers et al., 2008). Information regarding the chemical properties of humic substances and NOM are available in Appendix 3.

Chlorination experiments. All chlorination experiments were conducted at pH 4, 7 and 8 with an excess of organic precursor relative to hypochlorous acid to mimic the condition encountered in soils. The concentrations of 2-propanone and phenol were 4 mM and the concentration of humic acid was 100 mg L^{-1} (~4 mM C) and 500 mg L^{-1} (~20 mM C) for SOM. The concentration of hypochlorous acid was 500 μM. The solutions were prepared with pH 7 and 8 100 mM phosphate buffer solutions and the solutions at pH 4 were prepared with diluted phosphoric acid. The pH was measured after the reaction to verify its stability. The solutions containing the organic precursor were transferred in 40 ml vial and the hypochlorous acid was added. The vials were rapidly sealed with PTFE-lined screw caps. This method avoids the volatilization of chloroform during the reaction because of the lack of headspace. During the reaction time, the vials were agitated at room temperature (25°C). For each desired reaction time, the solution of one vial was quenched with 100 μL of an aqueous solution of sodium sulfite (Na_2SO_3) of 100 g L^{-1} and analyzed for chloroform concentration and isotope composition. Blanks of MilliQ water were also prepared under the same conditions. The samples were stored in dark at 4°C until analysis by GC-C-IRMS or GC-qMS. All experiments were done in triplicate.

GC/qMS. Chloroform was analyzed using gas chromatography Trace GC Ultra (Thermo Scientific) with quadrupole mass spectrometer DSQII (Thermo Scientific). After incubating at 60°C for 2 min, headspace samples (500 μL) were injected in split mode (1:10) by a Combi-PAL autosampler (CTC Analytics) onto a 60 m × 0.32 mm × 1.8 μm film thickness Zebron ZB 625 column (Phenomenex). The injector temperature was 250°C and the ion source temperature was 200°C. The oven temperature program was 150°C for 5 min. The analyses were carried out in single ion monitoring mode using the following *m/z*: 48, 50, 83, 85, 118 and 120.

GC/C/IRMS. The carbon isotopic ratios of chloroform were measured using gas chromatography Trace GC Ultra (Thermo Scientific) with GC combustion interface and an isotope ratio mass spectrometer (IRMS) Delta V Plus (Thermo Scientific). The GC/C/IRMS was equipped with a cryogenic focuser ATAS Optic 3 (GL Sciences) and coupled to a purge-and-trap concentrator Velocity XPT (Tekmar Dohrmann) and a liquid auto-sampler AquaTek70 (Tekmar Dohrmann). Aqueous samples (~25 mL) were purged with an N_2 flow of 40 ml min^{-1} and volatiles were trapped on a VOCARB 3000 trap (Supelco). After the extraction step, the trap was heated to 250°C for 3 min. Chloroform was thermally desorbed and transferred to the GC at 1.7 mL min^{-1} and trapped in the cryogenic focuser held at -100°C during 3 min with liquid N_2. The chromatographic separation was carried out with a 60 m × 0.25 mm × 1.4 µm film thickness DB-VRX column (Agilent). The GC oven temperature program used was as follow: 6 min at 40°C, 10°C/min to 175°C, hold for 1 min. Oxidation and reduction reactors of the combustion interface were maintained at 940 and 640°C, respectively. In order to reduce the uncertainty of $\delta^{13}C$, the aqueous samples were diluted to obtain constant peak amplitudes. All the $\delta^{13}C$ values were reported in ‰ relative to Vienna PeeDee Belemnite (V-PDB) defined as:

$$\delta^{13}C_{CF} = \left(\frac{R_{CF}}{R_{V-PDB}} - 1 \right) \times 1000 \tag{2}$$

Where R_{CF} and $R_{\mathrm{V\text{-}PDB}}$ are the carbon isotope ratios of chloroform and Vienna PeeDee Belemnite standard, respectively.

Data analysis. During chlorination of organic precursor, chloroform is produced via a multistep reaction. As in this study only chloroform was measured, $\delta^{13}C_{t=\infty}$ and AKIE values were determined using a simplified kinetic scheme previously described by Arnold et al. (Arnold et al., 2008). Briefly, the concentrations of chloroform as a function of time were fitted with a pseudo-first order kinetic equation using a nonlinear least-squares method implemented in *R* version 2.15.1. The initial concentration of precursor is selected to match the finale concentration of chloroform. Then, isotope ratios of chloroform versus time ($R_{\mathrm{CHCl_3},t}$) were fitted with the following equation:

$$R_{CHCl_3,t} = \frac{\left(1 + R_{t=0}\right)\left(1 - e^{-\frac{k}{1+AKIE}t}\right)}{\left(1 + \frac{1}{R_{t=0}}\right)\left(1 - e^{-\frac{k}{1+\frac{1}{AKIE}}t}\right)} \tag{3}$$

Where $R_{t=0}$ is $[(\delta^{13}C_{CHCl_3,t=\infty}/1000)+1]\times R_{\text{V-PDB}}$. AKIE is the fitting parameters and k is fixed at the value previously determined for the fit of the chloroform concentration versus time.

Modeling chloroform formation from 2-propanone. A numerical model of the chloroform formation from 2-propanone was developed to explore how intermediates compounds are formed and consumed on the way to chloroform and to determine the rate limiting step of the reaction in support of the interpretation of the isotope effect. Calculations were carried out for pH 8 as the rate constants available in the literature were obtained for this pH. The present model was developed on the basis of the kinetics and reaction rate constants determined by Guthrie and Cossar (Guthrie and Cossar, 1986). The model is based on the following set of ordinary differential equations (Figure 1):

$$\frac{dC_1}{dt} = -k_{12}C_1 + k_{21}C \qquad (4)$$

$$\frac{dC_2}{dt} = k_{12}C_1 - k_{21}C_2 - k_{23}C_2 \qquad (5)$$

$$\frac{dC_3}{dt} = k_{23}C_2 - k_{34}C_3 + k_{43}C_4 - k_{3P}C_3 \qquad (6)$$

$$\frac{dC_4}{dt} = k_{34}C_4 - k_{43}C_4 - k_{45}C_4 \qquad (7)$$

$$\frac{dC_5}{dt} = k_{45}C_4 - k_{56}C_5 - k_{5P}C_5 \qquad (8)$$

$$\frac{dC_6}{dt} = k_{56}C_5 - k_{67}C_6 \qquad (9)$$

$$\frac{dC_7}{dt} = k_{67}C_6 \qquad (10)$$

$$\frac{dP_1}{dt} = k_{3P}C_3 \qquad (11)$$

$$\frac{dP_2}{dt} = k_{5P}C_5 \qquad (12)$$

Where C_1, C_2, C_3, C_4, C_5, C_6 and C_7 are the concentration of 2-propanone, 2-propenolate, 1-chloro-2-propanone, 1-chloro-2-propenolate, 1,1-dichloro-2-propanone, 1,1,1-trichloro-2-propanone and chloroform, respectively. P_1 and P_2 are the concentration of 1-hydroxy-2-propanone and 1,1-dihydroxy-2-propanone which are by-products formed during the chlorination of 2-propanone (Figure 1). The kinetic constants were defined as follow according to Guthrie and Cossar (Guthrie and Cossar, 1986):

$$k_{12} = k_{12}^{-}\left[OH^{-}\right] \tag{13}$$

$$k_{23} = k_{23}^{0}\left[HOCl\right] + k_{23}^{-}\left[OCl^{-}\right] \tag{14}$$

$$k_{34} = 1.36\times10^{2}\left[OH^{-}\right] \tag{15}$$

$$k_{45} = k_{45}^{0}\left[HOCl\right] + k_{45}^{-}\left[OCl^{-}\right] \tag{16}$$

$$k_{56} = k_{56}^{0}\left[HOCl\right] + k_{56}^{-}\left[OCl^{-}\right]\left[OH^{-}\right] \tag{17}$$

$$k_{67} = k_{67}^{-}\left[OCl^{-}\right] \tag{18}$$

$$k_{3P} = k_{3P}^{-}\left[OH^{-}\right] \tag{19}$$

$$k_{5P} = k_{5P}^{-}\left[OH^{-}\right] \tag{20}$$

Where k^{-}_{12}=1.73×10^{-1} M^{-1} s^{-1}, k_{21}=4.1×10^{4} s^{-1}, k_{23}^{0}=1.7×10^{9} M^{-1} s^{-1}, k_{23}^{-}=2.0×10^{4} M^{-1} s^{-1}, k_{34}=1.36×10^{2} M^{-1} s^{-1}, k_{43}=7.8×10^{3} s^{-1}, k_{45}^{0}=1.7×10^{9} M^{-1} s^{-1}, k_{45}^{-}=2.0×10^{4} M^{-1} s^{-1}, k_{56}^{0}=1.7×10^{9} M^{-1} s^{-1}, k_{56}^{-}=1.3×10^{4} M^{-2} s^{-1}, k_{67}^{-}=48.2 M^{-} s^{-1}, k_{3P}^{-}=1.19 M^{-1} s^{-1} and , k_{5P}^{-}=4.27 M^{-1} s^{-1} (Guthrie and Cossar, 1986). Finally the concentration of hypochlorous acid and hypochlorite were calculated as follow:

$$\left[HOCl\right] = \frac{10^{-pH}\left[FAC\right]}{10^{-pH} + 10^{-pK_a}} \tag{21}$$

and

$$\left[OCl^{-}\right] = \frac{10^{-pK_a}\left[FAC\right]}{10^{-pH} + 10^{-pK_a}} \tag{22}$$

Where [*FAC*] is the free available chlorine concentration (total chlorine concentration) and where the *pKa* corresponds to7.48 (Sivey et al., 2010). The set of ordinary differential equations and the kinetic constants were implemented in a time-dependent solver (COMSOL Multiphysics version 4.2a). The concentration of chloroform and all intermediates were computed at pH 8 as function of time considering an initial concentration of 2-propanone (C_1) of 4 mM and [*FAC*]=0.5 mM.

▪ Results and Discussion

Chloroform formation rate constants and yields. The chloroform concentration trends for 2-propanone, phenol, humic acid and SOM determined for pH 4, 7 and 8 are shown in the Figure 2 and the chloroform formation rate constants and yields are given in Table 1. The reaction rate constants and chloroform yields of all precursors increase with increasing pH. The reaction rates constants of phenol, humic acid and SOM are within the same range whereas the kinetics of chloroform formation from 2-propanone is approximately one order of magnitude slower (Table 1). These formation rate constants are comparable to those measured in previous studies (Arnold et al., 2008; Boyce and Hornig, 1983; Deborde and von Gunten, 2008; Gallard and Von Gunten, 2002). However the chloroform yields are much lower than those previously reported in the literature because the reactions were carried out with an excess of organic matter.

Chlorination of model compounds. The $\delta^{13}C$ values of chloroform produced by chlorination of 2-propanone and phenol at pH 4, 7 and 8 are shown in the Figure 3. The $\delta^{13}C$ values of chloroform at *t*=∞,the AKIE values and the $\delta^{13}C$ values of all precursors are given in the Table 1.

Chloroform production from phenol displays a normal isotope effect at pH 4 and 7, whereas at pH 8 the process gives rise to an inverse isotope effect (Figure 1b and Table 1). For chloroform production from 2-propanone, an opposite pH-dependence is observed with a normal isotope effect at higher pH (7 and 8) and an inverse isotope effect at lower pH (4) (Figure 1a and Table 1). The AKIE values at pH7 and 8 are close to the AKIEs values measured by Arnold et al. for other ketones such as acetylacetone and acetophenone (Arnold et al., 2008). These results indicate that for both chloroform precursors the reaction mechanism and/or the rate limiting step in the sequence of reactions leading to chloroform changes as a function of pH.

Table 4. Chloroform formation upon chlorination of 2-propanone, phenol, humic acid and NOM from forest soil with 500 μM of sodium hypochlorite.

	pH	*k*	$CHCl_3$ yield [a]	$[CHCl_3]_{t=\infty}$	$\delta^{13}C$ precursor	$\delta^{13}C_{t=\infty}$ $CHCl_3$	$AKIE_{CHCl_3}$
	(-)	(s^{-1})	(-)	($\mu g\ L^{-1}$)	(‰ V-PDB)	(‰ V-PDB)	(-)
2-propanone	4	3.0×10^{-6}	6.3×10^{-6}	3.1	-25.1±0.1	-44.1±0.3[b]	0.9935±0.0007[b]
	7	4.1×10^{-5}	1.0×10^{-4}	50.3	-25.1±0.1	-45.7±0.2	1.0129±0.0020
	8	7.2×10^{-5}	3.0×10^{-4}	143.2	-25.1±0.1	-43.3±0.2	1.0189±0.0016
phenol	4	1.3×10^{-5}	1.1×10^{-5}	5.3	-28.7±0.1	-39.7±0.3	1.0156±0.0012
	7	1.8×10^{-4}	1.8×10^{-5}	7.0	-28.7±0.1	-33.5±0.2	1.0034±0.0005
	8	2.1×10^{-4}	2.2×10^{-5}	10.2	-28.7±0.1	-32.7±0.2	0.9900±0.0007
humic acid	4	1.6×10^{-5}	1.5×10^{-4}	79.1	-27.5±0.1	-35.4±0.2	-
	7	1.1×10^{-4}	1.7×10^{-4}	82.9	-27.5±0.1	-30.9±0.3	-
	8	2.0×10^{-4}	1.8×10^{-4}	90.2	-27.5±0.1	-27.4±0.3	-
SOM	4	2.4×10^{-5}	5.9×10^{-6}	14.7	-27.5±0.1	-39.5±0.2	-
	7	9.8×10^{-5}	7.5×10^{-6}	18.5	-27.5±0.1	-34.5±0.2	-
	8	1.2×10^{-4}	8.3×10^{-6}	20.7	-27.5±0.1	-30.3±0.2	-

[a] Yields are expressed as per mole of carbon in the precursor.

[b] Errors for the fitted $\delta^{13}C$ and AKIE values are 95% confidence interval as determined by *R*.

Chlorination of humic acid and SOM. The isotopic trends of chloroform produced by chlorination of humic acid and SOM at pH 4, 7 and 8 are shown in the Figure 7 and $\delta^{13}C$ values of chloroform at t=∞ are given in the Table 1. While model compounds show a steady isotope evolution towards a more enriched or depleted isotope composition, this is no longer the case for humic acid and SOM where the direction of the isotope evolution can change over time. For humic acids, the isotopic composition of chloroform formed at pH 4 and 7 tends to become more enriched in ^{13}C with the progress of the reaction. However, at pH 8 the $\delta^{13}C$ value of chloroform decreases during the first 200 min and then increase toward more enriched values (Figure 7a). For SOM, the chloroform formed at pH 4 tends to be progressively enriched in ^{13}C as function of time. Similarly as for humic acid, the $\delta^{13}C$ values of chloroform formed at pH 7 and 8 by chlorination of SOM decrease during the first 300 min of reaction and then increase toward more enriched values (Figure 7b). These results suggest that at an early stage of the reaction chloroform is likely formed by functional groups inducing inverse AKIE whereas after 200 to 300 min chloroform is formed by chlorination of functional groups inducing normal AKIE. As the AKIE associated with the formation chloroform may vary as function of time, the isotopic trend of chloroform formed by chlorination of humic acid and SOM cannot be described by a unique AKIE value. During chlorination, different functional groups present in the precursors can be involved in the formation of chloroform (Figure 8). Therefore, the isotopic trend of chloroform formed upon chlorination of humic acid and SOM reflect likely the combined AKIEs of the different functional groups involved in the reaction. In the following section the variation of AKIE as function of pH will be discussed

Mechanistic Insights

Model compounds. Variations of the AKIE values as function of pH have been observed for chloroform formed by chlorination of phenol and 2-propanone. In this section, the AKIE values associated with chloroform formation from model compounds will be discussed. The case of 2-propanone will be examined in detail as the reaction mechanism is known in detail. The formation mechanism of chloroform by chlorination of 2-propanone involves three steps: (*i*) the deprotonation at the α carbon, (*ii*) the chlorination of the α carbon and (*iii*) the elimination by neutral or alkaline hydrolysis of the trichlormethyl group (i.e. chloroform) formed upon chlorination (Figure 2) (Guthrie and Cossar, 1986). The AKIE values measured at pH 7 and 8 (1.0129 and 1.0189) are in the same range as the AKIE measured by Arnold et al. for the alkaline hydrolysis of 1,1,1-trichloro-2-propanone (1.014) and the AKIE measured for the hydrolysis of trichloroacetyl-containing compounds (AKIE from 1.014 to 1.019) (Arnold et al., 2008; [**II**]). The good agreement suggests that the hydrolysis of the trichlormethyl-group is the rate limiting step for the chloroform formation at pH 7 and 8 although one might rather expect that hydrolysis is rate limiting a low pH as the reaction is favored by the presence of OH^-. However, the modeling of the reaction kinetics supports the hypothesis of hydrolysis as the rate limiting step. As shown in Figure 5, 1,1,1-trichloro-2-propanone accumulates and only slowly releases chloroform. This can be explained by the fact that the keto-enol tautomerization of 2-propanone, 1-chloro-2-propanone and 1,1-dichloro-2-propanone is favored at pH 7 and 8 since the α carbon in more readily deprotonated (Figure 4a and 4b) (Bartlett, 1934; Guthrie and Cossar, 1986; Hegarty et al., 1998). Moreover, the electron withdrawing character of chlorine atom(s) exalts the acidity of hydrogen atom(s) remaining on the α carbon speeding up the formation of the enolate tautomers and therefore the chlorination of 1-chloro-2-propanone and 1,1-dichloro-2-propanone. Thus 1,1-dichloro-2-propanone is transiently formed and rapidly chlorinated to form 1,1,1-trichloro-2-propanone which then slowly releases chloroform be hydrolysis.

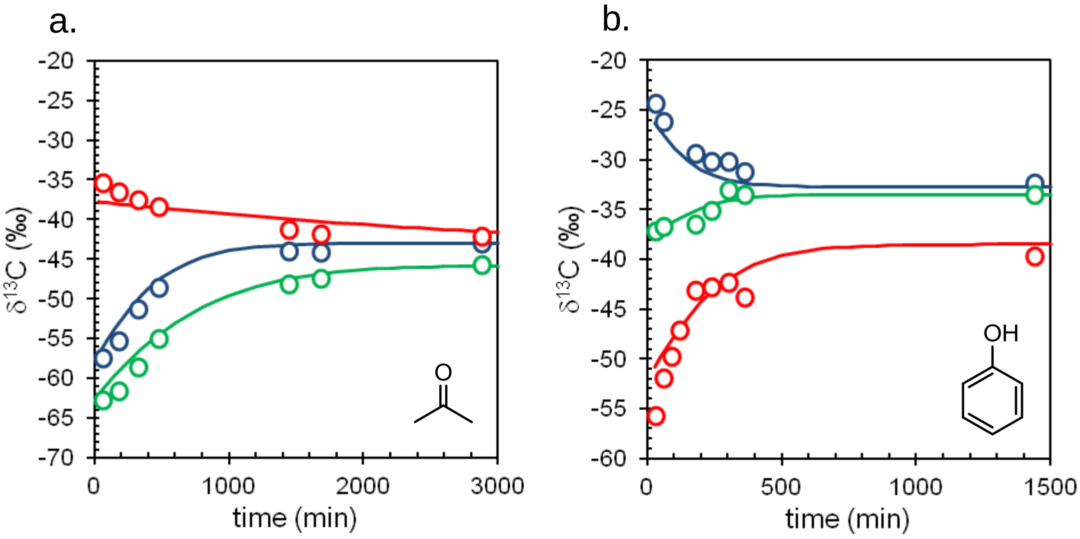

Figure 1. The δ^{13}C values of chloroform produced upon chlorination of (a) 2-propanone and (b) phenol at pH 4 (red circles), 7 (green circles) and 8 (blue circles). The blue, green and red lines are model fits calculated with the method developed by Arnold et al. (Arnold et al., 2008).

The formation of chloroform by chlorination of 2-propanone at pH 4 gives rise to an inverse AKIE (Figure 3a). This change in AKIE indicates that the rate limit step changes at pH 4, i.e. hydrolysis is no longer rate limiting, and that rate limiting step at pH 4 is associated with an inverse isotope effect. The observed inverse isotope effect suggests that the bond(s) to the carbon atom leading to chloroform become strengthened in the transition state compared to the ground state (Aelion M.C., 2010; Kohen and Limbach, 2006). Under acidic condition the electron-withdrawing inductive effect of chlorine atom(s) bonded to α carbon makes the protonation of the carbonyl-oxygen more difficult and therefore the tautomerization of 1-chloro-2-propanone and 1,1-dichloro-2-propanone is slowed down by the presence of chlorine atom(s) in the molecules (Figure 4c) (Smith and March, 2007). During tautomerization of 2-propanone, 1-chloro-2-propanone and 1,1-dichloro-2-propanone, the sp^3 α carbon atom (C−C) leading to chloroform is re-hybridized to *sp*^{2} (C=C) thereby inducing the strengthening of the carbon-carbon bond (Figure 4c). Although the hydrolysis of 1,1,1-trichloro-2-propanone is relatively slow under acidic condition, the inverse AKIE measured at pH 4 suggests that the tautomerization of 1-chloro-2-propanone and 1,1-dichloro-2-propanone is rate-limiting at pH 4 (Figure 4c). This conclusion is consistent with kinetic studies carried out previously by Dubois et al. and Guthrie and Cossar (Dubois et al., 1981; Guthrie and Cossar, 1986).

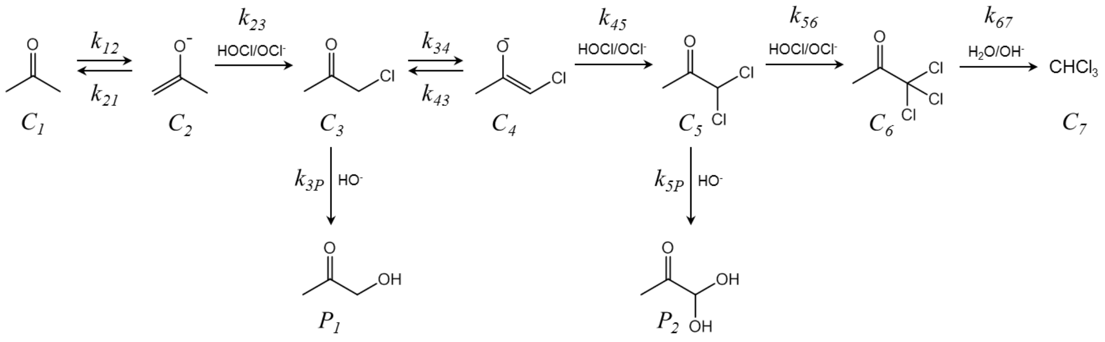

Figure 2. Reaction mechanism of chloroform formation by chlorination 2-propanone.

The chloroform formation from phenol gives rise to normal AKIEs at pH 4 and 7 and to an inverse AKIE at pH 8 (Table 1; Figure 3b). Similarly to 2-propanone, the normal AKIE observed at pH 4 (1.016) is close to a previously reported AKIE values for chloroform formation by hydrolysis of 2-propanone trichloroacetyl-containing compounds (AKIE from 1.014 to 1.019) (Arnold et al., 2008;[**II**]). Several studies have demonstrated that the formation of chloroform by hydrolysis of trichloroacetly-containing intermediates is favored under alkaline conditions. (Arnold et al., 2008; Boyce and Hornig, 1983; Breider et al., 2012). Thus, the isotope fractionation observed at pH 4 is consistent with the hydrolysis of a trichloroacetly-containing intermediate formed after the cleavage of the aromatic ring. At pH 7 the AKIE is much smaller likely because the normal isotope effect associated with the addition/elimination step is partly offset by an inverse isotope effect which is more clearly observed at pH 8. The inverse AKIE measured at pH 8 is in the same range as the value measured by Arnold et al. (Arnold et al., 2008). Similarly to 2-propanone, the inverse isotope effect observed at pH 8 is likely related to the re-hybridization of carbon atoms involved in chloroform formation.

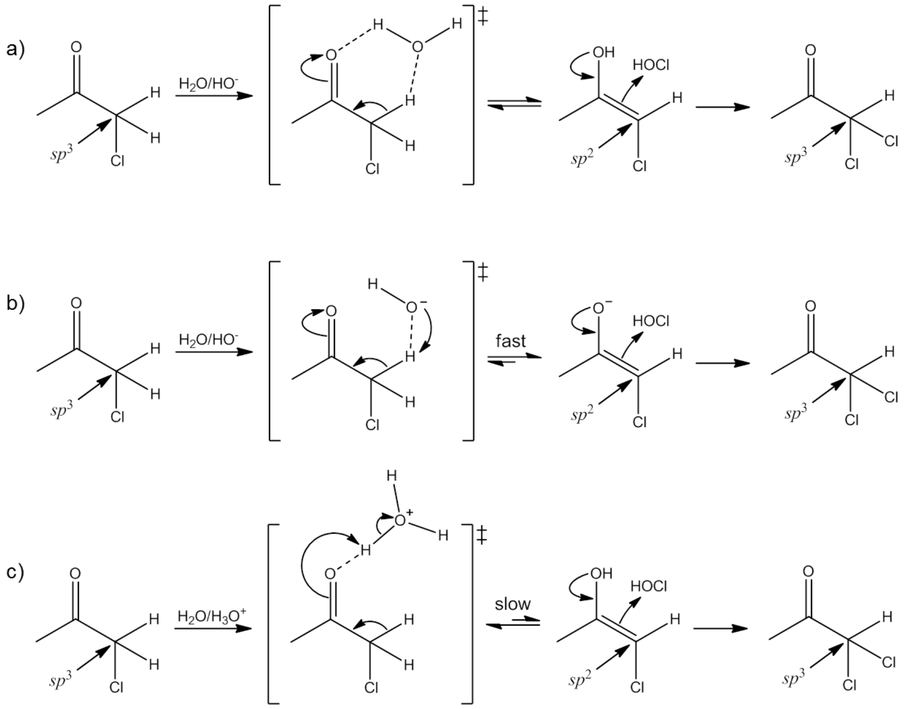

Figure 3. Carbon re-hybridization from sp^3 to sp^2 in during tautomerization of 1-chloro-2-propanone under (a) neutral (b) alkaline and (c) acidic conditions. The double dagger (‡)symbol represents the transition state.

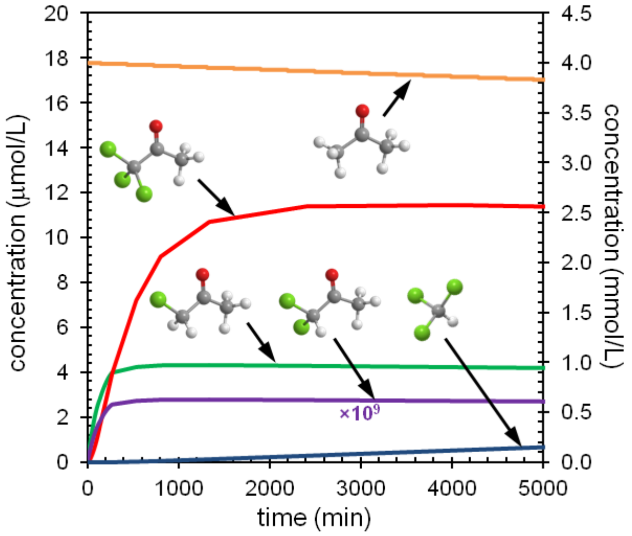

Figure 4. Model of the concentration of 1-chloro-2-propanone (green), 1,1-dichloro-2-propanone (purple), and 1,1,1-trichloro-2-propanone (red) in µmol L^{-1}, and chloroform (blue) and 2-propanone in mmol L^{-1} (yellow) at pH 8 as function of time [min]. In order to compare the concentration of 1,1-dichloro-2-propanone (purple) with those of the other compounds the concentration was multiplied by a factor of 1×10^9.

During the chlorination of the aromatic ring, the carbons atoms in ortho and para-position are transiently re-hybridized from sp^2 to sp^3 and then the aromatic ring form an arenium ion so called σ-complex (Figure 6). Although one carbon of the ring no longer has a conjugated *p* orbital, the σ-complex is relatively stable due to electron delocalization (resonance). The loss of the hydrogen atom bonded to chlorinated carbon will induce a second re-hybridization of the carbon atoms from *sp*^{3} and *sp*^{2}. This latter step induces a strengthening of the carbon-carbon bond which might explain the inverse AKIE observed at pH 8.

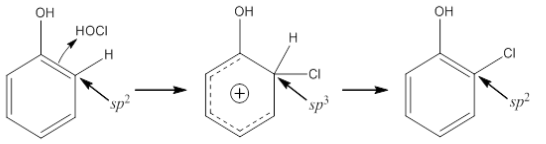

Figure 5. Carbon re-hybridization during chlorination of phenol.

The δ^{13}C values of chloroform produced from 2-propranone and phenol at steady-state are slightly depleted in ^{13}C compared to the initial isotopic composition of both precursors (Table 1). This deviation of the isotopic composition of chloroform might be induced by (*i*) an incomplete hydrolysis of the trichloroacetyl intermediates into chloroform or (*ii*) by an isotope sensitive branching of competing reaction pathways ([**II**]; Melander and Saunders, 1987). Indeed, during chlorination various chlorinated compounds are produced in parallel to chloroform. In the case of 2-propanone and phenol, by-products such as 1-hydroxy-2-propanone, 1,1-dihydroxy-2-propanone, chlorinated ketone and chlorinated aliphatic carboxylic acids are likely formed in parallel to chloroform (Boyce and Hornig, 1983; Deleer et al., 1985; Guthrie and Cossar, 1986). These parallel reactions will determine the concentration of the precursor available for chloroform formation and the isotopic composition of the precursors from which chloroform will be produced. Therefore the resulting isotope composition of chloroform can be more enriched or depleted in ^{13}C than the initial isotopic composition of precursors. However, from the present data it impossible to determine the isotope fractionation associated with such branching reactions.

Humic acid and SOM. The comparison of δ^{13}C values of chloroform at t=∞ and AKIE values of model compounds with the isotopic trends of chloroform formed by chlorination of humic acid and SOM provides additional insight into the formation mechanisms of chloroform from NOM. The chlorination of humic acid and SOM give rise to small variations in isotope ratios that change in direction with time (Table 6). These rather small AKIE might be explained by the superposition of opposed AKIEs induced by different functional groups present in humic acid and SOM. The Figure 7 shows that in some cases the AKIE for chloroform formation can be inverse at the beginning of the reaction and normal when the reaction approaches completion. Such inversions of the isotope effect can arise when an inverse AKIE associated with chloroform formation from a fast-reacting group is partly compensated by a normal AKIE related to chloroform formation from slow-reacting functional groups. These results suggest that at an early stage of the reaction chloroform is likely formed by chlorination of fast-reacting functional groups such as phenolic group and then by slow-reacting functional groups such as ketones. Nevertheless it cannot be excluded that that other fast-reacting functional group(s) giving rise to normal isotope effect(s), like acetylacetone or resorcinol, partly offset the AKIE induced by phenolic moieties at the beginning of the reaction (Arnold et al., 2008). These results suggest that the relative proportion of the functional group in NOM (e.g. phenolic vs. ketone groups) might strongly influence the carbon isotopic trends of chloroform. In addition, chloroform is more depleted in ^{13}C at steady-state compared to the original isotope signature of the precursors. These deviations of the δ^{13}C values of chloroform might be explained by the combined effect of (*i*) an isotope fractionation associated with branching reaction(s) on the way to the trichloroacetyl intermediates, (*ii*) an isotope fractionation associated with a partial formation of trichloroacetly-containing compounds or (*iii*) an isotope fractionation related to an incomplete hydrolysis of trichloroacetyl-containing compounds into chloroform ([**II**]).

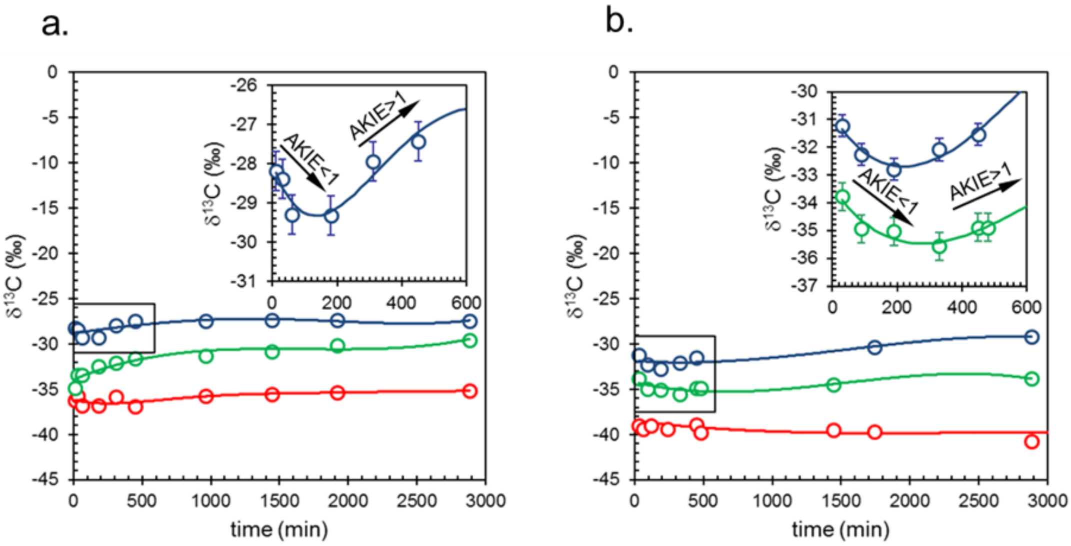

Figure 6. Carbon isotopic trends of chloroform formed at pH 4 (red circles), 7 (green circles) and 8 (blue circles) by chlorination of (a) humic acid and (b) SOM.

Conclusion. The main goals of this study were to better understand the mechanisms controlling the formation and the carbon isotopic composition of chloroform produced by chlorination of NOM from forest soils. The concentration and isotope data presented in this study provide additional insights into the mechanisms leading to chloroform and demonstrate that pH has strong influence on the isotopic composition of chloroform. It has been demonstrated that the rate limiting step(s) of the reaction leading to chloroform and therefore the isotopic trends of chloroform may vary as function of pH. Moreover, it has been shown that the formation of chloroform associated with the chlorination of phenol and 2-propanone induces opposed AKIEs. One of the most striking results of this study is the observation that AKIE associated with chloroform formation from 2-propanone changes from normal to inverse and for phenol from inverse to normal as the pH is lowered. Although further studies are required, the observed inverse AKIEs likely reflect the re-hybridization from *sp*^{3} to *sp*^{2} of the carbon atom leading to chloroform. The combination of the opposed AKIEs associated with the formation of chloroform from phenol and 2-propanone might explain the small AKIE values observed for chloroform produced from humic acid and SOM. These results confirm that phenolic and ketone moieties present in NOM are likely involved in the formation of chloroform and suggest that the relative proportion of these functional groups in NOM might influence of the carbon isotope composition of chloroform. Therefore the isotopic composition of natural chloroform formed in forest soils (i.e. pH 4 – 6) might be partly controlled by chemical composition of NOM, the hydrolysis of trichloroacetyl-containing intermediates and the tautomerization of ketone groups.

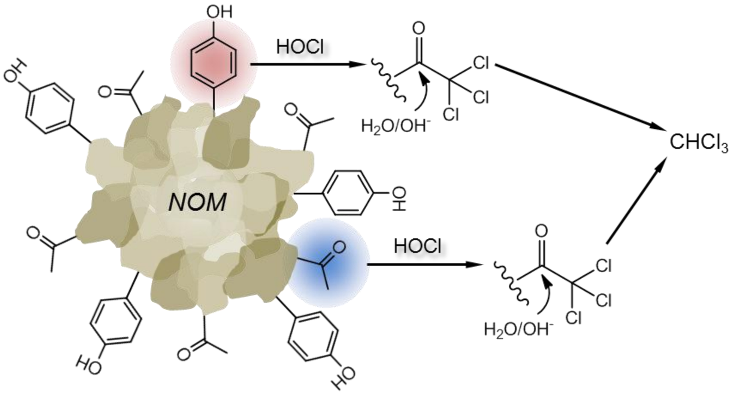

Figure 7. Conceptual model of the formation of chloroform from NOM.

▪Acknowledgments

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▪Supporting information

Additional information regarding the elemental composition, the carbon isotopic composition and the carbon distribution in humic substances and NOM are provided in the Appendix 3.

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Chapter IV

Investigating chloroperoxidase-catalyzed formation of chloroform from humic substances using chlorine stable isotope

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submitted to Environmental Science & Technology

ABSTRACT Chloroperoxidase (CPO) is suspected to play an important role in the biosynthesis of natural chloroform. The aims of the present study are to assess the mechanisms involved in the CPO-catalyzed chlorination of HS and to evaluate the δ^{37}Cl value of naturally produced chloroform.. The isotopes analysis have shown that the chlorination of HS in presence of high H_3O^+ and Cl^- concentrations induces large apparent kinetic isotope effect (AKIE=1.010-01.018) likely associated with the transfer of chlorine between two heavy atoms, whereas in presence of low H_3O^+ and Cl^- concentrations the formation of chloroform induces smaller AKIE. The large AKIE values measured for the formation of chloroform suggest that chlorination of HS by transiently formed HOCl is rate-limiting in presence of high H_3O^+ and Cl^- concentrations. The smaller AKIE values suggest the formation of a HOCl-ferriprotoporphyrin(IX) intermediate become rate-limiting in presence of low H_3O^+ and Cl- concentrations. These results seem to indicate that CPO-catalyzed formation of chloroform occurs via a chemo-enzymatic process in which HOCl is transiently formed and diffused out of the enzyme to react with HS. The concentrations of H_3O^+ and Cl^- in soils are generally relatively low. Therefore the formation of HOCl−ferriprotoporphyrin(IX) intermediate is likely rate-limiting in terrestrial environment. Thus, under environmental condition the δ^{37}Cl value natural chloroform should vary between -5‰ to -8‰ suggesting that isotopic composition of chloroform might be used to discriminate sources in the environment.

▪ Introduction

Chlorinated organic compounds are usually considered as anthropogenic contaminants. Nevertheless, over 4000 halogenated products have been isolated from natural sources of which about 1800 contain chlorine. Recent studies have shown that chloroform is frequently detected in soil and groundwater in absence of other anthropogenic contaminants (Albers et al., 2011). Hunkeler et al. have recently shown that the carbon isotopic signature of chloroform present in some forest soils and groundwater is consistent with the natural formation of chloroform from soil organic matter (SOM) (Hunkeler et al., 2012). It has been shown that chloroform can be formed in pure cultures of *Caldariomyces fumago*, *Mycena metata, Peniophora pseudopini* grown on SOM-glucose mixture and SOM (Hoekstra et al., 1998a). Numerous studies on natural organohalogens have suggested that halogenases and haloperoxidase excreted by some bacteria, fungi and plants could play an important role in biosynthesis of chlorinated organic compounds in various terrestrial environments (Asplund et al., 1993; Gribble, 2003; Hoekstra et al., 1998a). It has been be speculated that biological halogenations is used by organisms as a defense against infections or for the degradation of biomacromolecules such as lignin and humic substances (van Pee and Unversucht, 2003). Iron-containing chloroperoxidase (CPO) is an extracellular heme glycoenzyme containing ferriprotoporphyrin(IX) as the prosthetic group that is produced by the fungi such as *Caldariomyces fumago* (Sundaramoorthy et al., 1995a; Sundaramoorthy et al., 1995b, 1998). Several authors have shown that CPO can catalyze the chlorination of humic and fulvic acids in presence of hydrogen peroxide leading to the formation of chlorinated volatile organic compounds and chlorinated carboxylic acids (Asplund et al., 1993; Haiber et al., 1996; Hoekstra et al., 1998b, [**V**]). CPO, like all heme-dependent haloperoxidases, follows a reaction cycle that involves initial reaction of ferriprotoporphyrin(IX) (Fe(III)-heme(IX) complex) with H_2O_2 to produce Fe(IV)=O species, called compound I (Blasiak and Drennan, 2009). Compound I can oxidize chloride to produce an enzyme-bound ClO-ferriprotophorphyrin(IX) intermediate. The current hypothesis is that CPO expresses a chlorinating activity by the direct reaction of the enzyme-bound ClO with substrates (enzymatic reaction) (Figure 1c) or by the production of free HOCl (chemo-enzymatic reaction) (Figure 1a). Some studies have shown that certain substrates are chlorinated at higher rate than others and much higher reaction rates are obtained with CPO when compared to the chlorination with HOCl. These results suggest that the chlorine atom transfer to substrates likely occurs at the catalytic site (Dunford et al., 1987; Libby et al., 1996; Libby et al., 1982; Murphy, 2003; Wagenknecht and Woggon, 1997). However, Griffin and Neidleman-Geigert have observed free HOCl and Cl_2 in CPO chlorination mixture and no enantiomeric excess for chlorination of difference organic substances which suggest that the chlorination occurs likely with free oxidized chlorine species (Figure 1a) (Griffin, 1983; Griffin and Ashley, 1984; Lee et al., 1983; Neidleman and Geigert, 1987). To date, there is no clear evidence whether free HOCl or Cl_2 are formed (Figure 1a and 1b), or if the chlorination of organic substrates occurs directly at the catalytic site of CPO via the HOCl−ferriprotoporphyrin(IX) intermediate (Figure 1c).

Reddy et *al*. have shown that the product formed by abiotic (HOCl) and enzyme-catalyzed chlorination of 1,3,5-trimethoxybenzene and 3,5-dimethylphenol can be distinguished by their stable chlorine isotope signature (Reddy et al., 2002). The magnitude of the apparent chlorine kinetic isotope effect (Cl-AKIE) measured by Reddy et *al*. is much smaller for the abiotic chlorination compared to the CPO-catalyzed reaction suggesting that the mechanisms of both reactions are different.

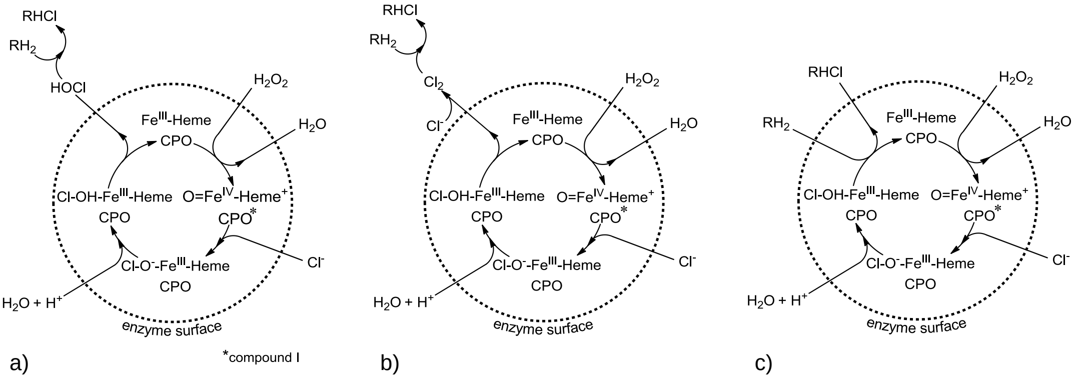

Figure 1 Formation of organochlorine via an enzymatic production of (a) hypochlorite or (b) molecular chlorine (*chemo-enzymatic reactions*), and (c) by direct reaction with the catalytic site of the chloroperoxidase (*enzymatic reaction*).

The aims of the present study are to characterize the chlorine isotope signature of chloroform formed by abiotic and enzymatic chlorination in order to (*i*) gain a better understanding of the mechanisms involved in the CPO-catalyzed chlorination of humic substances, (*ii*) to assess the effect of the pH and chloride concentration on the isotopic composition of chloroform and (*iii*) to constrain the chlorine isotope signature of chloroform formed by chemical and CPO-catalyzed chlorination in view of using isotopic data for distinguishing different sources of chloroform. The δ^{37}Cl values of chloroform formed by abiotic and enzymatic chlorination of humic substances were measured using gas chromatography-quadrupole mass spectrometry (GC-qMS) to assess if both formation pathways can be distinguished using chlorine stable isotopes. The variations of the pH and chloride concentration influence the speciation of chlorine and the rate of formation of the HOCl−ferriprotoporphyrin(IX) intermediate and thus the chlorination rate (Sivey et al., 2010; Thomas et al., 1970). Therefore, the effect of different Cl^- and H_3O^+ concentrations on the isotopic composition of chloroform formed by abiotic and enzymatic chlorination was evaluate. The results of these experiments were used to estimate the expected range of δ^{37}Cl values for chloroform naturally formed in forest soils. Moreover, chlorine isotope analysis of chloroform produced at different Cl^- concentration and pH enable to gain some insight about the potential variability of the chlorine isotope composition of chloroform in different systems. Finally, such chlorine isotope investigation of chloroform sources might contribute to better discriminate natural and industrial chloroform in the terrestrial environment.

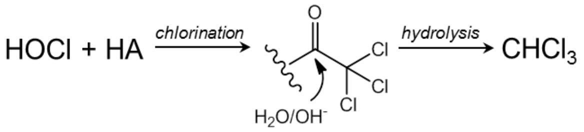

Figure 2 Formation and hydrolysis of trichloroacetyl-containing intermediates.

▪Materials and methods

Chemicals The following chemical were used as received: chloroform (Fluka 99.5%; Alfa Aesar 99%; Acros Organics 99.8%; Fisher Scientific >99%), sodium hypochlorite solution (Sigma-Aldrich, available chlorine ≥4%), phosphoric acid (Fluka, Seelze, Germany, 85%), sodium dihydrogen phosphate monohydrate (Merck, >99%), sodium sulfite (Sigma-Aldrich, >98%), nitric acid (Carlo Erba, Milan, Italy, 65%), Chloroperoxidase from *Caldariomyces fumago* (Sigma-Aldrich, $>1\times10^4$ U/mL), potassium chloride (Fluka, puriss. p.a.), hydrogen peroxide (Fluka, purssi. p.a.). Ultrapure water (18.2 MΩ cm at 25°C, Direct-Q UV-3; Millipore, Billerica, MA, USA) was used to prepare the aqueous solutions. The following humic substances from the International Humic Substance Society (St. Paul, MN, USA) were used for chlorination experiments: *Waskish* peat humic acid (IHSS, 1R107H), *Nordic reservoir* NOM (IHSS, 1R108N), *Pahokee* peat humic acid (IHSS, 1R103H), *Elliot* soil humic acid (IHSS, 1S102H).

Abiotic chlorination 5 mg of humic substances was added to a 42 mL vial containing 40 mL aqueous solution acidified to pH 2.5 or 4 with phosphoric acid. The reactions were carried out at pH 2.5 to assess the chlorine isotope signature of chloroform at the pH optimum of CPO and at pH 4 to reflect conditions in acidic soils. The reaction was initiated by adding gradually 1 mL of an aqueous solution of 0.02 M NaOCl over a 1.5 h interval (666 µl h^{-1}) with a syringe pump NE-400 (New Era Pump System Inc., Farmingdale, NY, USA) in order to mimic the CPO-catalyzed chlorination. The remaining volume was completed with pure water and the vials were sealed with Teflon septum caps and agitated for 24 h at room temperature (~25°C). After 24 h, the samples were quenched with 100 µL of sodium sulfite solutions (100 g L^{-1}) to stop the reaction. Samples were preserved at 4°C in darkness until analysis (within one week). In order to assess the effect of chloride concentration on the δ^{37}Cl values of chloroform formed at pH 2.5 and 4 by chlorination of *Elliot* soil humic acid, the chloride ion content of the reaction mixture was varied from 0.001 to 1 M by adding KCl with a δ^{37}Cl value of 2.08±0.11‰. All experiments were performed in duplicate with an excess of humic substances relative to NaOCl in order to be in the same condition as for the enzymatic chlorination. The isotopic signature of NaOCl used for the chlorination was determined by treating it with an excess of H_2O_2 and the resulting NaCl were converted into methyl-chloride and analyzed using continuous flow isotope ratio mass spectrometry (CF-IRMS) (δ^{37}Cl=-0.15±0.06‰; *n*=4).

CPO-catalyzed chlorination 5 mg of humic acid was added to 42 mL vials filled with 40 mL of an aqueous solution containing 0.375 g L^{-1} of KCl and acidified to pH 2.5 or 4 with phosphoric acid. The reactions were carried out at pH 2.5 to assess the chlorine isotope signature of chloroform at pH optimum of CPO and at pH 4 to reflect conditions in acidic soils. Then 5 μL of an aqueous suspension of CPO of 1×10^4 units mL^{-1} was added and the vials were stirred for 30 min. Subsequently, 1 mL of 0.0129 M H_2O_2 was added gradually over a 1.5 h interval using a syringe pump NE-400 (New Era Pump System Inc., Farmingdale, NY, USA). The solution was stirred for 1 h, and 1 mL of H_2O_2 solution was then added over a second 1.5 h interval. The remaining volume was completed with pure water and the vials were sealed with Teflon septum caps and agitated for 24 h at room temperature (~25°C). Samples were preserved at 4°C in darkness until analysis (within one week). In order to assess the effect of chloride concentration on the $\delta^{37}Cl$ values of chloroform formed by CPO-catalyzed chlorination of *Elliot* soil humic acid at pH 2.5 and 4, the chloride ion content of the reaction mixture was varied from 0.0005 to 0.25 M by adding KCl. All experiments were performed in duplicate with an excess KCl. To determine the chlorine isotopic composition of KCl, the inorganic Cl^- was transformed into methyl-chloride and analyzed using continuous flow isotope ratio mass spectrometry ($\delta^{37}Cl$=2.08±0.11‰; *n*=6).

Concentration and chlorine isotopes analysis For concentration and chlorine isotope analysis, 15 ml of each sample was transferred in 20 ml headspace vials closed with PTFE-lined caps. The concentration and the $\delta^{37}Cl$ values were measured using a GC Agilent 7890A coupled to a quadrupole mass selective detector (GC-qMS) Agilent 5975C (Agilent, Santa Clara, CA). Headspace injections were performed using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland). After incubating at 60°C for 2 min, headspace samples (1000 μL) were injected in split mode (1:20) onto a 30 m × 0.25 mm DB5 column with a 0.25 μm film thickness (Agilent, Santa Clara, CA, USA). The column flow rate (helium) was 1.2 mL min^{-1}. The injector temperature was 250°C and the ion source temperature was 230°C. The analyses were carried out in single ion monitoring (SIM) mode using the two heaviest fragments from isotopologues containing zero and one ^{37}Cl atom ($[^{12}C^{1}H^{35}Cl^{35}Cl]^+$: *m/z* 83; $[^{12}C^{1}H^{35}Cl^{37}Cl]^+$: *m/z* 85) using positive electron impact ionization (EI+ at 70 eV). An optimal scan time of 50 ms with a delay time of 10 ms was determined. Data were recorded using ChemStation software (version E.02.01.1177) and integrated using ChemStation integrator. The average concentrations of chloroform were calculated on the basis of triplicate measurements while the average $\delta^{37}Cl$ values were determined on the basis of ten injections of the same samples.

Calculation and calibration of chlorine isotopic ratios The isotope ratios were calculated from mass spectra using the ion abundance. The electron ionization of chloroform generate charged molecular and fragment ions with three, two or one chlorine atoms. For an ion containing *n* chlorine atoms, the probability P_z of an isotopologue containing *z* ^{37}Cl and (*n* - *z*) ^{35}Cl atoms is:

$$P_z = \frac{n!}{z!(n-z!)} \left(X_{^{37}Cl} \right)^z \left(X_{^{35}Cl} \right)^{n-z} \quad (1)$$

Where $X_{^{37}\mathrm{Cl}}$ and $X_{^{35}\mathrm{Cl}}$ are the relative abundances of ^{37}Cl and ^{35}Cl, respectively. As P_z is proportional to the area A_z under the mass trace of the corresponding isotopologue in the chromatogram, the ratio of areas from two isotopologues containing one and zero ^{37}Cl atom, respectively is (Aeppli et al., 2010):

$$R_{Cl} = \frac{X_{^{37}Cl}}{X_{^{35}Cl}} = \frac{1}{n} \frac{A_1}{A_0} \quad (2)$$

Where R_{Cl} is the chlorine isotopic ratio (^{37}Cl/^{35}Cl). The mass spectrum of chloroform is characterized by two main peaks corresponding to the fragment ions containing the two chlorine atoms. As the abundance of the molecular ion is very low, the chlorine isotopic composition of chloroform was calculated only from the two main peaks corresponding to *m/z* of 85 $[^{12}\mathrm{C}^{1}\mathrm{H}^{35}\mathrm{Cl}^{37}\mathrm{Cl}]^+$ and of 83 $[^{12}\mathrm{C}^{1}\mathrm{H}^{35}\mathrm{Cl}^{35}\mathrm{Cl}]^+$:

$$\delta^{37}Cl = \left(\frac{\frac{1}{2} \frac{A_{m/z85}}{A_{m/z83}}}{R_{std}} - 1 \right) \times 1000 \quad (3)$$

The raw δ^{37}Cl values determined by GC-qMS were converted to the international Standard Mean Ocean Chlorine scale (SMOC) using two chloroform standards. The isotope composition of the chloroform standards was determined by isotope ratio mass spectrometry (IRMS) at the University of Waterloo (Canada) after conversion of chloroform to methyl chloride (Holt et al., 1997). The apparent kinetic isotope effect (Cl-AKIE) of the overall reaction leading to chloroform which can be expressed as the ratio of $^{35}k/^{37}k$ was determined using the following equation (Melander and Saunders, 1987):

$$Cl\text{-}AKIE = \frac{^{35}k}{^{37}k} = \frac{\log(1-f)}{\log\left[1 - \left(f \frac{R_{CHCl_3}}{R_{Cl}} \right)\right]} \quad (4)$$

Where f is the fraction of chlorine incorporated in the organic substrate, R_{CHCl3} is the isotope ratio of chloroform and R_{Cl} is the isotope ratio of KCl in the case of the enzymatic chlorination or NaOCl in the case of the abiotic chlorination (Melander and Saunders, 1987). The fraction f was determined by subtracting the concentration of KCl or NaOCl after reaction of the initial concentration of the chlorine. The initial and remaining concentrations of Cl^- were measured by ion chromatography (Dionex DX-120, Sunnyvale, CA, USA). In the case of the abiotic chlorination, NaOCl was treated with an excess of H_2O_2 and the resulting Cl^- ions were also analyzed by ion chromatography. The $\delta^{37}Cl$ and AKIE values measured for the abiotic chlorination of humic substances were plotted as function of the concentration of chloride. However, as the activity of the CPO is directly proportional to product of Cl^- and H_3O^+ concentrations, the AKIE values measured for the enzymatic chlorination were plotted as function of Cl^- time H_3O^+ concentration (Murali Manoj, 2006; Thomas et al., 1970).

▪Results and Discussion

Abiotic and enzymatic chloroform production at different pH

The concentrations of chloroform produced by abiotic chlorination of humic substances at pH 2.5 range between 0.85 and 2.18 μM, while at pH 4 the concentrations varies between 6.60 and 10.57 μM. The concentrations of chloroform formed by CPO-catalyzed chlorination at pH 2.5 range between 0.51 and 0.75 μM whereas the concentrations range between 1.05 and 1.85 μM at pH 4 (Table 1). For abiotic and CPO-catalyzed chlorination of humic substances more chloroform is produced at pH 4 compared to pH 2.5. Recent studies have shown that chloroform is likely formed from SOM via hydrolysis of trichloroacetyl-containing intermediates (Figure 2) (Albers et al., 2010a, b; Deleer et al., 1985; [**II**]). Although these trichloroacetyl-containing intermediates can be hydrolyzed by water molecules, it has been demonstrated that the release of chloroform by hydrolysis of trichloroacetyl-containing compounds is favored in presence of OH^- anions (Figure 2) (Albers et al., 2010a, b; [**II**]). Thus, at low pH the hydrolysis of trichloroacetyl-containing intermediates is likely the rate-limiting step of the reaction leading to chloroform since the chlorination step is promoted under acidic condition. Therefore, the higher chloroform production at pH 4 might be due to a higher rate of hydrolysis of trichloroacetyl-containing intermediates compared to pH 2.5.

Chlorine isotope composition of abiotic and enzymatic chloroform at different pH The $\delta^{37}Cl$ values of chloroform formed by abiotic and CPO-catalyzed chlorination of humic substances are given in Table 1. The average $\delta^{37}Cl$ values of chloroform formed by enzymatic chlorination is -8.4±0.3‰ at pH 2.5 and -5.7±0.1‰ at pH 4, whereas the average $\delta^{37}Cl$ value of chloroform produced by abiotic chlorination is -9.3±0.5‰ at pH 2.5 and -6.3±0.2‰ at pH 4. The $\delta^{37}Cl$ value of chloroform formed by abiotic chlorination of humic substances is in the same range as chloroform formed by CPO-catalyzed chlorination. For both formation processes, chloroform produced at pH 4 is enriched in ^{37}Cl by about 2.8‰ compared to chloroform formed at pH 2.5. The isotope signatures of chloroform formed by chlorination of humic substances are distinctly different from the $\delta^{37}Cl$ values of industrial chloroform previously published in the literature and analyzed in this study (median $\delta^{37}Cl_{industrial}$= -3.0‰) (Table 2). Industrial chloroform is generally produced by radical chlorination of methane whereas abiotic and enzymatic chlorination of humic substances results of an electrophilic attack of humic substances by HOCl. Thus the differences in the isotopic signature between industrial chloroform and chloroform produced by chlorination of humic substances likely reflect differences in the formation processes.

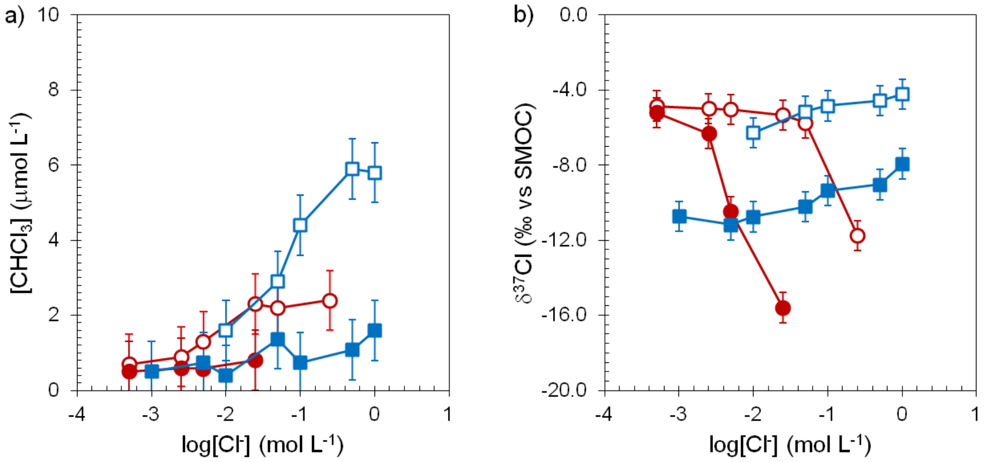

Figure 3 (a) Concentration of chloroform formed by abiotic (squares) and CPO-catalyzed (circles) chlorination of *Elliot* soil at pH 2.5 and 4 as function of the logarithm of the Cl^- concentrations. (b) $\delta^{37}Cl$ values of chloroform formed by abiotic (squares) and CPO-catalyzed (circles) chlorination at pH 2.5 and 4 as function of the logarithm of the Cl^- concentrations. The reactions carried out at pH 2.5 are represented by red circles in the case of the abiotic chlorination and by blue squares for the CPO-catalyzed chlorination. The reaction carried out at pH 4 are represented by white circles and squares for the abiotic and the CPO-catalyzed chlorination, respectively.

Table 1. Concentrations, δ^{37}Cl and Cl-AKIE values of chloroform produced by abiotic and enzyme-catalyzed chlorination of different humic substances at pH 2.5 and 4 ([Cl$^-$]=5×10^{-3}M).

precursor	pH	[CHCl$_3$] (μM) (*n*=3)	δ^{37}Cl$_{CHCl_3}$ (‰ SMOC) (*n*=10)	*f* (*n*=3)	Cl-AKIE
Enzymatic chlorination (CPO)					
Elliot soil humic acid	2.5	0.51±0.01	-8.7±0.2	0.039±0.019	1.0125±0.0001
Elliot soil humic acid	4.0	1.05±0.01	-5.7±0.2	0.026±0.010	1.0073±0.0001
Pahokee peat humic acid	2.5	0.51±0.01	-8.6±0.3	0.041±0.007	1.0123±0.0001
Pahokee peat humic acid	4.0	1.44±0.08	-5.6±0.2	0.019±0.007	1.0069±0.0001
Waskish peat humic acid	2.5	0.52±0.04	-8.1±0.3	0.014±0.008	1.0113±0.0001
Waskish peat humic acid	4.0	1.78±0.10	-5.9±0.2	0.011±0.009	1.0078±0.0001
Nordic reservoir NOM	2.5	0.72±0.01	-8.1±0.3	0.029±0.010	1.0114±0.0001
Nordic reservoir NOM	4.0	1.84±0.08	-5.8±0.1	0.018±0.009	1.0076±0.0001
Abiotic chlorination (NaOCl)					
Elliot soil humic acid	2.5	1.73±0.21	-8.6±1.1	0.002±0.002	1.0100±0.0003
Elliot soil humic acid	4.0	6.60±0.54	-6.4±0.9	0.002±0.002	1.0060±0.0002
Pahokee peat humic acid	2.5	2.18±0.23	-9.4±1.2	0.003±0.002	1.0115±0.0004
Pahokee peat humic acid	4.0	10.57±1.13	-6.1±0.8	0.002±0.002	1.0055±0.0002
Waskish peat humic acid	2.5	0.85±0.19	-9.4±0.9	0.003±0.002	1.0115±0.0002
Waskish peat humic acid	4.0	8.77±0.10	-6.5±0.7	0.002±0.002	1.0061±0.0002
Nordic reservoir NOM	2.5	1.34±0.11	-9.8±1.1	0.002±0.002	1.0122±0.0003
Nordic reservoir NOM	4.0	7.80±0.74	-6.4±0.6	0.002±0.002	1.0060±0.0002

At pH 2.5 the abiotic and CPO-catalyzed chlorination exhibit larger Cl-AKIEs values (1.0101 to 1.0125) compared to the same reaction carried out at pH 4 (1.0055 to 1.0078) (Table 1). Similarly to pH, the variation of the chloride concentration in solution might induce changes of Cl-AKIE since the chloride concentration directly influence the speciation of chlorine and in the case of the enzymatic chlorination the chloride concentration can influence the rate of formation of the HOCl−ferriprotoporphyrin(IX) intermediate and thus the chlorination rate (Murali Manoj, 2006; Sivey et al., 2010; Wagenknecht and Woggon, 1997).. Therefore, to assess the combined effects of pH and chloride concentration on the δ^{37}Cl and Cl-AKIE values of chloroform, chlorination experiments were carried out at pH 2.5 and 4 with different chloride concentrations for Elliott soil. The results of these experiments are presented in the following section.

Table 2. δ^{37}Cl values of different industrial chloroform.

Supplier	$\delta^{37}Cl_{CHCl_3}$ (‰ SMOC)	±1σ	*n*	method	reference
Fluka	-2.90	0.59	70	GC-qMS	this study
Fluka	-3.02	0.17	17	GC-IRMS	this study
Alfa-aesar	-5.30	0.60	70	GC-qMS	this study
Alfa-aesar	-5.41	0.25	8	GC-IRMS	this study
Fisher	-3.46	0.62	10	GC-qMS	this study
Accros	-4.37	0.61	10	GC-qMS	this study
unknown	-1.51	-	1	GC-IRMS	Holt et al., 1997
unknown	-1.53	-	1	GC-IRMS	Holt et al., 1997
unknown	0.32	0.08	2	GC-IRMS	Jendrzejewski et al. 2001

Effect of Cl^- concentration on the ^{37}Cl values For abiotic and CPO-catalyzed chlorination of Elliot soil humic acid, the concentration of chloroform formed increases with the increasing chloride concentration (Figure 3a). This increase of the chloroform concentration is likely due to the formation of Cl_2 which is a strong chlorinating agent. The increase of the concentration of Cl^- drives the equilibrium between HOCl and Cl_2 to the right (reaction 1).

$$HOCl + Cl^- + H^+ \rightleftarrows Cl_2 + H_2O \quad [1]$$

Thus, the relative contribution of Cl_2 to the chlorination increases with the chloride concentration and therefore the elevated Cl^- concentration enhances the chloroform. For the enzymatic reaction, the increase of the chloroform concentration with the increasing chloride concentration might be also due to the activation of the catalytic site of the enzyme in presence of high chloride concentration (Murali Manoj, 2006; Thomas et al., 1970). The chloroform formed by abiotic chlorination tends to be more enriched in ^{37}Cl with the increasing chloride concentration while chloroform produced by CPO-catalyzed chlorination tends to be more depleted in ^{37}Cl (Figure 3b). Moreover, independently of the chloride concentration, the isotopic composition of chloroform produced by both processes is shifted toward more enriched δ^{37}Cl values at pH 4 compared to pH 2.5. For an identical chloride concentration, Cl-AKIE values for abiotic chlorination are about half as large at pH 4 compared to pH 2.5 (Figure 4). Moreover, the Cl-AKIE measured at pH 2.5 and 4 tend to slightly decrease with the increasing chloride concentrations. For the enzymatic chlorination of Elliot soil humic acid, the Cl-AKIE values were plotted as a function of Cl^- time H_3O^+ concentration as the activity of the CPO is directly proportional to product of Cl^- and H_3O^+ concentrations (Figure 5) (Murali Manoj, 2006; Thomas et al., 1970). When plotted in this way, all Cl-AKIE values follow the same trend independent of pH. At low Cl^- and H_3O^+ concentrations, the Cl-AKIE is fairly constant (AKIE≈1.0075) (Figure 5). However, when the product of Cl^- and H_3O^+ concentrations ($[Cl^-]\times[H_3O^+]$) exceeds 1×10^{-5} mol^2 L^{-2} Cl-AKIE values strongly increase (Figure 5). The possible reasons of the variations of the AKIEs values as function of pH and chloride concentration are discussed in detail in the following section.

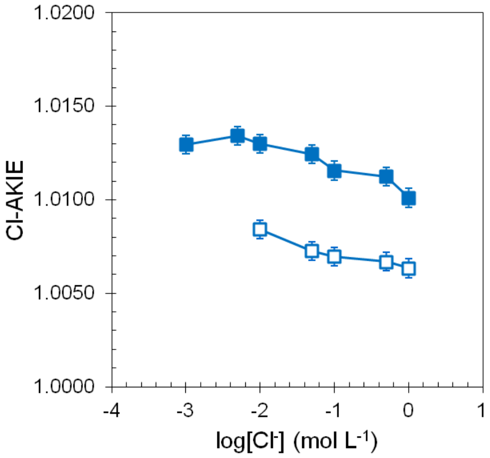

Figure 4 Cl-AKIE values for chloroform formed by abiotic chlorination of *Elliot* soil humic acid at pH 2.5 (blue squares) and 4 (white squares) as function of the logarithm of the Cl^- concentrations.

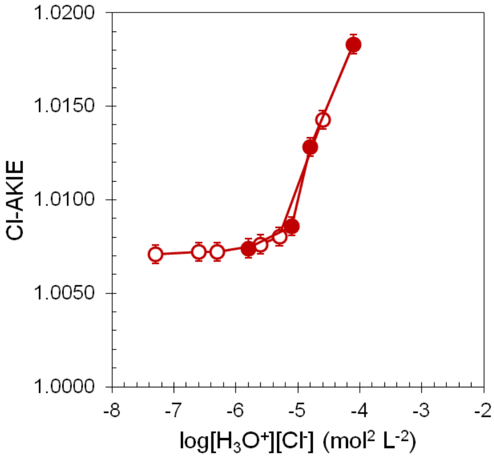

Figure 5 Cl-AKIE values for chloroform formed by CPO-catalyzed chlorination of *Elliot* soil humic acid at pH 2.5 (red circles) and 4 (white circles) as function of the logarithm of the product of H_3O^+ and Cl^- concentrations.

Potential explanation of Cl-AKIE associated to chloroform formation

Abiotic chlorination The Cl-AKIE values calculated for the formation of chloroform reflect the overall isotopic effect associated with all reaction steps leading to chloroform in which chlorine bonds are formed or cleaved. In the case of the abiotic chlorination, this includes (*i*) KIEs associated with the chlorination of the humic substance and (*ii*) and EIE related to the speciation of chlorine in solution. The formation of chloroform implies the incorporation of at least three chlorine atoms in the organic precursor. Thus, the measured Cl-AKIEs likely correspond to multiple chlorine KIEs (Sicinska et al., 2005; Swiderek and Paneth, 2012). At pH 2.5 the abiotic formation of chloroform from different humic substances exhibits large C-AKIEs values ranging from 1.0100 to 1.0122 (Table 1). A recent study has shown using molecular modeling that reactions in which chlorine atom is passed between two heavy atoms and is shielded from interactions with solvent molecules can induce KIEs at least as large as those measured for the formation of chloroform at pH 2.5 (Swiderek and Paneth, 2012). Therefore, the large Cl-AKIE values measured at pH 2.5 might originate from the transfer of the chlorine atom bonded to the oxygen of the hypochlorous acid to a carbon atom present in the substrate without any interaction with the surrounding water molecules. The isotopic composition of chloroform is shifted toward more enriched δ^{37}Cl values at pH 4 compared to pH 2.5 (Table 1 and Figure 3b) corresponding to larger Cl-AKIE values at pH 2.5 than 4 (Figure 3b and 4). A diminution of the pH from 4 to 2.5 favors the formation of Cl_2 (reaction 1) relative to HOCl and thereby enhances the chlorination of humic substances due to the higher reactivity of Cl_2 (Sivey et al., 2010). The displacement of the equilibrium (reaction 1) toward Cl_2 can influence the magnitude of the Cl-AKIE if the formation of Cl_2 is associated with an EIE. The EIE associated with this equilibrium (reaction 1) was calculated from reduced partition function ratio for ^{35}Cl and ^{37}Cl-containing molecules using vibrational frequencies determined by Schauble using an *ab initio* force field model (Schauble, 2004; Schauble et al., 2003) (see Supporting Information). The comparison of EIE associated with the HOCl/Cl_2 equilibrium (EIE=1.0048) with the isotopic shift observed between chloroform formed at pH 2.5 and pH4 ($\Delta\delta^{37}$Cl≈4.5‰) (Figure 3b) suggests that at pH 4 mainly HOCl contributes to chlorination while at pH 2.5 Cl_2 dominates and the isotope ratios of these two species are offset by 4.8‰. The progressive reduction of the Cl-AKIE associated with the abiotic chlorination as function of the chloride concentration might be due to the incorporation of heavy chlorine atoms from KCl into chloroform (Figure 3). Chloride from KCl can be incorporated indirectly in chloroform by isotope exchange reactions between chloride and the chlorination agents (i.e. HOCl and Cl_2). An increase of chloride concentration will drive the reaction 1 to the right. Cl_2 will be more strongly enriched in ^{37}Cl with an increasing chloride concentration since the added KCl (δ^{37}Cl=2.08±0.10‰) is enriched in ^{37}Cl compared to HOCl (δ^{37}Cl=-0.15±0.06‰). As Cl_2 is a strong chlorinating agent which reacts faster than HOCl, part of the chlorine-carbon bonds will be formed by reaction between Cl_2 enriched in ^{37}Cl and the organic substrate (Sivey et al., 2010; Sivey and Roberts, 2012). Therefore chloroform becomes progressively enriched in ^{37}Cl with the increasing of the chloride concentration (Figure 3b).

CPO-catalyzed chlorination At low H_3O^+ and Cl^- concentrations , the Cl-AKIE associated with the formation of chloroform by CPO-catalyzed chlorination range between 1.007 and 1.008 whereas Cl-AKIE values become much larger when $[Cl^-]\times[H_3O^+]$ is $>1\times10^{-5}$ mol^2 L^{-2} (Figure 5). This shift of the Cl-AKIE values of chloroform might reflect a change of the rate limiting step of the reaction since the chlorinating activity of CPO is directly proportional to the product of the H_3O^+ and Cl^- concentrations. From the X-ray crystal structure of CPO, Sundaramoorthy et al. have identified glutamate (Glu183) as part of a proton relay of a system including histidine (His105) and aspartate (Asp106) (Sundaramoorthy et al., 1995b). Glu183 which is located adjacent to the heme of CPO might be at the origin of some of the solution and pH dependence of the CPO activity (Sundaramoorthy et al., 1995b). During formation of the HOCl−ferriprotoporphyrin(IX) intermediate, chloride anions compete with a carboxyl group of Glu183 for the sixth coordinated position of the heme (Figure 6) (Thomas et al., 1970; Wagenknecht and Woggon, 1997). When the pH is lowered, the carboxyl group of Glu183 becomes protonated (pK_a=4.27) allowing the chloride anions to compete more favorably (Figure 6). In these conditions, the formation of the HOCl−ferriprotoporphyrin(IX) intermediate is fast and therefore the chlorination of humic substances by transiently formed hypochlorous acid is likely the rate-limiting step of the reaction. This suggests that at low pH and high Cl^- concentration the rate-limiting step of the CPO-catalyzed chlorination is comparable to that of the abiotic process. Therefore the large Cl-AKIE measured in presence of high Cl^- and H_3O^+ ions concentrations might be associated with the chlorination step in which the chlorine atom present in the transiently formed free hypochlorous acid is transferred to the organic substrate (Figure 6).

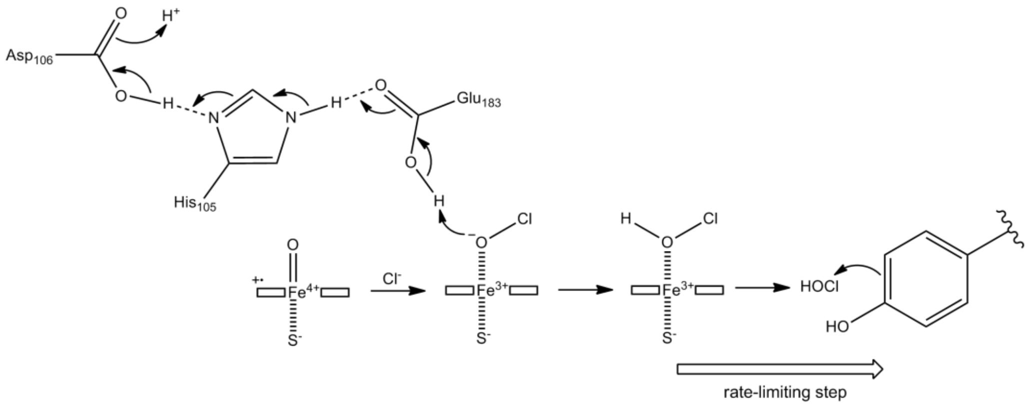

Figure 6 Proposed rate-limiting steps for CPO-catalyzed chlorination in presence of high Cl^- and H_3O^+ concentrations.

Inversely in presence of low Cl^- and H_3O^+ ions concentrations the formation of the HOCl−ferriprotoporphyrin(IX) intermediate becomes rate-limiting since the deprotonated carboxyl group of Glu183 repel entry of Cl^- owing to unfavorable electrostatic interactions (Figure 7) (Wagenknecht and Woggon, 1997). This reaction step might be associated with a smaller Cl-AKIE as the isotope effect associated with the incorporation of the Cl atom is partly compensate by the preferential desolvatation of Cl^-. Indeed previous studies have shown that chlorine isotope effects measured in protic solvents such as water tend to be smaller than isotope effects associated with identical reaction in dipolar aprotic solvent (Cromartie and Swain, 1976a, b). During this reaction step, chloride anions can strongly interact with water molecules and thus the changes in chloride bonding to solvent are likely more important than the changes in bonding to carbon at transition state (Cromartie and Swain, 1976a, b).

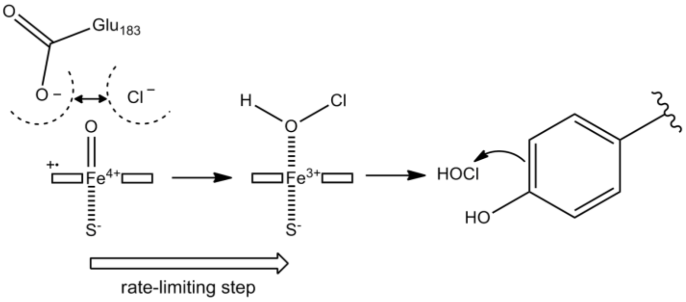

Figure 7 Proposed rate-limiting steps for CPO-catalyzed chlorination in presence of low Cl^- and H_3O^+ concentrations.

During the formation of the chlorine-oxygen bond of the HOCl−ferriprotoporphyrin(IX) intermediate in water at least one hydrogen bond to chloride must be broken before chloride can approach the reactive oxygen atom (Chandrasekhar et al., 1985; Melander and Saunders, 1987). As the desolvation of chloride in protic solvent is faster for the heavy isotope, the isotope effect associated with incorporation into HOCl-ferrinprotoporphyrin(IX) is therefore partly compensated (Melander and Saunders, 1987)On the basis of the measured Cl-AKIE, the present study suggests that humic substances are chlorinated by free hypochlorous acid formed by CPO. This hypothesis is also supported by crystal structures of CPO from *Caldariomyces fumago* measured by Kuehnel et al, showing that the catalytic site constituted by the ferriprotoporphyrin(IX) group is not accessible directly (Kuehnel et al., 2006; Wang et al., 2011). Indeed, Kühnel et *al*. have shown using high resolution X-ray synchrotron diffractometry that narrow (Ø~4 Å) and wide (Ø~10 Å) channels connect the protein surface with the heme of CPO (Kuehnel et al., 2006). Thus small organic molecules and ions such as chloride can reach the heme by diffusion through these channels. However, humic substances have a diameter ranging from ~100 Å to ~2000 Å and a molecular weight comprised between ~10 kDa up to ~300 kDa, it can therefore be excluded that humic substances can penetrate inside CPO and react directly at the catalytic site (Balnois et al., 1999; Beckett et al., 1987; Christl et al., 2000; Logan and Wagenseller, 2000; Rice et al., 1999; Sutton and Sposito, 2005). These results support that the enzyme-bound HOCl−ferriprotoporphyrin(IX) intermediate release HOCl which in turn diffuse through channels to the solution. The free HOCl released from catalytic site can then react directly with an organic substrate similarly to the abiotic chlorination with NaOCl (Figure 1a).

Implications for chloroform sources assessment The results of the present study enable to gain some insight about the potential variability of δ^{37}Cl values of chloroform naturally produced by CPO-catalyzed chlorination in different environment. Natural chloroform produced in terrestrial environmental is essentially formed in temperate and boreal forest soils where the pH ranges between 4 and 7 and Cl^- concentration may varies from 10 up to 400 µgCl^- g^{-1}soil in coastal forest soils (Johansson et al., 2003). As under typical environmental conditions the concentrations of H_3O^+ and Cl^- are very low compared to the batch experiments, it can therefore be expected that the formation of the HOCl−ferriprotoporphyrin(IX) intermediate is likely rate-limiting in forest soils (KIE between 1.006 and 1.007). Therefore in the case where natural chloroform would be produced in forest soils by microorganism excreting extracellular iron-containing CPO and with chloride from atmospheric deposition with δ^{37}Cl value between -1‰ and 1‰, the δ^{37}Cl value of natural chloroform should ranges between -5‰ and -8‰ (Kaufmann et al., 1984). Thus, natural and industrial chloroform sources might be distinguished from the chlorine isotope composition since the δ^{37}Cl values of industrial chloroform range between 0.32 and -5.4‰ with a median value of -3.0‰ while natural chloroform is expected to be more depleted in ^{37}Cl. Recently Chan et al. have demonstrated that the biodegradation of industrial chloroform produce δ^{13}C values in the same range as natural chloroform, underscoring the need to evaluate signature at contaminated sites with care (Chan et al., 2012). Although further studies are required, dual carbon-chlorine stable isotope investigation of sources might also contribute in this context to better discriminate natural and industrial chloroform in the terrestrial environment.

▪Acknowledgements

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▪Supporting information

Additional materials concerning the error analysis of AKIE and the isotope effect associated with equilibrium between HOCl and Cl_2 are available in Appendix 2. Additional information regarding the elemental composition, the carbon isotopic composition and the carbon distribution in humic substances and NOM are provided in the Appendix 3.

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Chapter V

Position-Specific Carbon Isotope Analysis of Trichloroacetic Acid by Gas Chromatography/Isotope Ratio Mass Spectrometry

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ABSTRACT Trichloroacetic acid (TCAA) is an important environmental contaminant present in soils, water and plants. A method to determine the carbon isotope signature of the trichloromethyl position in TCAA using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) was developed and tested with TCAA from different origins. Position-specific isotope analysis (PSIA) can provide direct information on the kinetic isotope effect for isotope substitution at a specific position in the molecule and/or help to distinguish different sources of a compound. The method is based on the degradation of TCAA into chloroform (CF) and CO_2 by thermal decarboxylation. Since thermal decarboxylation is associated with strong carbon isotope fractionation (ε=-34.6±0.2‰) reaction conditions were optimized to ensure full conversion. The combined isotope ratio of CF and CO_2 at the end of the reaction corresponded well to the isotope ratio of TCAA confirming the reliability of the method. A method quantification limit (MQL) determined for TCAA of 18.6 µg/L was reached. TCAA produced by enzymatic and non-enzymatic chlorination of natural organic matter (NOM) and some industrially produced TCAA were used as exemplary sources. Significant different PSIA isotope ratios were observed between industrial TCAA and TCAA produced by chlorination of NOM. This highlights the potential of the method to study the origin and the fate of TCAA in the environment.

▪ Introduction

Trichloroacetic acid (TCAA) is an important environmental contaminant widely distributed around the world, especially in forest soils (Berg et al., 2000; Hoekstra, 2003; Scholer et al., 2003). TCAA has also been found in remote locations such as in 100 year old glaciers and firn ice in Antarctica (von Sydow et al., 1999). TCAA can originate from anthropogenic and natural sources. TCAA is known as a disinfection by-product formed during drinking water chlorination and has been also detected in effluents from paper manufacturing (US-EPA, 2001; McCulloch, 2002; Ahlers et al., 2003). The atmospheric oxidation of perchlorethylene and 1,1,1-trichloroethane emitted from industries may lead to the formation of TCAA as well (US-EPA, 2001). During many years, TCAA has been widely used as herbicide in agriculture and polymerization catalyst in the chemical industry (US-EPA, 2001). This compound is of environmental concern because of its high phytotoxicity, causing chlorosis. In addition, TCAA may have direct effects on human health such as toxic and mutagenic effects on liver and heart (Golfinopoulos, 2003). Regarding natural sources, studies by Matucha et al. and Heal et al. suggest that TCAA may be formed in soil via chlorination of organic material, analogous to the production of chloroacetic acids from humic substances during drinking water treatment (Laturnus et al., 2005; Matucha et al., 2007b; Heal et al., 2010). Laboratory studies suggest that TCAA is produced when humic or fulvic acids are incubated with chloroperoxidase (CPO) in presence of chloride and H_2O_2 (Haiber et al., 1996; Niedan et al., 2000). Compound-specific isotope analysis (CSIA) is increasingly used to evaluate the source and fate of different classes of organic compounds such as chlorinated solvent (e.g. PCE, TCE and DCE), PCBs and aromatic compounds (e.g. BTEX and PAHs) (Aelion M.C., 2010). As the isotopic composition of a compound often varies as a function of its source, CSIA can be used to distinguish different sources of a given contaminant. It can be also used to study the fate of organic compounds in the environment (e.g. biodegradation, vaporization, diffusion) (Aelion M.C., 2010). So far, CSIA has found little application for evaluating the origin and fate of TCAA, partly due to the lack of a simple analytical method. Because of its low Henry's constant (7.4.104 mol/kg atm; Bowden et al., 1998) and high water solubility (81.7 g/L H_2O at 20°C, Sigma-Aldrich), aqueous samples of TCAA cannot be analyzed by gas chromatography / combustion / isotope ratio mass spectrometry (GC/C/IRMS) using common extraction methods for volatile organic compounds (Bowden et al., 1998). Wong et al. have developed an analytical procedure to measure the $\delta^{13}C$ values of chloroacetic acids using derivatization with acidic methanol with a known isotopic signature (Wong et al., 2003). Chloroacetic acids are transformed into chloroacetic acid methyl ester, extracted with pentane and then concentrated by gentle blowdown using nitrogen gas before analysis. Although the effect of derivatization on the isotope ratio can be accounted for using a carbon mole balance, this method is labor intensive. An alternative method of sample pretreatment is thermal decarboxylation that leads to the production of chloroform (CF) which can be easily analyzed by GC/C/IRMS. Albers et al. have demonstrated that the thermal decarboxylation of TCAA has to be carried out at a pH<3 to minimize the interference with trichloroacetyl-containing compounds (Albers et al., 2010a). At pH above 3 trichloroacetyl containing compounds can be hydrolysed and form CF and carboxylic acid as well. The thermal decarboxylation method provides position-specific isotope ratios. This can be an advantage when fingerprinting different TCAA sources as they may have a same average isotope ratio (δ^{13}C-CSIA) but different position-specific isotope ratios (δ^{13}C-PSIA). Furthermore, it is possible to access directly the effect of isotope substitution at the trichloromethyl-position on the reaction rate (i.e. hydrolysis).

Here an analytical procedure to determine the carbon position-specific isotope signature of trichloromethyl group in TCAA using thermal decarboxylation is described. Moreover, this study examines the isotopic composition of TCAA produced by chlorination of NOM to evaluate the ability of the method to study TCAA produced by different processes.

▪ Materials and Methods

Chemicals and reagents. The following chemicals were used as received: trichloroacetic acid (Sigma-Aldrich, 99.0% purity; Acros Organics, 99%; Fisher Scientific, analytical reagent grade), sodium hypochlorite solution (Sigma-Aldrich, available chlorine ≥4%), phosphoric acid (Fluka, 85%), sodium dihydrogenphosphate monohydrate (Merck, >99%), sodium sulfite (Sigma-Aldrich, >98%), nitric acid (Carlo Erba, 65%), Chloroperoxidase from *Caldariomyces fumago* (Sigma-Aldrich, >10000 U/mL), potassium chloride (Fluka, puriss. p.a.), hydrogen peroxide (Fluka, purssi. p.a.). Ultrapurified water (18.2 MΩ cm at 25°C, Direct-Q UV-3 Millipore) was used to prepare the aqueous solutions. The following humic substances from the International Humic Substance Society and soil were used for TCAA formation experiments: Suwannee river NOM (IHSS, 1R101N), Nordic reservoir NOM (IHSS, 1R108N), Pahokee peat humic acid (IHSS, 1R103H), Elliot soil humic acid (IHSS, 1S102H) and coniferous forest soil (F-horizon, Tisvilde, Denmark).

Quantification of reaction yield and isotope fractionation during thermal decarboxylation. Vials of 42 mL volume were completely filled with aqueous solutions of industrial TCAA (Sigma Aldrich) prepared from a stock solution with water acidified at pH2. Initial concentrations of TCAA were varied (from ~10 mg/L to ~50 μg/L) taking into account the reaction kinetics and decarboxylation time such that 17 nmol CF was expected to be formed in 42 mL of sample (Verhoek, 1934). As thermal decarboxylation of TCAA is a slow process at room temperature, the experiment was conducted at 65°C using a thermostatized water bath. The decarboxylation was conducted at rather low temperature to avoid an overpressure in the vials. The thermal decarboxylation was stopped after 2, 4, 8, 24, 32, 48, 56, 72, 96 and 120 hours by cooling the vials to 4°C. Based on the kinetic constants determined by Zhang et al. the conversion of TCAA into CF is expected to be nearly complete after 120h of reaction (Zhang and Minear, 2002). The δ^{13}C-PSIA value of CF released by thermal decarboxylation of TCAA was measured in triplicate for each time using the purge-and-trap method described below.

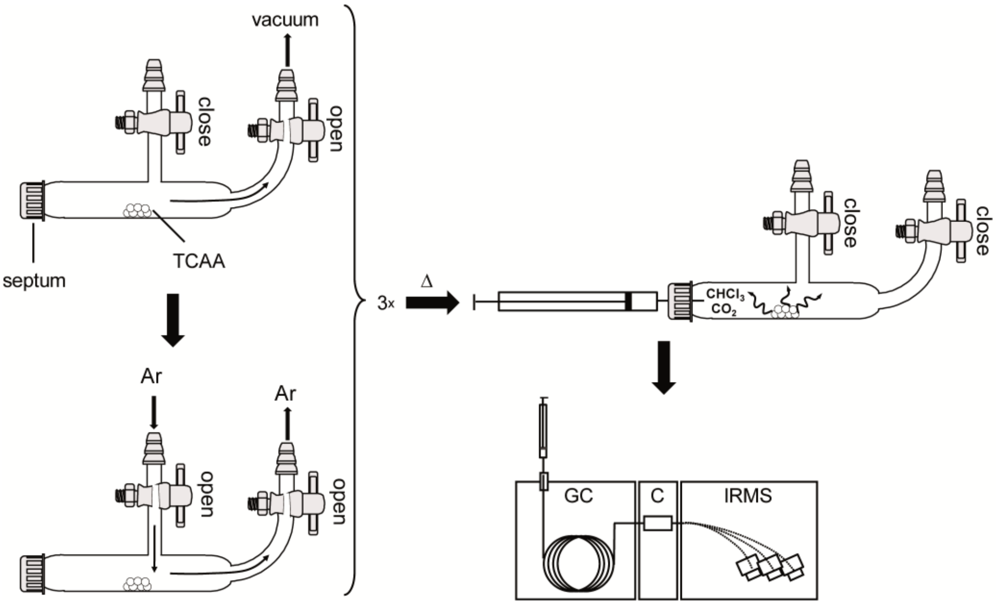

Figure 1. Procedure for the carbon isotope analysis of CO2 released by thermal decarboxylation of TCAA.

To establish an isotope balance of the decarboxylation products for quality control, the isotope ratio of CO2 produced during the decarboxylation of TCAA was measured using a glass gas trap (Figure 1). 10 mg of pure TCAA (Sigma Aldrich) was introduced in the gas trap. The atmospheric CO_2 present in the gas trap was purged during 2min using a vacuum pump ME2 (Vacuubran, Wertheim, Germany) and the air was replaced by pure argon. This step was repeated three times in order to insure that no traces of atmospheric CO_2 remains in the gas trap. The gas trap was placed in an oven at 90°C during 24h and the CO_2 produced was sampled through a septum with a 2 mL gas tight syringe and analyzed by GC/C/IRMS.

Determination of enrichment factor. Isotope fractionation associated with the release of CF can be quantified by the following isotope fractionation factor α:

$$\alpha = \frac{\dfrac{d^{13}CF}{d^{12}CF}}{\dfrac{^{13}TCM}{^{12}TCM}} \tag{1}$$

Where $d^{13}CF$ and $d^{12}CF$ is the amount of instantaneously produced CF with ^{13}C and ^{12}C, respectively; ^{13}TCM and ^{12}TCM the remaining amount of trichloromethyl-carbon with ^{13}C and ^{12}C, respectively. Isotope fractionation can also be expressed in terms of an enrichment factor ε, which is defined by

$$\varepsilon = (\alpha - 1) \times 1000 \qquad (2)$$

The isotope ratio of the accumulating CF that is measured in the experiment, ε, is expected to evolve according to the following relationship:

$$\overline{R}_{CF} = R_{TCM,f=0} \frac{1 - f^{\left(\frac{\varepsilon}{1000}+1\right)}}{1 - f} \qquad (3)$$

where RTCM, *f*=0 is the final isotope ratio of the trichloromethyl-carbon position and f the remaining fraction of TCAA ($f = C/C_0$). The CF concentration was determined on the basis of the peak area of *m/z* 44 using a five point calibration with external standards and then f was derived from the CF concentrations. The CF peak area of *m/z* 44 is indeed proportional to the TCAA concentration (r^2=0.9985). The measured isotope ratios of the accumulating CF were fitted with the equation 3 by varying the enrichment factor ε such that the sum of squared residuals is minimized (least square method). Since only one carbon atom is present in CF, the fractionation factor α can directly be related to the kinetic isotope effect (KIE) according to:

$$KIE = \frac{1}{\alpha} \qquad (4)$$

Production of TCAA from NOM. TCAA was formed by chlorination of forest soil and four humic substances with sodium hypochlorite. 40 mg of soil or 5 mg of humic substances was added in 42 mL vial containing 40 mL of 100 mM phosphate buffer and the reaction was initiated by adding 100 μL of an aqueous solution of 0.02 M NaOCl and the remaining volume was completed with pure water. The chlorination was carried out at pH4 in order simulate the conditions encountered in forest soils. The vials were sealed with Teflon septum caps and agitated during 24h at room temperature (~25°C). After 24h, the samples were quenched with 100 μL of sodium sulfite solutions (100 g/L) to stop the reaction. Forest soil and Elliott soil humic acids were also chlorinated with CPO and potassium chloride. 40 mg of soil or 5 mg of humic acid was added in 42 ml vials filled with 40ml of 100mM pH4 phosphate buffer solution containing 0.375 mg/L of KCl. 5 μL of an aqueous suspension of CPO >10^4 units/mL was added and the vials were stirred during 30min. 1 mL of H_2O_2 0.0129M was added progressively over a 1.5h interval. The solution was stirred for 1h, and then 1 mL of H_2O_2 solution was added over a second 1.5h interval.

Pretreatments of TCAA samples. The pH of the samples containing TCAA produced by chlorination of NOM was adjusted to ≥12 and the samples were purged during 30 min with pure N_2 to remove chloroform formed by the hydrolysis of trichloroacetyl-containing compounds (Albers et al., 2010a). Then, the pH of the samples containing industrial

TCAA or TCAA formed by chlorination of NOM was set to ≤2 with concentrated HNO_3 and the vials were filled with pure water such that no headspace remained. Finally the vials were heated in an oven at 65°C during 120h to completely convert the TCAA into CF by thermal decarboxylation. All samples were stored in the dark at 4°C until analysis with purge-and-trap GC/C/IRMS. The δ^{13}C-PSIA values of the trichloromethyl position of TCAA produced by chlorination of organic matter were compared with the δ^{13}C-PSIA values of synthetic TCAA from different suppliers.

^{13}C analysis of CF from thermal decarboxylation of TCAA. Isotope ratios of CF produced during the TCAA thermal decarboxylation were measured with a Thermo Trace gas chromatograph coupled to a Thermo Delta XP isotope ratio mass-spectrometer via a GC Combustion III interface (Thermo Fisher Scientific, San Jose, USA). The gas chromatograph was equipped with a cryogenic focuser Optic 3 (ATAS-GL, Veldhoven, Netherlands) and coupled to a purge-and-trap concentrator Velocity XPT (Teledyne Tekmar Dohrmann, Mason, USA) with a liquid auto-sampler AquaTek70 (Teledyne Tekmar Dohrmann, Mason, USA). Aqueous samples (25 mL) were purged with an N2 flow of 40 mL/min and volatiles were trapped on a VOCARB 3000 trap (Supelco, Bellefonte, USA) at 35°C. After the extraction step, the trap was heated to 250°C for 3 min. Chloroform was thermally desorbed and transferred to the gas chromatograph. The chromatographic separation was carried out with a DB-VRX column (60 m × 0.25 mm × 1.4 μm, Agilent, Santa Clara, USA). The column flow of the gas chromatograph was maintained constant at 1.7 mL/min. The following gas chromatograph oven temperature program was used: 6 min at 40°C, 10°C/min to 175°C, 175°C held for 1 min. The cryogenic focuser was set to -100°C during 3 min with liquid N_2. Oxidation and reduction reactors of the combustion interface were maintained respectively at 940°C and 650°C. In order to maximize the accuracy of the measured δ^{13}C values, the samples were diluted to obtain constant peak amplitudes. The purge efficiency was determined by measuring the peak area of *m/z* 44 for different purge time and was compared with theoretical extraction yield predicted by the following equation (Jochmann et al., 2006):

$$\frac{Area^{total}_{mass\,44}}{Area^{t}_{mass\,44}} = 1 - e^{\left(-\frac{K_{aw}G}{V_w}\right)t} \times 100 \qquad (5)$$

where and are respectively the peak area of *m/z* 44 for total extraction and at a specified time t. Vw is the volume of the aqueous sample in ml, G the purge gas flow rate in ml/min and Kaw the air-water partition constant ($K_{aw(CHCl_3)}$=0.13 at 20°C) (Jochmann et al., 2006). For the determination of δ^{13}C values of CO_2 the GC was fitted with an injection valve with a 250 μL loop (VICI, Houston, USA) and a Rt-QSplot column (30 m, 0.32 mm ID, 10 μm, Restek, Bellefonte, USA). The GC oven temperature was maintained at 35°C.

δ^{13}C values of pure solid TCAA samples were determined in triplicate using an elemental-analyzer coupled with a stable isotope mass spectrometer (Finnigan MAT Delta S). All δ^{13}C values were reported in ‰ relative to Vienna PeeDee Belemnite (V-PDB) defined as:

$$\delta^{13}C = \left(\frac{R}{R_{std}} - 1 \right) \times 1000 \tag{6}$$

▪Results and Discussion

Evaluation of decarboxylation time and enrichment factor. The decarboxylation yields of TCAA (Sigma-Aldrich) at different reaction times are shown in Figure 2a. TCAA is completely converted into CF after 120h of reaction. At the beginning of the decarboxylation reaction, CF formed is strongly depleted in 13C (Figure 2b). Afterwards CF becomes progressively enriched in 13C as the reaction proceeds. After 120h, the isotope signature of CF (δ^{13}C-PSIA=-40.4±0.5‰, *n*=3) is similar to the δ^{13}C-CSIA values of TCAA used for the experiment (δ^{13}C-CSIA=-41.3±0.2‰, *n*=3).

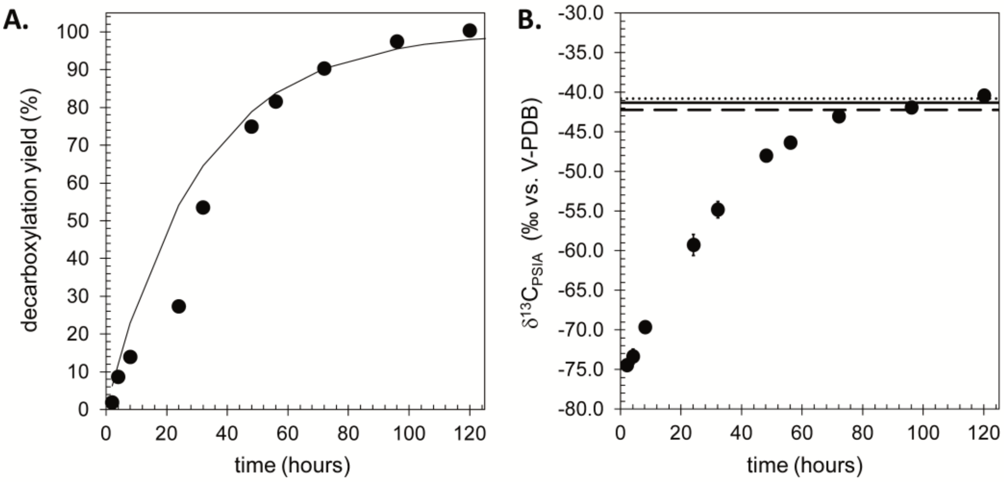

Figure 2. (a) Measured (black dots) and modeled (black line) decarboxylation yields calculated using the decomposition rate constants determined by Zhang and Minear (Zhang and Minear, 2002). (b) Measured (black dots) isotope signature of chloroform released during the thermal decarboxylation of TCAA versus time. The full line corresponds to the δ^{13}C value of TCAA, the dashed line to the δ^{13}C value of the carboxyl position in TCAA, and the dotted line to the calculated δ^{13}C value of the trichloromethyl position in TCAA. The error bars correspond to the standard deviation.

The carbon isotope ratio of CO_2 generated from complete thermal decarboxylation of TCAA amounts to -41.8±0.9‰ (*n*=6). The δ^{13}C value of TCAA calculated based on the δ^{13}C-PSIA values of the trichloromethyl and carboxyl positions (-41.1±1.0‰) corresponds well to the measured δ^{13}C value using the EA/IRMS method (-41.3±0.2‰)

confirming the accuracy of the analytical methods. The isotope enrichment factor obtained using equation 3 (Figure 3) amounts to ε=-34.6±0.2‰ (r^2=0.9878) corresponding to a KIE=1.036±0.002. Hence, thermal decarboxylation is associated with a normal kinetic isotope effects (KIE>1). The calculated KIE value is consistent with the Streitwieser semi-classical limit for the isotope effect for cleavage of a C−C bond (KIEC-C=1.049) (Aelion M.C., 2010).

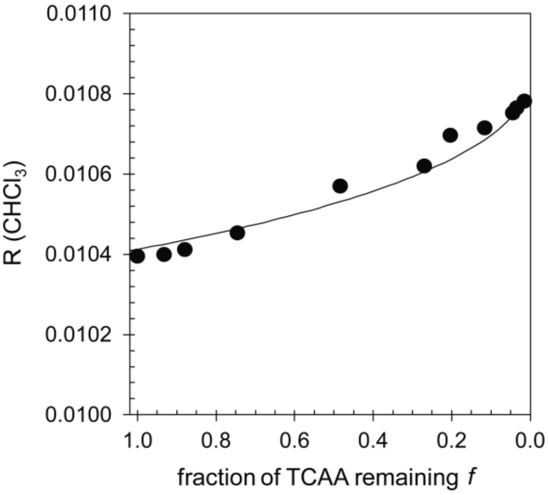

Figure 3. Measured (black dots) and modeled (black line) carbon isotope signatures of accumulated CF.

The somewhat smaller KIE values calculated for the thermal decarboxylation may be an indication that in the transition state the C−C bond is not completely broken. Furthermore it is expected that C-Cl bonds are strengthened in the transition state leading to an inverse secondary KIE that partly offsets the primary KIE. The measured KIE is in the same range as the KIE observed by Lindsay et al. for thermal decarboxylation of malonic acid, and is within the typical range KIE=1.03-1.06 of reactions for which the decarboxylation is entirely rate determining (Lindsay et al., 1951; Oleary and Limburg, 1977; Mundle and Kluger, 2009; Mundle et al., 2010). Therefore, the KIE of thermal decarboxylation of TCAA is consistent with the chemical mechanism proposed by Atkins et al. where at pH~2 TCAA (pK_a=0.64) is deprotonated and the C−C bond of the trichloroacetate anion is cleaved in a rate-limiting step (Atkins et al., 1984). The heterolytic cleavage of the C−C bond leads to the formation of CO_2 and trichloromethanide ion which is rapidly converted in CF by protonation.

Determination of purge time and method detection limit. The purge-and-trap extraction efficiency for CF is quite well predicted by the dynamic phase equilibrium model (r^2=0.964, Figure 4). The peak area of *m/z* 44 reaches the maximum between 10 and 15 min and decrease for purge time longer than 15 min. This decrease suggests chloroform breakthrough in the trap. The shift to a lower δ^{13}C-PSIA value at 20 min purge time could originate from this breakthrough as heavy isotopologues might elute preferentially from the trap (Figure 4) (Jochmann et al., 2006). Between 5 and 15 min purge time constant δ^{13}C-PSIA values are observed. On the basis of these measurements, 10min is an optimal purge time as a high accuracy and a high sensitivity is reached.

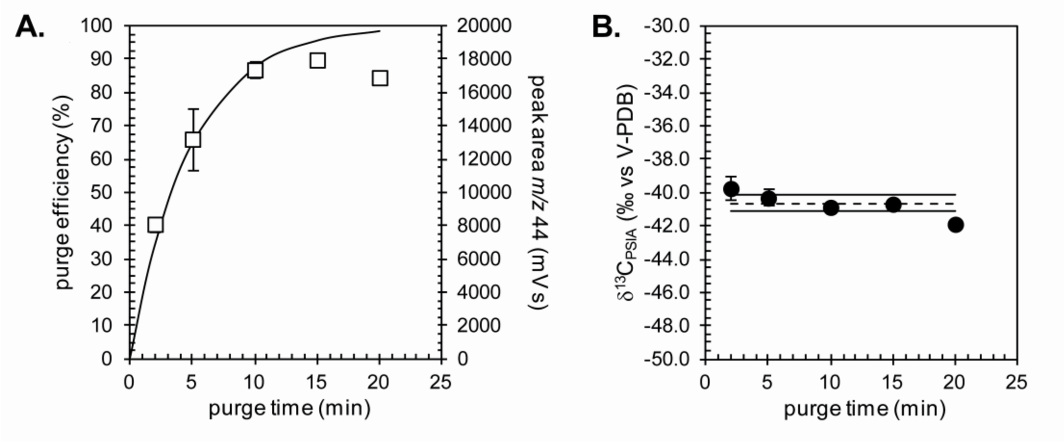

Figure 4. Measured (white squares) and theoretical (solid line) purge efficiency during CF extraction. (b) δ^{13}C-PSIA values of the extracted CF (black dots), mean δ^{13}C-PSIA value of the trichloromethyl position (dashed line) and interval of ±0.5% around it (full lines).

The method quantification limit (MQL) was quantified using the method developed by Jochmann et al. using standards with different concentrations of TCAA prepared from a stock solution (Jochmann et al., 2006). The reproducibility was determined by calculating the mean and standard deviation of all δ^{13}C values for which the concentration is equal and above the calculated MQL. A MQL of 18.6 µg/L (5 nmol carbon) and a reproducibility expressed as standard deviation (Figure 5) of ±0.5‰ (*n*=12) are obtained for CF released from TCAA. The calculated MQL value is very close to the MQL determined by Jochmann et al. for chlorinated and brominated methanes using a purge-and-trap method (Jochmann et al., 2006). According to McCulloch, the concentrations of TCAA in soils are very variable and, while 60% of the determinations were less than 0.5 µg/kg, the remainder spanned a wide range (up to 150 µg/kg) (McCulloch, 2002). The concentrations of TCAA in water are also very variable and range between 0.009 µg/L (e.g. natural water) to 7600 µg/L (e.g. industrial effluent) (McCulloch, 2002). Therefore, the position specific isotope analysis should be possible on a wide range of samples. However, a lower quantification limit could be reached using larger sample volume or using an anion exchanger to pre-concentrate aqueous TCAA.

Demonstration of applicability. TCAA was produced by chlorination of NOM with hypochlorite and by CPO-induced chlorination. The trichloromethyl position of TCAA produced from soil and humic substances with hypochlorite gives δ^{13}C-PSIA values between -23.9 to -32.3‰ (Table 1). These isotopic ratios (mean δ^{13}C-PSIA=-27.8±0.5‰) are in the same range as the isotope signature of soil and humic substances (mean

δ^{13}Cprecursor=-26.5±0.1‰) used in the experiments and are not influenced by the quantity of hypochlorite added for the chlorination (Figure 6) (IHSS).

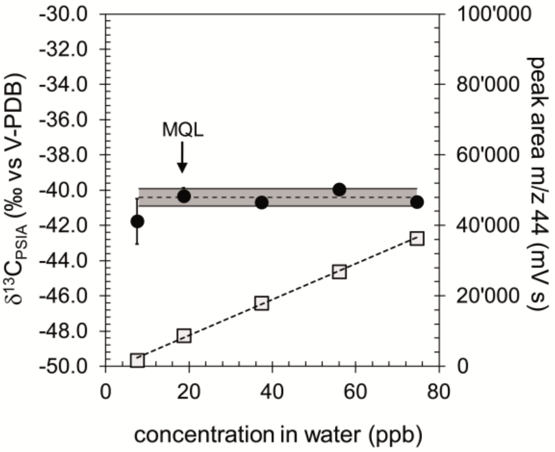

Figure 5. δ^{13}C-PSIA values of TCAA (black dots), peak area of *m/z* 44 (white squares), mean d13CPSIA value of TCAA (dashed line) and interval of ±0.5% around it (full lines).

Except TCAA produced from Nordic reservoir NOM, all δ^{13}C-PSIA values of the trichloromethyl position of TCAA are slightly depleted in ^{13}C compared to the precursor isotopic composition. This offset is likely due to a difference between the isotopic composition of functional groups involved in the reaction and the bulk isotopic ratios of the humic substances. These δ^{13}C values are in the same range as the results obtained by Bergamaschi et al. ($\delta^{13}C_{CF}$=-30.1‰, $\delta^{13}C_{NOM}$=-26.2‰) which show that the CF produced upon chlorination of agricultural water drainage is slightly depleted in ^{13}C compared to isotope signatures of dissolved organic matter (Bergamaschi et al., 1999). The TCAA produced by CPO-induced chlorination of forest soil and humic acid is slightly enriched in 13C compared to the isotopic composition of the precursors and TCAA produced with hypochlorite. The small difference of δ^{13}C-PSIA observed between the enzymatic and the non-enzymatic chlorination may be explained by a different degree of reaction advancement between the CPO-induced ($[CF]_{CPO}$: 10 − 40 μg/L) and non-enzymatic chlorination ($[CF]_{NaOCl}$: 80 − 150 μg/L). The relatively good agreement of the δ^{13}C values for CPO-induced and non-enzymatic chlorination is consistent with the hypothesis that CPO reacts with H_2O_2 and oxidizes chloride to hypochlorite which in turn chlorinates the NOM. The δ^{13}C-PSIA values of the three industrial TCAA samples ranges between -38.9 and -43.8‰, and hence are strongly depleted in ^{13}C compared to TCAA produced by enzymatic and non-enzymatic chlorination of NOM (Table 1). These relatively low δ^{13}C-PSIA values may result from the use of ^{13}C-depleted reactant during the synthesis of TCAA and the purification processes (e.g. re-crystallization, extraction) inducing isotope fractionation. The variation of the δ^{13}C-PSIA values among the different suppliers suggests that TCAA have been produced from different carbon feed stock with different isotopic signature (Wong et al., 2003).

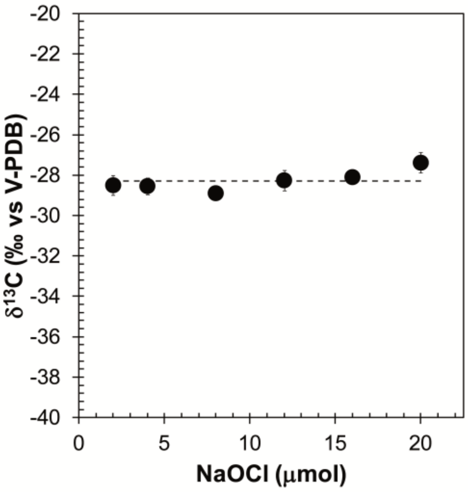

Figure 6. δ^{13}C-PSIA values of the extracted TCAA produced from forest soil with different amount of hypochlorite (black dots), mean δ^{13}C-PSIA value of the trichloromethyl position (dashed line).

While the samples of industrial TCAA analyzed in the present study have a distinctly different δ^{13}C compared to TCAA from NOM, three TCAA samples analyzed by Wong et al. have δ^{13}C-CSIA values (δ^{13}C between -27.5‰ and -29.0‰) that overlap with TCAA produced from NOM (Wong et al., 2003). Hence, when using isotope analysis for TCAA source identification, it is important to test the plausibility ofthe results with additional information such as concentration patterns, similarly as for industrial solvents studies where different sources can sometimes also have similar carbon isotope signatures. The Table 1 shows the δ^{13}C-PSIA and δ^{13}C-CSIA values of three synthetic TCAA measured by GC/C/IRMS and EA/IRMS, respectively.

Conclusions. This study demonstrates that thermal decarboxylation combined with isotope analysis of the released CF can be potentially used as a routine method for carbon isotope analysis of TCAA. Indeed, this novel method makes it possible to determine with high precision the carbon isotope signature of the trichloromethyl position of TCAA at ppb level without labor intensive pretreatment. Hence, the study provides the basis for a more widespread application of isotope analysis to evaluate the origin and fate of TCAA in the environment. The wide range of carbon isotope signatures observed in this study underlines the potential of the method for distinguishing different sources of TCAA although further work is needed to characterize the effect of different processes on the TCAA isotope composition.

Table 1. δ^{13}C-CSIA values (‰) of different TCAA sources and precursors. δ^{13}C-PSIA values (‰) of the trichloromethyl position of different industrial TCAA and TCAA produced by chlorination of NOM.

	CSIA (EA/IRMS)				PSIA (GC/C/IRMS)	
Sample	$\delta^{13}C_{precursor}$ (‰ V-PDB)	1σ (‰ V-PDB)	$\delta^{13}C_{TCAA}$ (‰ V-PDB)	1σ (‰ V-PDB)	$\delta^{13}C_{trichloromethyl}$ (‰ V-PDB)	1σ (‰ V-PDB)
Industrial TCAA						
Fisher Scientific	-	-	-37.9	±0.2	-38.9	±0.7
Acros Organics	-	-	-39.5	±0.3	-43.8	±0.1
Sigma-Aldrich	-	-	-41.3	±0.2	-40.4	±0.5
Chlorination with NaOCl						
Pahokee peat	-26.5	±0.1	-	-	-27.0	±0.4
Nordic reservoir	-27.8	±0.1	-	-	-27.4	±0.5
Suwannee river	-28.0	±0.1	-	-	-32.3	±0.5
Elliott soil	-22.6	±0.1	-	-	-23.9	±0.4
Forest soil	-27.5	±0.1	-	-	-28.5	±0.4
Chlorination with CPO						
Elliott soil	-22.6	±0.1	-	-	-20.2	±0.8
Forest soil	-27.5	±0.1	-	-	-27.3	±0.3

While TCAA from Sigma-Aldrich and Fisher Scientific only show small differences between δ^{13}C-PSIA and δ^{13}C-CSIA values ($\Delta\delta^{13}C_{Sigma\text{-}Aldrich}$=0.9‰ and $\Delta\delta^{13}C_{Fisher\ Scientific}$=1.0‰) indicating little intra-molecular δ^{13}C variations, the difference is substantial for Acros Organics-TCAA ($\Delta\delta^{13}C_{Acros\ Organics}$=4.3‰). The occurrence of intra-molecular variations in δ^{13}C suggests that PSIA can potentially provide additional discrimination between TCAAs from different sources.

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▪Supporting information

Additional information regarding the elemental composition, the carbon isotopic composition and the carbon distribution in humic substances and NOM are provided in the Appendix 3.

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Conclusions

Investigating the origin chloroform in soils and groundwater using carbon and chlorine stable isotopes analysis

Concluding remarks and future directions

Although organochlorines are often considered as compounds of anthropogenic origin, there is evidence for the natural occurrence of chlorinated organic compounds in the terrestrial and aquatic environment. Abiotic and enzymatic processes occurring in different environmental compartments may lead to the formation of various chlorinated compounds. The main focus of this thesis was the natural formation of chloroform and its related precursors in the terrestrial environment. Although the natural formation of chloroform in some forest soils and peatlands was rather well establish, it is difficult to demonstrate a natural origin of chloroform in groundwater as chloroform can be transported over extended distances from anthropogenic sources.

In this thesis it has been demonstrated that compound-specific isotope analysis (CSIA) of chloroform constitute a tool of choice to discriminate anthropogenic and natural of chloroform sources. The $\delta^{13}C$ of chloroform at the water table (-22.0‰) of forest sites corresponded well to the $\delta^{13}C$ of soil gas chloroform (-22.8 and -26.2‰) demonstrating that chloroform maintains its characteristic isotope signature during transport through the unsaturated zone. At three forested sites, the $\delta^{13}C$ of groundwater ($\delta^{13}C$ from -22‰ to -27 ‰) was close to the values of soil gas chloroform indicating a natural origin of chloroform. This conclusion is plausible as the three sampling sites were located within spruce and pine forest which are associated with soils that are favorable for chloroform production. In contrast, chloroform from the landfill (-42.1‰) and urban (-47.0‰) sampling sites have clearly an anthropogenic signature ($\delta^{13}C$ from -43‰ to -63‰) consistent with concentrations higher than at the forested sites. Chloroform was detected in groundwater samples as old as 35 years demonstrating that chloroform can persist over extended periods in oxic groundwater. Although the isotope analysis of groundwater samples collected in spruce forest indicates that the origin of chloroform can still be determined based on isotope composition even if chloroform was transported over an extensive distance,. the fate of chloroform in the terrestrial environment remain unclear. The transport of chloroform through the unsaturated zone and in groundwater is likely associated with different physico-chemical processes such as diffusion, solvatation and adsorption. As no chemical bond is formed or cleaved during these physico-chemical processes, it is likely that the isotope fractionation induced by the transport of chloroform is not sufficient to significantly modify the $\delta^{13}C$ values. However, the biodegradation of chloroform by reductive dechlorination might likely induce a large enrichment of chloroform in ^{13}C. Indeed, recently Chan et al. have demonstrated that the biodegradation of industrial chloroform by *dehalobacter* cultures produces $\delta^{13}C$ values in the same range as natural chloroform, underscoring the need to evaluate the isotope signature at contaminated sites with care. Although CSIA can be a valuable approach for source fingerprinting of chloroform, the $\delta^{13}C$ data must be interpreted within the detailed hydrogeological and geochemical context of the site. Recent analytical advances in chlorine isotope analysis suggest that dual isotope analysis ($\delta^{13}C$ and $\delta^{37}Cl$) could help to better discriminate natural and industrial chloroform sources in the environment. Such dual isotopic approach might be particularly helpful to separate natural chloroform from industrial chloroform that was partly degraded. Indeed, in this case the carbon isotopic signature might be similar to natural chloroform; however the chlorine isotopic composition of industrial chloroform might be more enriched in ^{37}Cl compared to natural chloroform since the C-Cl bonds containing a ^{35}Cl atom are preferentially cleaved during the reductive dechlorination of chloroform. In this thesis a method to analyze the chlorine isotope composition of chloroform using gas chromatography quadrupole mass spectrometry (GC-qMS) has been developed. It was shown that in the case where natural chloroform would be produced in forest soils by microorganisms excreting extracellular iron-containing chloroperoxidase with chloride from atmospheric deposition, the δ^{37}Cl value of natural chloroform should ranges between -5‰ and -8‰. Thus, natural and industrial chloroform sources might be also distinguished from the chlorine isotope composition since the median δ^{37}Cl value of industrial chloroform is -3‰ while natural chloroform is expected to be more depleted in ^{37}Cl. To date, the method quantification limit for chlorine isotope analysis of chloroform by GC-qMS is about 60 μg L^{-1}. Therefore, further analytical developments are still required to reach lower quantification limit to determine the chlorine isotope composition of chloroform in environmental samples. Moreover, other enzymatic and abiotic chlorination processes must still be investigated in order to better characterize the chlorine isotope signature of natural chloroform sources. In addition to source fingerprinting, CSIA is as well a powerful tool to gain insight into the mechanisms leading to the formation and the degradation of various organic contaminants. The carbon isotope analysis of trichloromethyl groups in trichloroacetyl-containing compounds present in forest soils has shown that these groups are strongly enriched in ^{13}C compared to natural chloroform. Chlorination experiments combined with a mathematical model have revealed that the trichloroacetyl-containing compounds are reaction intermediates which are subsequently degraded into chloroform by hydrolysis. The enriched δ^{13}C values of the trichloromethyl position in trichloroacetyl-containing compounds might explain why natural chloroform has a similar isotope composition as organic matter (NOM) despite a large carbon isotope fractionation during its release. Nevertheless, a deviation of the isotope signature of chloroform compared to NOM cannot be excluded. Indeed, the isotopic composition of chloroform might slightly vary if a reaction step leading to a normal or an inverse isotope effect preceded the hydrolysis of the trichloroacetyl-containing compounds. In the case where a large normal kinetic isotope effects would be associated with a reaction step preceding the hydrolysis, the reaction might lead to the formation of light chloroform in the same range as anthropogenic chloroform. Nevertheless, the results presented in this thesis suggest that the hydrolysis of trichloroacetyl-containing compound is likely rate-limiting in forest soils (low pH); therefore the isotopic deviation induced by a reaction step preceding the hydrolysis is likely limited. The measurement of the apparent kinetic isotope effects (AKIE) associated with the formation of chloroform from model compounds, humic acid and NOM has also enabled to take a step forward towards a better understanding of the mechanisms leading to chloroform. It has been demonstrated that the AKIE for chloroform formation from model compounds representing NOM functional groups are strongly pH-dependent. The comparison of the AKIE associated with the chlorination of model compounds and NOM has confirmed that phenolic compounds and ketone groups might be partly responsible for chloroform formation upon chlorination of NOM in forest soils. Nevertheless, other groups such as resorcinol, guaiacol and hydroquinones are likely involved in the formation of chloroform. NOM is constituted of heterogeneous and redox-sensitive natural organic macromolecules. In environments with changing redox conditions, such as capillary fringes of soils, peatland and tundra, NOM may act as an electron carrier by accepting electrons under anoxic condition, and upon reaeration by donating electrons to oxygen. Thus, the redox conditions of soil can affect the reactivity of some functional groups present in humic substances. Indeed, under anoxic conditions quinone groups can accept electrons and form hydroquinones which are highly reactive in presence of hypochlorous acid. Therefore, the environmental redox conditions must be taken into consideration in determining the reactivity of the functional group leading to the formation of natural organochlorines.

Chlorine isotope analysis of chloroform can provide complementary information regarding the mechanisms involved in the chlorination of NOM. The chlorination mechanism of the fungal enzyme chloroperoxidase is debated since more than thirty years. The analysis of the AKIE associated with the abiotic and chloroperoxidase-catalyzed chlorination suggests that the enzymatic chlorination of NOM occurs via the formation and the diffusion of free hypochlorous acid. Moreover, the δ^{37}Cl data suggest that the formation of the HOCl-Fe(III)-protoporphyrin complex might be rate-limiting in forest soils.

Although the carbon and chlorine isotope analysis of chloroform has provided valuable information on the mechanisms involved in the formation of natural organochlorines further experiments and fields measurements are still needed to better understand the mechanisms of chloroform in environment. A number of research questions regarding the natural formation and the fate of chloroform and its related precursors remain to be elucidated. The more important questions among these are the following:

(1) *What are the main structural characteristics of trichloroacetyl-containing compounds?* (2) *How these structural characteristics influence the fate of trichloroacetyl-containing compounds in soil and groundwater?* (3) *What are the factors controlling the hydrolysis and the subsequent release of chloroform from trichloroacetyl-containing compounds?* (4) *Do trichloromethyl groups present in trichloroacetyl-containing compounds could be massively liberated as chloroform by pH rising subsequent to forest clearfelling?* (5) *What is the effect of redox conditions on the chlorination of natural organic matter?* (6) *Does chloroform can be biodegraded in soil?* (7) *Do chlorinated organic compounds formed by enzyme-catalyzed chlorination have the same chlorine isotopic signature as those produced by iron-catalyzed chlorination?*

Appendices

Investigating the origin chloroform
in soils and groundwater using carbon and chlorine
stable isotopes analysis

Appendix 1

Demonstrating a Natural Origin of Chloroform in Groundwater Using Stable Carbon Isotopes

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Validation of the analytical system. As stable isotope analysis of chloroform in groundwater requires the purge of large volume water samples, it is import to verify that the measured isotope ratio is independent of the amount that is injected in the GC-C-IRMS and to evaluate the method quantification limit (MQL). For this reason the isotope ratio of chloroform were measured for samples at different concentrations. The tested concentration range shows a linear relationship between the concentration and the peak area of mass 44 (Figure S1). The MQL was determined for a standard deviation of ±0.8‰ according to the method described in the Material and Method section. For isotope analysis of chloroform by P&T-GC-IRMS using 2 L sample volumes, the MQL is 0.6 μg/L (2 nmol C injected). This result demonstrates that purging of large volumes samples makes it possible to reach very low detection limits. The reproducibility determined for the range between the MQL (0.6 μg/L) and the highest tested concentration (3 μg/L) are respectively -47.2±0.6‰ (n=25). Compared to direct gas injection of chloroform which gives δ^{13}C value of -47.9±0.2‰ (n=7), the isotopic ratios of large volume samples measured by P&T are slightly enriched in ^{13}C of about 0.7‰. If 20% of chloroform is transferred into gas phase, the calculated gas/water enrichment factor is 1.63‰.

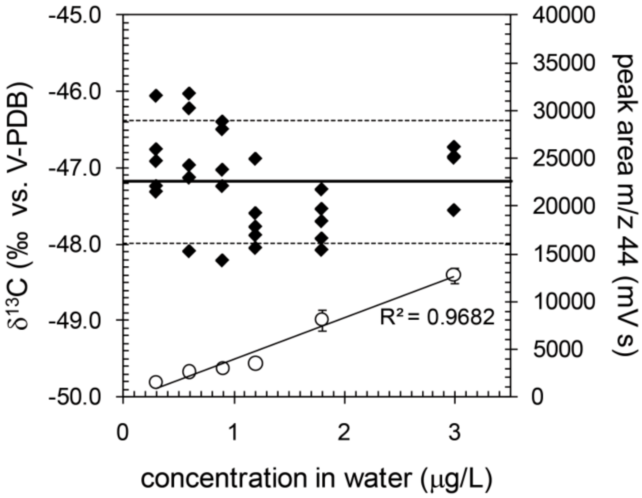

Figure S1: Linearity test. The white circles and black diamond's represent respectively the peak area of mass 44 and the δ^{13}C values of chloroform measured at different concentrations. The bold black line and the doted lines correspond respectively to the mean δ^{13}C value of chloroform and to an uncertainty of 0.8‰ around the mean δ^{13}C value.

This value is close to the isotope enrichment factor determined by Hunkeler and Aravena for chloroform in distilled water (ε_{gw}=1.46±0.34‰). Therefore, an explanation for this isotope enrichment of chloroform might be incomplete chloroform removal and to the water/gas partitioning (Hunkeler and Aravena, 2000). Since the isotope ratios of chloroform determined by these two methods are not significantly different, the δ^{13}C values of chloroform in the samples were not corrected for this. These results confirm that it is possible to obtain precise and accurate $\delta^{13}C$ values of chloroform from large volume samples even at low concentration. Use of a P&T system equipped with a 2 L bottle extends the capabilities of the technique for the isotopic analysis of VOCs at trace level in water.

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Appendix 2

Investigating chloroperoxidase-catalyzed formation of chloroform from humic substances using chlorine stable isotope

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submitted to *Environmental Science and Technology*

Error analysis on Cl-AKIE. In this annex we will discuss the influence of an uncertainty in the measured relative isotope composition of chloroform and also the influence of an uncertainty in the remaining fraction of chloride on the chlorine apparent kinetic isotope effect (Cl-AKIE) of chloroform formed by abiotic and chloroperoxidase-catalyzed chlorination. For this situation the Cl-AKIE can be calculated using the following equation (Melander and Saunders, 1987):

$$\text{Cl-AKIE} = \frac{^{35}k}{^{37}k} = \frac{\log(1-f)}{\log\left[1-\left(f\,\frac{R_{CHCl_3}}{R_{Cl}}\right)\right]} \tag{1}$$

1-f denotes the remaining fraction of chloride and $R_{\mathrm{CHCl_3}}/R_{\mathrm{Cl}}$ corresponds to the ratio of isotope ratios of chloroform and chloride. In order to simplify the error analysis on Cl-AKIE, decimal logarithms have been replaced by natural logarithms.

$$\text{Cl-AKIE} = \frac{^{35}k}{^{37}k} = \frac{\ln(1-f)}{\ln\left[1-\left(f\,\frac{R_{CHCl_3}}{R_{Cl}}\right)\right]} \tag{2}$$

In the paper IV the $R_{\mathrm{CHCl_3}}/R_{\mathrm{Cl}}$ ratios were measured by gas chromatography quadrupole mass spectrometry and by isotope ratio mass spectrometry. In the case of a heavy-atom isotope the *R* ratios are subject to an uncertainty of about ±0.3% (Melander and Saunders, 1987). *f* is obtained by the analysis of chloride concentrations by ion liquid chromatography. The latter may introduce some coupling between the uncertainty in the *R* ratios and in *f*, but in order the simply the analysis of errors we have neglected this coupling in the following equations (Melander and Saunders, 1987). To determine the influence of the above mentioned uncertainties on Cl-AKIE, the partial derivative of the equations 2 are used:

$$\frac{\partial\left(^{35}k/^{37}k\right)}{\partial\left(R_{CHCl_3}/R_{Cl}\right)} = \frac{f}{1-\left(f\,\frac{R_{CHCl_3}}{R_{Cl}}\right)}\,\frac{\ln(1-f)}{\left(\ln\left[1-\left(f\,\frac{R_{CHCl_3}}{R_{Cl}}\right)\right]\right)^2}$$

(3)

and

$$\frac{\partial\left({}^{35}k/{}^{37}k\right)}{\partial f}=\frac{\dfrac{\dfrac{R_{CHCl_3}}{R_{Cl}}}{1-\left(f\dfrac{R_{CHCl_3}}{R_{Cl}}\right)}\ln\left(1-f\right)-\dfrac{1}{1-f}\ln\left[1-\left(f\dfrac{R_{CHCl_3}}{R_{Cl}}\right)\right]}{\left(\ln\left[1-\left(f\dfrac{R_{CHCl_3}}{R_{Cl}}\right)\right]\right)^2}$$

(4)

The relative error on Cl-AKIE induced by the relative deviation of the $R_{\mathrm{CHCl_3}}/R_{\mathrm{Cl}}$ ratios can be estimated as follows:

$$\varepsilon_R=\frac{\Delta\left({}^{35}k/{}^{37}k\right)}{{}^{35}k/{}^{37}k}=\frac{1}{\text{Cl-AKIE}}\frac{\partial\left({}^{35}k/{}^{37}k\right)}{\partial\left(R_{CHCl_3}/R_{Cl}\right)}\Delta\left(R_{CHCl_3}/R_{Cl}\right)$$

(5)

Where $\Delta(R_{\mathrm{CHCl_3}}/R_{\mathrm{Cl}})$ is the relative deviation of the R ratios. The relative error on Cl-AKIE cause by the absolute deviation of f (Δf) can be calculated using the following equation:

$$\varepsilon_f=\frac{\Delta\left({}^{35}k/{}^{37}k\right)}{{}^{35}k/{}^{37}k}=\frac{1}{\text{Cl-AKIE}}\frac{\partial\left({}^{35}k/{}^{37}k\right)}{\partial f}\Delta f \qquad (6)$$

The figure 1 show how the influence of an uncertainty in the measured relative isotope composition of chloroform and also the influence of an uncertainty in the remaining fraction of chloride on Cl-AKIE considering, Cl-AKIE=1.0100, $\Delta(R_{\mathrm{CHCl_3}}/R_{\mathrm{Cl}})=0.000165$ (±0.5‰) and $\Delta f=0.1$ (10%). These results demonstrate that the influence of the uncertainties of f and $R_{\mathrm{CHCl_3}}/R_{\mathrm{Cl}}$ on the Cl-AKIE is relatively small if these latter parameters are measured in presence of a large excess of chloride (small f value).

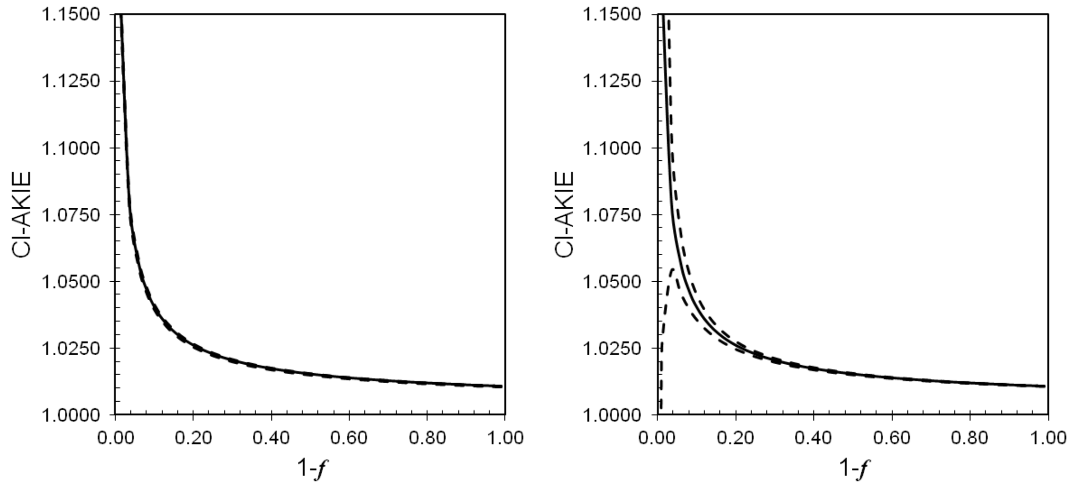

Figure 1.Deviation of Cl-AKIE values of chloroform as function of the remaining fraction of chloride (1-*f*). a) The dashed lies represent the error on the Cl-AKIE value caused by the uncertainty of the measured relative isotope composition of chloroform ($\Delta(R_{\mathrm{CHCl_3}}/R_{\mathrm{Cl}})=0.002$). b) The dashed lies represent the error on the Cl-AKIE value caused by the uncertainty in the remaining fraction of chloride ($\Delta f=0.1$).

Isotope effect associated with the equilibrium between HOCl and Cl_2.

Hypchlorous acid (HOCl) can form molecular chlorine (Cl_2) in presence of H^+ and Cl^- in solution (reaction 1). This chemical equilibrium is associated with isotope exchange reactions, where ^{35}Cl and ^{37}Cl swap between Cl^-, HOCl and Cl_2 (reactions 2−4):

$$HOCl + Cl^- + H^+ \rightleftarrows Cl_2 + H_2O \qquad [1]$$

$$HO^{35}Cl + {}^{37}Cl^- \rightleftarrows HO^{37}ClO + {}^{35}Cl^- \qquad [2]$$

$${}^{35}Cl^{35}Cl + {}^{37}Cl^- \rightleftarrows {}^{35}Cl^{37}Cl + {}^{35}Cl^- \qquad [3]$$

$$HO^{35}Cl + {}^{35}Cl^{37}Cl \rightleftarrows HO^{37}Cl + {}^{35}Cl^{35}Cl \qquad [4]$$

The reduced partition function ratio (*RPFR*), often expressed as *s/s'*×*f*, corresponds to the isotope fractionation factor *α* (or sometimes *β*) between a pair of isotopologues and a dissociated free atom. For example in the case of chlorine isotopes in HOCl it corresponds to:

$$\frac{s}{s'}f = \alpha_{HOCl-Cl} = \frac{\dfrac{HO^{37}Cl}{HO^{35}Cl}}{\dfrac{^{37}Cl}{^{35}Cl}} = \frac{\left[\dfrac{^{37}Cl}{^{35}Cl}\right]_{HOCl}}{\left[\dfrac{^{37}Cl}{^{35}Cl}\right]_{Cl}} \qquad (1)$$

Generally the fractionation factor can be related to equilibrium constants for exchange reaction using[1]:

$$n\ X^{L}_{m}A + m\ X^{H}_{n}A \leftrightarrow n\ X^{H}_{m}A + m\ X^{L}_{n}A \tag{2}$$

$$\alpha_{XA-XB} = \left[\frac{S^{H}_{X_mA}}{S^{L}_{X_mA}}\right]^{1/n} \left[\frac{S^{L}_{X_nB}}{S^{H}_{X_nB}}\right]^{1/m} K^{\frac{1}{mn}} \tag{3}$$

or

$$K_{eq} = \alpha_{XA\text{-}XB}{}^{mn} \times \left[\frac{S^{L}_{X_mA}}{S^{H}_{X_mA}}\right]^{m} \left[\frac{S^{H}_{X_nB}}{S^{L}_{X_nB}}\right]^{n} \tag{4}$$

Where $S^{H}{}_{X_mA}$, $S^{L}{}_{X_mA}$, $S^{H}{}_{X_mB}$ and $S^{L}{}_{X_mB}$ are the molecular symmetry numbers for each reactant and product molecule and K_{eq} is the equilibrium constant.
In the following, it is shown that also for substances containing more than two atoms of the element of interest; *RPFR* and α represent the isotope fractionation factor and not an isotopologue fractionation factor.

In case of Cl_2, the following exchange reaction can be written:

$${}^{35}Cl\,{}^{35}Cl + {}^{37}Cl^{-} \rightleftarrows {}^{35}Cl\,{}^{37}Cl + {}^{35}Cl^{-} \tag{5}$$

For this exchange reaction, we can write equilibrium constant according to equation 3, taking into account that only one atom is exchanged (*n*=1, *m*=1):

$$K_1 = \frac{{}^{35}Cl\,{}^{37}Cl\ \times\ {}^{35}Cl}{{}^{35}Cl\,{}^{35}Cl\ \times\ {}^{37}Cl} = 2 \times \alpha_{Cl_2\text{-}Cl} \tag{6}$$

Where 2 originate from symmetry numbers and α is the fractionation factor (RPFR).
In case of

$${}^{35}Cl\,{}^{37}Cl + {}^{37}Cl^{-} \rightleftarrows {}^{37}Cl\,{}^{37}Cl + {}^{35}Cl^{-} \tag{7}$$

$$K_2 = \frac{{}^{37}Cl\,{}^{37}Cl\ \times\ {}^{35}Cl}{{}^{35}Cl\,{}^{37}Cl\ \times\ {}^{37}Cl} = \frac{\alpha_{Cl_2\text{-}Cl}}{2} \tag{8}$$

Assuming that replacing the first or second heavy isotope is associated with the same isotope effect.

Or by dividing 6 by 8:

$$\frac{{}^{35}Cl\,{}^{37}Cl}{{}^{35}Cl\,{}^{35}Cl} \Big/ \frac{{}^{37}Cl\,{}^{37}Cl\ \times}{{}^{35}Cl\,{}^{37}Cl} = 4 \tag{9}$$

The overall isotope fractionation factor between Cl_2 and Cl^- is given by:

$$\alpha_{eff}=\frac{\left[\frac{^{37}Cl}{^{35}Cl}\right]_{Cl_2}}{\left[\frac{^{37}Cl}{^{35}Cl}\right]_{Cl}}=\frac{\frac{2\cdot{}^{37}Cl^{37}Cl+{}^{37}Cl^{35}Cl}{2\cdot{}^{35}Cl^{35}Cl+{}^{37}Cl^{35}Cl}}{\frac{^{37}Cl}{^{35}Cl}}=\frac{\frac{2\cdot{}^{37}Cl^{37}Cl/{}^{37}Cl^{35}Cl+1}{2\cdot{}^{35}Cl^{35}Cl/{}^{37}Cl^{35}Cl+1}}{\frac{^{37}Cl}{^{35}Cl}}$$

$$=\frac{\frac{1/2\cdot({}^{35}Cl^{37}Cl/{}^{35}Cl^{35}Cl)+1}{2\cdot{}^{35}Cl^{35}Cl/{}^{37}Cl^{35}Cl+1}}{\frac{^{37}Cl}{^{35}Cl}}=\frac{\frac{\frac{^{35}Cl^{37}Cl+2\cdot{}^{35}Cl^{35}Cl}{2\cdot{}^{35}Cl^{35}Cl}}{\frac{2\cdot{}^{35}Cl^{35}Cl+{}^{35}Cl^{37}Cl}{^{37}Cl^{35}Cl}}}{\frac{^{37}Cl}{^{35}Cl}}=\frac{\frac{^{37}Cl^{35}Cl}{2\cdot{}^{35}Cl^{35}Cl}}{\frac{^{37}Cl}{^{35}Cl}} \quad (10)$$

Or

$$2\cdot\alpha_{eff}=\frac{\frac{^{37}Cl^{35}Cl}{^{35}Cl^{35}Cl}}{\frac{^{37}Cl}{^{35}Cl}} \quad (11)$$

Comparing 11 with 6 shows that:

$$\alpha_{eff}=\frac{\left[\frac{^{37}Cl}{^{35}Cl}\right]_{Cl_2}}{\left[\frac{^{37}Cl}{^{35}Cl}\right]_{Cl}}=\alpha_{Cl_2-Cl} \quad (12)$$

Hence the fractionation factor between HOCl and Cl_2 is given by:

$$\alpha_{Cl_2\text{-}HOCl}=\frac{\left[\frac{^{37}Cl}{^{35}Cl}\right]_{Cl_2}}{\left[\frac{^{37}Cl}{^{35}Cl}\right]_{HOCl}}=\frac{\alpha_{Cl_2\text{-}Cl}}{\alpha_{HOCl\text{-}Cl}}=\frac{\beta_{Cl_2}}{\beta_{HOCl}} \quad (13)$$

Then, the fractionation factor α of the reactions 1 and 2 can be calculated from *RPFR* for the normal mode molecular vibration frequencies of ^{35}Cl and ^{37}Cl-containing molecules using equation 14 and 15.[3-5]

$$\alpha_{HOCl-Cl}=\frac{Q_{37}}{Q_{35}}\cdot\frac{\sigma_{37}}{\sigma_{35}}=\prod_{i}^{l}\frac{u_{37}}{u_{35}}\cdot\frac{e^{-\frac{1}{2}u_{37}}}{e^{-\frac{1}{2}u_{35}}}\cdot\frac{1-e^{-u_{35}}}{1-e^{-u_{37}}} \quad (14)$$

Where Q_{35} and Q_{37} are the reduced partition function ratios relative to the separated atoms for ^{35}Cl and ^{37}Cl-containing molecules, respectively. l is the number of degrees of freedom for vibration modes (l=3N-5 for a linear molecule and N is the number of atoms in the molecule). σ_{35} and σ_{37} are the symmetry numbers for the two isotopically substituted species. In addition, $u_i = hc\omega_i/kT$, where ω_i corresponds to each of the i^th^ normal frequencies, h is Plank's constant, k is Boltzman's constant, c is the speed of light, and T is the absolute temperature in Kelvin. As Cl_2 contains two equivalent atoms then $\alpha_{Cl_2\text{-}Cl}$ is calculated using a more general expression[6-7]:

$$\alpha_{Cl_2-Cl} = \left(\prod_i^l \frac{u_{37}}{u_{35}} \cdot \frac{e^{-\frac{1}{2}u_{37}}}{e^{-\frac{1}{2}u_{35}}} \cdot \frac{1-e^{-u_{35}}}{1-e^{-u_{37}}} \right)^{\frac{1}{n}} \tag{15}$$

Where n is a normalizing factor that accounts for multiple substitutions in molecules containing more the one chlorine atom. Recently, Schauble et al. have estimated the vibrational frequencies of ^{35}Cl and ^{37}Cl-containing molecules using *ab initio* force field model (HF/6-31Gd theory level)[1]. The ratio of isotope ratios ($\alpha_{eq.}$) of HOCl and Cl_2 at 25°C (298 K) calculated from the vibrational frequencies determined by Schauble et al. are 1.0085 and 1.0037, respectively. Thus, the isotope fractionation factor $\alpha_{Cl_2\text{-}HOCl}$ associated with the reaction 4 equal 1.0048. Therefore, the isotope ratios of HOCl and Cl_2 should be offset by 4.8‰ at equilibrium.

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Appendix 3

Chemical properties of the humic substances and NOM

Table 1. ^{13}C NMR estimates of carbon distribution in humic substances and NOM

sample	Carbonyl 220-190 ppm	Carboxyl 190-165 ppm	Aromatic 165-110 ppm	Acetal 110-90 ppm	Heteroaliphatic 90-60 ppm	Aliphatic 60-0 ppm
IHSS Standards						
Aquatic NOM						
Suwannee River[#]	8	20	23	7	15	27
Norid Lake[#]	8	21	19	5	16	31
Humic acid						
Elliott soil[#]	6	18	50	4	6	16
Pahokee peat[#]	5	20	47	4	5	19
Forest soil (Denmark)						
Forest soil NOM	<0.5	7.8	23	9	35	24
Forest soil humic acid	1.7	8	31	8	31	19

[#] K. A. Thorn, D. W. Folan, and P. MacCarthy (1989), *Characterization of the International Humic Substances Society standard and reference fulvic and humic acids by solution state carbon-13 (^{13}C) and hydrogen-1 (^{1}H) nuclear magnetic resonance spectrometry*, U.S. Geological Survey, Water-Resources Investigations Report 89-4196, Denver, CO, 93 pp.

Table 2. Elemental composition and stable isotopic ratios of humic substances and NOM

sample	C $\%_{w/w}$	H $\%_{w/w}$	O $\%_{w/w}$	N $\%_{w/w}$	δ^{13}C ‰ V-PDB
IHSS Standards					
Aquatic NOM					
Suwannee River	52.47	4.19	42.69	1.10	-28.0
Norid Lake	53.17	5.67	nd	1.10	-27.8
Humic acids					
Elliott soil	58.13	3.68	34.08	4.14	-22.6
Pahokee peat	56.37	3.82	37.34	3.69	-26.0
Forest soil (Denmark)					
Forest soil NOM	55	5.8	37	2.0	-27.5
Forest soil humic acid	54	4.8	38	2.2	-27.5